MABGEL 1:

C2F5, C4E10 & C2G12 as a vaginal microbicide

Georgina Claire Morris

MA (Cantab.), MBBS (Lond.), MRCP(UK)

Thesis submitted in candidature for the degree of Doctor of Medicine (Medical Sciences)

University of York and University of Hull

Hull York Medical School

December 2012

ABSTRACT

Topical microbicides are being developed as a female-controlled method for preventing HIV-1 infection. Non-antiretroviral (ARV)-based candidates may be advantageous given increasing levels of ARV resistance in low and middle- income countries.

MABGEL 1 was a phase 1 trial designed to evaluate the pharmacokinetics and safety of a vaginal microbicide containing the broadly HIV-1 neutralizing monoclonal antibodies (mAbs) C2F5, C4E10 and C2G12 in a hydroxyethylcellulose-based gel vehicle. It was the first study of topical mAb application to the human female genital tract.

Twenty-eight healthy women were randomised to apply either high dose Mabgel (containing 20mg/g of each mAb) (n= 10), low dose Mabgel (containing 10mg/g of each mAb) (n=9) or placebo gel (n=9). Doses (2.5ml) were applied over 12 consecutive days. Genital tract sampling was performed at baseline, 1 hour, 8 hours and 24 hours post 1st dose and 12 and 36 hours post 12th dose with serum samples collected at baseline, 8 hours post 1st dose and 12 hours post 12th dose. Safety was assessed through participant report and clinical examination, including colposcopy.

Residence half-lives (t ½) in vaginal secretions (Weck-Cel samples) were estimated to be between 4 and 5.5 hours for C4E10 and C2F5. In contrast, vaginal levels of C2G12 did not conform to a single overall exponential decay, displaying a more rapid initial rate of decline, which then slowed at lower concentrations. The estimated early t ½ of C2G12 was 1.4 hours (95% CI 1.2 to 1.8). There was no evidence of systemic absorption.

Daily vaginal application of up to 50g of each mAb over 12 days was safe. Although adverse events (AEs) were reported by all but 1 participant, 95 % were mild, none were serious and only 4 were moderate. There was no statistically significant difference in the number of AEs reported per participant between the 3 study arms.

Although there are a number of caveats, results demonstrate 'proof of principle' of the potential for combinations of HIV-1 neutralizing mAbs to be used as a coitally-dependent microbicide.

OVERVIEW OF MAIN CONTENTS

1.	**INTRODUCTION**	**22**
2.	**MABGEL1: CLINICAL TRIAL DESIGN AND METHODOLOGY**	**92**
3.	**PHARMACOKINETIC RESULTS AND ANALYSES**	**135**
4.	**SAFETY RESULTS AND ANALYSES**	**225**
5.	**QUALITATIVE INTERVIEW SUB-STUDY**	**260**
6.	**SUMMARY, OVERALL CONCLUSIONS, AND FUTURE DIRECTIONS**	**280**
REFERENCES		**298**

CONTENTS

ABSTRACT 2
OVERVIEW OF MAIN CONTENTS 3
CONTENTS 4
LIST OF FIGURES 8
LIST OF TABLES 10
ABREVIATIONS 12
DEDICATION 19
ACKNOWLEDGEMENTS 20
DECLARATION 21

1. INTRODUCTION 22

1.1 OVERVIEW OF THE STATE OF THE HIV-1 PANDEMIC 22
1.2 BASIC VIROLOGY OF HIV-1 24
1.2.1 Viral genes, life-cycle and targets for intervention 25
1.2.2 Structure of HIV-1 envelope glycoproteins 30
1.2.3 HIV-1- target cell tropism and entry 31
1.2.4 Reverse transcription and genetic heterogeneity 32
1.3 BASIC ANATOMY AND PHYSIOLOGY OF THE HUMAN FEMALE GENITAL TRACT 33
1.3.1 The human menstrual cycle 36
1.3.2 Hormonal contraception 39
1.4 DEFENSES AGAINST HIV-1 INFECTION IN THE FGT 40
1.4.1 Initial barriers to HIV-1 infection 40
1.4.2 Cytokines and chemokines and pathogen recognition 42
1.4.3 Natural Killer Cells 44
1.4.4 Adaptive immunity 45
1.5 HIV-1 TRANSMISSION VIA THE FGT 51
1.5.1 Factors influencing efficiency of of HIV-1 transmission 51
1.5.2 Early events in HIV-1 transmission and dissemination 55
1.6 STRATEGIES FOR PREVENTING SEXUAL TRANSMISSION OF HIV-1 61
1.6.1 Treatment of HIV infected individuals to prevent transmission 63
1.6.2 Interventions to reduce individuals' susceptibility to HIV 66
1.6.3 Potential Role of Microbicides in HIV prevention 69
1.6.4 Microbicide Development 70
1.6.5 Early Microbicide Candidates 71
1.6.6 Antiretroviral microbicides and oral pre-exposure prophylaxis 74
1.6.7 Current non-ARV microbicides 83
1.6.8 Broadly neutralizing monoclonal antibodies against HIV-1 84

2. MABGEL1: CLINICAL TRIAL DESIGN AND METHODOLOGY 92

2.1 OVERVIEW OF CLINICAL TRIAL DESIGN AND CONDUCT 92
2.1.1 Involvement of the author in trial design, conduct and data interpretation 92

2.2 RESEARCH GOVERNANCE AND APPROVALS 92
2.3 RESEARCH QUESTION AND RATIONALE 93
2.3.1 Primary Study Objective: Assessment of local mAb pharmacokinetics following vaginal application 93
2.3.2 Primary Study Objective: Determination of evidence of systemic absorption of the mAbs following vaginal application 95
2.3.3 Secondary Study Objective: Assessment of the local and systemic safety of the mAbs when applied vaginally 96
2.4 SUB-STUDIES 99
2.4.1 Investigation of the reliability of the Weck-Cel technique for collection of vaginal secretions (for antibody quantification) post gel application and comparison of participant self-sampling to sampling by clinicians 99
2.4.2 Investigation of the impact of Mabgel on vaginal flora 101
2.4.3 Investigation of participants' experience of taking part in the clinical trial 103
2.5 SELECTION OF STUDY PARTICIPANTS 103
2.5.1 Number and Source of Study Participants 103
2.5.2 Informed Consent and Screening Procedures 103
2.5.3 Eligibility 104
2.6 STUDY INTERVENTIONS 106
2.6.1 Randomisation and blinding 106
2.6.2 The Study Gels 107
2.6.3 Administration of the Study Gel (Gel Dosing) 108
2.6.4 Measures of Compliance and Adherence 108
2.7 PHARMACOKINETIC EVALUATIONS 109
2.7.1 Pharmacokinetic Outcome Measures 109
2.7.2 Pharmacokinetic Sampling Procedures 110
2.7.3 Sampling windows 114
2.8 SAFETY EVALUATIONS 115
2.8.1 Safety-Related Outcome Measures 115
2.8.2 Safety-Related Definitions and Classifications 115
2.8.3 Procedures for Assessing Safety 117
2.9 DOSING INTERRUPTIONS, WITHDRAWALS AND STUDY TERMINATION 118
2.10 FINANCIAL RE-IMBURSEMENT OF STUDY PARTICIPANTS 119
2.11 LABORATORY PROCEDURES 120
2.11.1 Screening and Safety Specimens 120
2.11.2 Initial Processing and Storage of Specimens for mAb Analysis 121
2.11.3 ELISAs for Quantifying mAb Concentrations 124
2.11.4 Specimens for Vaginal Flora Sub-study 125
2.12 DATA MANAGEMENT AND QUALITY ASSURANCE 126
2.12.1 Data Management and Trial Monitoring 126
2.12.2 Trial Management Group 127
2.12.3 Data and Safety Monitoring Committee (DSMC) 127
2.13 PLANNED STATISTICAL ANALYSES 128
2.13.1 Final Statistical Analysis Plan 128
2.13.2 Analysis Populations 129
2.13.3 Data Derivations and Data Handling 129
2.13.4 Pharmacokinetic Analyses 130

2.13.5 Safety Analyses *132*
2.13.6 Sub-Studies *133*

3. PHARMACOKINETIC RESULTS AND ANALYSES 135

3.1 DATA SETS ANALYSED 135
3.2 DATA ANALYSIS AND REPORTING 135
3.3 STUDY PARTICIPANTS 137
3.3.1 Accrual and Retention *137*
3.3.2 Baseline Characteristics *137*
3.3.3 Screen Failures *139*
3.4 BLINDING AND RANDOMISATION 139
3.5 COMPLIANCE WITH PROTOCOL 140
3.5.1 Gel usage and sexual abstinence *140*
3.5.2 Deviations Relating to Sample Collection or Analysis *141*
3.6 PHARMACOKINETIC SAMPLING AND ELISA DATA 142
3.6.1 Samples Obtained *142*
3.6.2 mAb Concentrations Detected on ELISAs *143*
3.6.3 Dilution Factor Correction For mAb Concentrations Detected in Weck-Cel Samples *144*
3.7 STATISTICAL ANALYSIS OF PHARMACOKINETIC DATA 145
3.7.1 Handling of Missing Data *145*
3.7.2 Primary and Secondary Pharmacokinetic End-Point Analyses *145*
3.7.3 Analysis of mAb half-lives *155*
3.7.4 Analysis of Variability in Weck-Cel Sampling and relationship between sample weight and mAb concentration *166*
3.8 SUMMARY AND DISCUSSION OF PHARMACOKINETIC RESULTS 180
3.8.1 mAb Pharmacokinetics *180*
3.8.2 mAb Concentrations detected in vaginal secretions sampled using Weck-Cels (primary study end-points) *181*
3.8.3 mAb concentrations in other samples (secondary end points) *182*
3.8.4 mAb Half-lives *183*
3.8.5 Variability in Weck- Cel sample volumes *183*
3.8.6 Limitations of study design and procedures *188*
3.8.7 Comparison of mAb concentrations and t ½ with those from other studies *196*
3.8.8 Putative mechanisms governing the elimination of the mAbs from the vaginal lumen *200*
3.8.9 Possible explanations for the difference in pharmacokinetics between 2G12 and the MPER mAbs *213*

4. SAFETY RESULTS AND ANALYSES 225

4.1 MAIN SAFETY DATA 225
4.1.1 Adverse Events *225*
4.2 STATISTICAL ANALYSIS OF MAIN SAFETY DATA 240
4.2.1 Analyses of All AEs Regardless of Attribution to Study Gel Use *241*
4.2.2 Analyses of AEs Attributed as Being At Least Possibly Related to Study Gel Use *242*
4.2.3 Summary and Discussion of Main Safety Results *243*

4.3 Results and Analyses From the Vaginal Flora Sub-Study 246
4.3.1 Data Relating to Quantitative PCR for Lactobacillus Species, Nugent Scores and Vaginal pH 246
4.3.2 Evaluation and Statistical Analysis of Lactobacillus PCR Results 246
4.3.3 Discussion of Methods Used to Assess Vaginal Flora 253
4.3.4 Comparison Between Clinical Assessment, Nugent Score and PCR Methodologies in Our Trial 256

5. QUALITATIVE INTERVIEW SUB-STUDY 260

5.1 Rationale behind the qualitative interview sub-study 260
5.2 Research Objectives 260
5.3 Methodology 261
5.3.1 General conduct of the interviews 261
5.3.2 Interview topic guide 262
5.3.3 Data Analysis 265
5.4 Results 266
5.4.1 Trial Recruitment 266
5.4.2 Discussing the trial with others 267
5.4.3 Adequacy and suitability of information provided before and during the trial 268
5.4.4 Experience of Clinical Trial Visits and Procedures 270
5.4.5 Compliance with study procedures 274
5.4.6 Experience of using the gel 275
5.4.7 Closing comments and suggestions to improve future trials 276
5.4.8 Discussion and conclusions 277

6. SUMMARY, OVERALL CONCLUSIONS, AND FUTURE DIRECTIONS 280

6.1 Summary 280
6.2 Mabgel: a feasible microbicide for the ‘real-world’? 281
6.2.1 Stability 281
6.2.2 Safety 282
6.2.3 Effectiveness 283
6.2.4 Production Costs 291
6.2.5 Acceptability 292
6.2.6 Novel anti -HIV-1 NAbs 295
6.2.7 Conclusion 296

REFERENCES 298

LIST OF FIGURES

Figure 1-1 The HIV-1 virion 26
Figure 1-2 The HIV-1 RNA genome 26
Figure 1-3 HIV-1 Viral Life-Cycle 28
Figure 1-4 Schematic Representation of the crystal structure of gp120 both unbound and bound to CD4 31
Figure 1-5 Diagramatic representation of the human female genital tract 35
Figure 1-6 Sagittal view of the human female pelvis 36
Figure 1-7 Human female menstrual cycle 38
Figure 1-8 Typical structure of an antibody 47
Figure 1-9 Initial steps in the establishment of HIV-1 infection in the FGT mucosa 60
Figure 1-10 Binding sites of NAbs on gp120 and gp41 85
Figure 2-1 Median mAb concentrations in Weck-Cel secretion samples in macaques 95
Figure 3-1 CONSORT Diagram Depicting the Passage of Participants Through the MABGEL 1 Clinical Trial 136
Figure 3-2 Median Concentrations (Unadjusted for Dilution Factors) of C2F5, C4E10, C2G12 Detected in Weck-Cel Samples Over Time in Participants Randomised to High Dose Mabgel Arm (20µg/ml of each mAb) 149
Figure 3-3 Median Concentrations of C2F5, C4E10, C2G12 (Unadjusted for Dilution Factors) Detected in Weck-Cel Samples Over Time in Participants Randomised to Low Dose Mabgel Arm (10µg/ml of each mAb) 149
Figure 3-4 Median Concentrations (Adjusted for Dilution Factors) of C2F5, C4E10, C2G12 Detected in Weck-Cel Samples Over Time in Participants Randomised to High Dose Mabgel Arm (20µg/ml of each mAb) 151
Figure 3-5 Median Concentrations (Adjusted for Dilution Factors) of C2F5, C4E10, C2G12 Detected in Weck-Cel Samples Over Time in Participants Randomised to Low Dose Mabgel Arm (20µg/ml of each mAb) 152
Figure 3-6 C2F5 Concentration Since Application of 1st Dose of Mabgel 156
Figure 3-7 Log_e of C2F5 Concentration Since Application of 1st Dose of Mabgel 156
Figure 3-8 Concentration of C4E10 Since Application of 1st Dose of Mabgel 158
Figure 3-9 Log_e of C4E10 Concentration Since Application of 1st Dose of Mabgel 159
Figure 3-10 Concentration of C2G12 Since Application of 1st Dose of Mabgel 160
Figure 3-11 Log_e of C2G12 Concentration Since Application of 1st Dose of Mabgel 160
Figure 3-12 Concentration of C2F5 (Adjusted for Dilution Factor) Since Application of 1st Dose of Mabgel 161
Figure 3-13 Log_e of C2F5 Concentration (Adjusted for Dilution Factor) Since Application of 1st Dose of Mabgel 162
Figure 3-14 Concentration of C4E10 (Adjusted for Dilution Factor) Since Application of 1st Dose of Mabgel 163
Figure 3-15 Log_e of C4E10 Concentration (Adjusted for Dilution Factor) Since Application of 1st Dose of Mabgel 163
Figure 3-16 Concentration of C2G12 (Adjusted for Dilution Factor) Since Application of 1st Dose of Mabgel 164

Figure 3-17 Log_e of C2G12 Concentration (Adjusted for Dilution Factor) Since Application of 1st Dose of Mabgel 165
Figure 3-18 Relationship between variability of weights of samples obtained at the same time-point in the same woman and magnitude of the mean sample weight (in grams) 167
Figure 3-19 Relationship between variability of weights of samples obtained at the same time-point in the same woman and log $_{10}$ of the mean sample weight 167
Figure 3-20 Relationship Between the weights of paired samples obtained at the same time-point in the same woman 168
Figure 3-21a and b – Distribution and within visit variability of raw sample weights (residuals) 170
Figure 3-22 Distribution and within visit variability of log_{10} transformed sample weights (residuals) 170
Figure 3-23 Scatter plot of mean log_{10} Weck-Cel sample weight against log_{10} C2F5 concentration detected by ELISA (unadjusted for dilution factor) 176
Figure 3-24 Scatter plot of mean Weck-Cel sample weight against C2F5 concentration detected by ELISA (unadjusted for dilution factor) 177
Figure 3-25 Scatter plot of mean log_{10} Weck-Cel sample weight against log_{10} C4E10 concentration detected by ELISA (unadjusted for dilution factor) 177
Figure 3-26 Scatter plot of mean Weck-Cel sample weight against C4E10 concentration detected by ELISA (unadjusted for dilution factor) 178
Figure 3-27 Scatter plot of mean log_{10} Weck-Cel sample weight against log_{10} C2G12 concentration detected by ELISA (unadjusted for dilution factor) 178
Figure 3-28 Scatter plot of mean Weck-Cel sample weight against C2G12 concentration detected by ELISA (unadjusted for dilution factor) 179
Figure 3-29 Putative mechanisms governing elimination of mAbs from the vaginal lumen 201
Figure 3-30 Atypical structure of 2G12 218
Figure 3-31 Structures of 2F5 and 4E10 219
Figure 3-32 Concentrations of C2F5, C4E10 and C2G12 after incubation with human vaginal secretions at pH 4.0 224
Figure 4-1 Box Plot of *L. crispatus* Counts By Study Arm and Visit 251
Figure 4-2 Box Plot of *L. iners* Counts By Study Arm and Visit 251
Figure 4-3 Box Plot of *L. jensenii* Counts By Study Arm and Visit 252
Figure 4-4 Box Plot of *L. gasseri* Counts By Study Arm and Visit 252

LIST OF TABLES

Table 1-1 HIV genes, proteins and their main functions 27
Table 1-2 Current licensed antiretroviral agents 29
Table 1-3 Human immunoglobulin classes and major characteristics 48
Table 1-4 Biological co-factors which facilitate HIV-1 transmission 54
Table 1-5 Strategies for preventing sexual transmission of HIV-1 62
Table 1-6 1st Generation HIV-1 Microbicides 72
Table 1-7 Specific anti-HIV-1 agents with potential for use as microbicides or oral PrEP 82
Table 1-8 Summary of mucosal SHIV challenge studies in macaques involving anti-HIV-1 neutralizing mAbs 2F5, 4E10 and 2G12 +/-b12 88
Table 1-9 Summary of clinical trials using C2F5, C4E10 and C2G12 to date 90
Table 2-1 Median, minimum and maximum mAb Concentrations detected using Weck-Cels in macaques (CEA study THE0703) following application of 20mg/g Mabgel 94
Table 2-2 Study Schedule 98
Table 2-3 Composition of Study Gels 107
Table 2-4 Scoring System for Bacterial Morphotypes on Gram-stained Vaginal Smears 125
Table 2-5 *Lactobacillus* Specific PCR Primers 126
Table 3-1 Summary of Baseline Characteristics of Enrolled Participants 138
Table 3-2 Median, Minimum and Maximum Values for Concentrations of Each of C2F5, C4E10, C2G12 Detected in Weck-Cel Samples By Study Arm (Unadjusted Values) 147
Table 3-3 Median, Minimum and Maximum Values for Concentrations of Each of C2F5, C4E10, C2G12 Detected in Weck-Cel Samples By Study Arm (After Adjustment for Dilution Factors) 150
Table 3-4 Median, Minimum and Maximum Values for Concentration of Each of C2F5, C4E10, C2G12 Detected in CVL Samples By Study Arm 153
Table 3-5 Median, Minimum and Maximum Values for Concentration of Each of C2F5, C4E10, C2G12 Detected in CVL Samples By Study Arm 154
Table 3-6 ANOVA table 1 for $\log_{10}$ transformed weights of repeated Weck-Cel samples 172
Table 3-7 Mean (and standard deviation) of paired Weck-Cel sample weights, in grams, by visit and treatment arm 174
Table 3-8 ANOVA table 2 for $\log_{10}$ transformed weights of repeated Weck-Cel samples 174
Table 3-9 Regression slope of $\log_{10}$ mAb concentration on $\log_{10}$ Weck-Cel Sample Weight 180
Table 3-10 Median mAb Concentrations (µg/ml) detected using Weck-Cels (before and after adjustment for dilution factors) in participants in the High Dose (20mg/g) Mabgel arm compared with those in macaques in the CEA THE0703 study 197
Table 4-1 AE Distribution by Study Arm 227
Table 4-2 Severity of All AEs by Study Arm 241
Table 4-3 Mean and Standard Deviation of All AEs Per Study Arm 241
Table 4-4 Attribution of Likely Causality of AEs By Study Arm 242
Table 4-5 Severity of AEs Attributed as Being At Least Possibly Related to Study Gel Use by Study Arm 243

Table 4-6 Mean and Standard Deviation of Number of AEs Attributed As Being At Least Possibly Related to Use of Study Gel Per Study Arm 243
Table 4-7 Number (%) of women with specific *Lactobacillus* species by visit and study arm 248
Table 4-8 Average (mean and median) *Lactobacillus* species counts per ml of elution buffer by study arm and visit 250

ABREVIATIONS

Abreviation	Explanation/Terminology in Full
AAV	Adeno-associated virus
aCL	Anti-cardiolipin
ADCC	Antibody-dependent cellular cytotoxicity
ADVI	Antibody-dependent cell-mediated virus inhibition
ADR	Adverse drug reaction
AE	Adverse event
Ab	Antibody
Ag	Antigen
AID_{50}	50 % animal infectious doses
AIDS	Acquired Immunodeficiency Syndrome
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APC	Antigen presenting cell
API	Active pharmaceutical ingredient
APOBEC3G	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G
APTT	Activated partial thromboplastin time
AR	Adverse reaction
ARV	Antiretroviral
B cell	B lymphocyte
BV	Bacterial vaginosis
C1 to C5	Conserved regions 1 to 5 of HIV-1 gp120
CCL	CC-Chemokine ligand
CCR5	CC-Chemokine receptor type 5
CD	Cluster of differentiation
CDC	United States Centres For Disease Control
CDR	Complementary-determining region of an antibody
CEA	French Alternative Energies and Atomic Energy Commission (Commissariat à l'énergie atomique

	aux énergies alternatives).
C_H (1-3)	Constant domains (1 to 3) of Ig heavy chain
CHO	Chinese Hamster Ovary
CI	Chief Investigator
CIN	Cervical intraepithelial neoplasia
C_L	Constant domain of Ig light chain
COCP	Combined (oestrogen and progestagen containing) oral contraceptive pill
CRF	Case report form
CRF	Circulating recombinant form (of HIV-1)
CT	Chlamydia trachomatis
CTA	Clinical Trials Authorisation
CTL	$CD8^+$ Cytotoxic T lymphocyte
CVF	Cervico-vaginal fluid
CVL	Cervico-vaginal Lavage
CXCL	CX- Chemokine ligand
CXCR4	CX-Chemokine receptor type 4
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMC	Data Monitoring Committee
DMPA	Depot medroxyprogesterone acetate
DNA	Deoxyribonucleic acid
EC	Epithelial cell
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbant assay
EMPRO	European Microbicides Programme
Env	Envelope (HIV transmembrane and surface proteins (gp41and gp120)
EUDRACT	European Union Drug Regulatory Agency clinical trial
EUROPRISE	European Vaccines and Microbicides Enterprise
Fab	Fragment of antigen binding region of an antibody
FBC	Full blood count
Fc	Fragment, crystallisable region of an antibody

FcR	Fc Receptor
FcRn	Neonatal Fc receptor
FcRn-KO	FcRn knockout
FDA	United States Food and Drug Administration
FGT	Female genital tract
FSH	Follicle-stimulating hormone
Gag	HIV structural protein
GALT	Gut-associated lymphoid tissue
GC	Gonorrhoea
GCP	Good Clinical Practice
GI	Gastrointestinal
GMP	Good Manufacturing Practice
GP	General Practitioner
gp41	41 kDa HIV transmembrane glycoprotein
gp120	120 kDa HIV surface glycoprotein
gp 160	160kDa HIV envelope glycoprotein (gp120/41) precursor
GU	Genitourinary
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEC	Hydroxyethyl cellulose
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HI-FCS	Heat inactivated fetal calf serum
HIVIG	HIV immune globulin (polyclonal IgG from HIV positive individuals)
HLA	Human leucocyte antigen
HPTN	HIV prevention trials network
HPV	Human papillomvirus
HSV-2	Herpes simplex virus type 2
HYMS EMU	Hull York Medical School Experimental Medicine Unit
IB	Investigator’s brochure
IC_{50}	Antibody concentration needed to achieve 50% virus neutralization *in-vitro*

IC_{90}	Antibody concentration needed to achieve 90% virus neutralization *in-vitro*
ID	Identification number
IDU	Injection drug use
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular administration
IMP	Investigational medicinal product
IN	HIV Integrase enzyme
INR	International normalised ratio
ISG	IFN-stimulated gene
ISRCTN	International standard randomised controlled trial number
ITT	Intention-to-treat
IUCD	Intrauterine contraceptive device (copper containing)
IUS	Intrauterine contraceptive system (progestagen containing)
IV	Intravenous administration
IVR	Intravaginal ring
LFTs	Liver function tests
LH	Luteinizing hormone
LTR	Long terminal repeat
mAbs	Monoclonal antibodies
MALT	Mucosa-associated lymphoid tissue
MCP	Macrophage chemotactic protein
mDC	Myeloid dendritic cell
MDP	Microbicides development programme
MIP	Macrophage inflammatory protein
MHC	Major histocompatibility complex
MHRA	Medicines and Healthcare products Regulatory Agency
MPER	Membrane proximal external region
MRC CTU	Medical Research Council Clinical Trials Unit

MSM	Men who have sex with men
MUC	Glycoprotein constituent of mucin
ND	Not detectable
N9	Nonoxynol 9
NALT	Nasal-associated lymphoid tissue
NAb	Neutralizing antibody
Nef	Negative regulatory factor (HIV accessory protein)
NF-κB	Nuclear factor kappa B
NHP	Non-human primate
NHS	National Health Service
NK cell	Natural Killer cell
NKR	NK cell receptor
NMB	Non-menstrual bleeding
NNRTI	Non-nucleotide reverse transcriptase inhibitor
NRTI	Nucleotide reverse transcriptase inhibitor
OE	On examination
ORF	Open reading frame
OTC	Over the counter
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PD	Pharmacodynamics
pDC	Plasmacytoid dendritic cell
PEP	Post-exposure prophylaxis
PHI	Primary HIV infection
PI	Protease Inhibitor
pI	Isoelectric point
PID	Pelvic inflammatory disease
pIgR	Polymeric immunoglobulin receptor
PIS	Participant Information Sheet
PHI	Primary (acute) HIV
PK	Pharmacokinetics
PMN	Polymorphonucleocytes
Pol	HIV enzyme precursor protein
POP	Progestagen containing oral contraceptive pill

PrEP	Pre-exposure prophylaxis
PRR	Pattern recognition receptor
PT	Prothrombin time
PV	Per vagina
(q) PCR	(Quantitative) polymerase chain reaction
QOL	Quality of life
QP	Qualified person
R5	CCR-5 using (tropic) HIV-1 isolate
R & D	Research and Development
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
RBCs	Red blood cells
RCT	Randomised controlled trial
RES	Reticuloendothelial syndrome
REV	Regulator of virion expression (HIV regulatory protein)
RNA	Ribonucleic acid
RR	Relative risk
RT	Reverse transcriptase (HIV enzyme)
RTI	Reverse transcriptase inhibitor
SAE	Serious adverse event
SAP	Statistical analysis plan
SAR	Serious adverse reaction
SC	Secretary component
SC	Subcutaneous administration
SDF-1	Stromal cell derived factor-1
SGA	Single genome amplification
SID	Screening Identification Number
SIV	Simian immunodeficiency virus
SHIV	Simian human immunodeficiency virus
SLP1	secretory leucocyte protease inhibitor-1
SOP	Standard operating procedure
SP	Study physician
STI	Sexually transmitted infection
SUSAR	Suspected unexpected serious adverse reaction
Tat	Transcriptional activator (HIV regulatory protein)

$TCID_{50}$	50% tissue culture infectious dose
T cell	T lymphocyte
TER	Transepithelial resistance
Tfh	Follicular T-helper cell
Th	Helper $CD4^{+}$ T cell
TLR	Toll-like receptor
TMF	Trial master file
TMG	Trial Management Group
TRIM5α	Tripartite motif 5 alpha
TSC	Trial Steering Committee
U and Es	Urea, creatinine and electrolytes
ULN	Upper limit of normal range
UM	Unmeasurable
URAI	Unprotected receptive anal intercourse
URVI	Unprotected receptive vaginal intercourse
V1 to V5	Variable regions of HIV-1 gp120
V_c	Central volume of distribution
VEC	Vaginal-ectocervical tissue model
V_H	Variable region of Ig heavy chain
VL	Viral load
Vpr	Viral protein R (HIV accessory protein)
Vpu	Viral protein U (HIV accessory protein)
V_{ss}	Steady state volume of distribution
WBC	White blood cell
Weck-Cel	Weck-Cel ophthalmic sponge
WHO	World Health Organization
WT	Wild-type
X4	CXCR-4 using (tropic) HIV-1 isolate

DEDICATION

To my parents, Joan and Barry, without whose sacrifices, love, support and encouragement I would never have attained a university education or made it through the early years as a junior doctor.

ACKNOWLEDGEMENTS

A big thank you to everyone who was involved in the Mabgel Trial (investigators, research staff and participants) and to everyone who has supported me and enabled me to complete this thesis, either directly, or indirectly.

In particular, thank you to:

Charles for all of your advice, supervision and encouragement and for providing me with the opportunity to undertake this research project.

Paul Kaye and Rob Newton for your support, advice and time as part of my Thesis Advisory Panel.

Rebecca Wiggins, Carol Taylor, Nicola Marshall, Nelisa Poshai and David Thompson who worked alongside me on the HYMS EMU throughout the Mabgel 1 Trial (including some very early mornings and late nights) and who I now consider to be surrogate members of my family.

Martin Bland, Brigitta Vcelar and Vicky Jespers, as co –investigators, for their crucial oversight of laboratory and statistical analyses, and for all their advice and support.

Sarah Woodhall, Deborah Phillips, Caroline Mozley and the Monitoring and Data Management team from Covance for the roles they played in ensuring the study was conducted smoothly and to the highest standard, and to Katrein Schäfer and Sarah Douglas for their helpful contributions.

Amy Evans, Janet Wilson, Ian Fairley and all of my other friends and colleagues who have provided support and encouragement when there seemed to be no light at the end of the tunnel.

And lastly and most importantly to Daniel, for his love, understanding, patience, IT trouble-shooting and culinary skills and for those endless weekends and evenings spent with a boring girlfriend glued to her computer.

DECLARATION

I confirm that this work is original and that if any passage(s) or diagram(s) have been copied from academic papers, books, the internet or any other sources these are clearly identified by the use of quotation marks and the reference(s) is fully cited. I certify that, other than where indicated, this is my own work and does not breach the regulations of HYMS, the University of Hull or the University of York regarding plagiarism or academic conduct in examinations. I have read the HYMS Code of Practice on Academic Misconduct, and state that this piece of work is my own and does not contain any unacknowledged work from any other sources.

I confirm that any patient information obtained to produce this piece of work has been appropriately anonymised.

Georgina Claire Morris

December 2012

1. INTRODUCTION

1.1 Overview of the state of the HIV-1 pandemic

Thirty years have passed since the first cases of Acquired Immunodeficiency Syndrome (AIDS) were recognised by the United States Centres for Disease Control (CDC) [1,2] and there have now been over 30 million deaths from AIDS worldwide [3,4]. The past three decades have seen remarkable scientific and clinical progress in identifying the underlying causative agent, the retrovirus HIV-1 (human immunodeficiency virus type 1), understanding its role in pathogenesis and developing interventions to halt its progression [5,6]. Rapid and accurate HIV diagnosis is now possible at point-of-care and disease can be monitored through the measurement of CD (cluster of differentiation) 4-expressing T lymphocytes ($CD4^+$ T cells), the main target cell depleted by HIV infection, and HIV ribonucleic acid (RNA) copy number, or Viral load (VL), in plasma [7,8]. Since 1996, combinations of antiretroviral agents (ARVs), known as highly active antiretroviral therapy (HAART), have been available in high-income countries with the ability, provided adherence is good, of suppressing HIV replication to below the limits of detection (VL < 50 copies/ml) in plasma [9]. The prognosis of individuals receiving such treatment has been transformed and, with early diagnosis and guideline-recommended regimens, an HIV-1-infected individual can now achieve a near normal life-expectancy [10]. Between 2001 and 2010, the number of people in resource-poor regions who had access to HIV treatment increased by 22 fold [3]. In 2011, another 1.4 million people started ARVs in these regions, bringing the total receiving treatment to 8 million [4]. However, although this represents a huge achievement, there are still currently another 9 million individuals who are not on treatment, but who require it based on clinical and CD4-count ≤ 350 cells/mm^3 thresholds, and there were 1.7 millon deaths from AIDS in 2011 [4,11].

Despite some significant advances we are a long way from controlling the HIV-1 pandemic. Unfortunately, opportunities to try to limit the spread of HIV in the early years after its discovery were not taken up, with the responses provided by most governments and global non-government organisations in the 1980s and 1990s being ‘too little, too

late' [12]. This, coupled with increasing survival, mean there are now estimated to be 34.2 million people living with HIV-1 world-wide, 97 % of whom reside in low or middle income countries [4]. Despite a gradual decline in the global annual HIV-1 incidence of almost 1/4 in the past decade, there are still 2.3 million new infections in adults per year, with 70% occurring in sub-Saharan Africa [3]. Indeed, for every 2 people started on ARVs, another 3 become newly infected [4]. At the current rate of growth, the costs attributable to caring for the HIV-1 infected population are not sustainable. Thus, development and implementation of effective HIV prevention strategies is a global research priority [13].

HIV-1 infections can be acquired through exposure to infected body fluids via three major routes. Firstly, blood to blood, i.e. through transfusions, sharing needles and needle-stick injuries. Secondly, vertically from mother to child, either in-utero, during delivery or through breast feeding. Thirdly, via sexual intercourse. In adults, although HIV-1 transmissions are increasing in association with injection drug use (IDU), especially in Eastern Europe and Central Asia, over 80% of infections are acquired through sex [3,4]. High rates of transmission continue to be seen in men who have sex with men (MSM), who were the initial population in whom HIV-1 was first detected in high-income countries. However, the overwhelming majority of infections world-wide occur in heterosexuals [4].

Young women, especially in Africa, appear particularly vulnerable to acquiring HIV. Globally, around half of all people living with HIV-1 are female. In sub-Saharan Africa, women account for almost 60 % of HIV-1 infected adults, however, in the 15 to 24 year age group, this proportion rises to almost 3/4 [3]. In many of the most highly-affected regions, young women are up to 3 times more likely to be HIV-1 infected than men of the same age [4]. World-wide, women under 25 account for over a 1/4 of the total new HIV infections in those aged 15 or older [3]. The explanation for the excess of infections in young women is multi-factorial with inter-generational partnerships, gender inequalities, hygiene practices, sexually transmitted infections (STIs), hormonal contraceptive use and innate biological susceptibility all playing a role [4, 14, 15]. Understanding these factors and how they interact to enhance HIV-1 transmission will be crucial to developing successful HIV prevention strategies for this important target population [16].

A growing number of interventions have shown potential in reducing sexual transmission of HIV-1 [17]. Behavioural strategies, which are aimed largely at reducing high risk sexual encounters, include increasing HIV testing and knowledge of HIV serostatus, delaying coitarche, reducing the number and concurrency of sexual partnerships and encouraging correct and consistent condom use. Biomedical methods, ideally used as an adjunct to the above, include measures both to reduce donor infectiousness, and to decrease recipient susceptibility. Earlier initiation of ARV treatment of HIV infected individuals to reduce onward transmission is a particularly promising strategy. Examples of interventions which aim to directly protect HIV seronegative individuals are medical male circumcision, the use of systemic or topical ARVs (as pre-exposure prophylaxis (PrEP)), non-ARV-based topical microbicides, and, ultimately, once developed, an effective prophylactic vaccine.

This thesis is primarily concerned with the first-in-human evaluation of a novel vaginal microbicide, MABGEL, containing three HIV-1 neutralizing monoclonal antibodies (mAbs) and its potential role in preventing HIV-1 transmission to women. The aims of the remainder of this introductory chapter are to: 1) Provide relevant background information regarding the virology and life-cycle of HIV-1, anatomy and physiology of the human female genital tract (FGT) and innate and adaptive mucosal immune defenses; 2) Give an overview of HIV-1 transmission via the FGT, including factors which influence its efficiency; 3) Discuss the current status of biomedical HIV prevention strategies and the rationale behind the development of an anti-HIV-1 mAb-based vaginal microbicide.

1.2 Basic virology of HIV-1

HIV-1 is a human lentivirus of the *Retroviridae* family [18,19]. It is closely related to HIV-2 and both HIV viruses are believed to have originated in West-Central Africa as zoonoses from the Simian Immunodeficiency Viruses (SIV) affecting chimpanzees (SIV_{cpz}) and sooty mangabeys (SIV_{smm}) respectively [20, 21]. In comparison to HIV-1, HIV-2, is generally less pathogenic and has largely remained confined to its region of origin [22].

Phylogenetic analysis of HIV-1 viral nucleotide and amino acid sequences has identified three separate groups, known as M (main), O (outlier) and N (Non-M, non-O), that are thought to have arisen through separate cross-species transmission events. Viruses in

group M, the only group to become widely established, have been further divided into subtypes (or clades), and circulating recombinant forms (CRFs) have also arisen through co-infections with two or more distinct subtypes [23]. HIV-1 subtypes are largely geographically separated, with subtype B predominating in MSM populations in high income countries (Europe, USA, Japan, Australia), South and Central America and the Carribean, C in Southern Africa and Asia (responsible for > 50% of all infections worldwide), A and D in Central and Eastern Africa, and CRF01_ AE and CRF02_ AG in Thailand and Central Africa [24]. However, increased migration and air travel has resulted in the global dispersal of most subtypes.

1.2.1 Viral genes, life-cycle and targets for intervention

The general organization of the HIV-1 viral particle (virion) and genome are shown in Figure 1-1 and Figure 1-2. HIV-1 genes, resultant proteins and their main functions are outlined in Table 1-1. Each virion contains two copies of the full-length HIV-1 RNA genome. The HIV-1 genome is approximately 9 kb in length and is flanked at each end by single long terminal repeats (LTR). These are required for integration into the host cell nuclear deoxyribonucleic acid (DNA) and contain promoter sequences necessary for gene expression, including binding sites for the viral protein Tat and host transcription factors such as nuclear factor kappa B (NF-κB) [25]. Three of the nine open reading frames (ORFs) encode the Gag, Gag-Pol and Env polyproteins (later cleaved into individual proteins by the viral protease). The *gag* gene products are core structural proteins responsible for the assembly of the virion. In contrast, *pol* encodes three enzymes essential for viral replication: reverse transcriptase (RT) (an RNA-dependant DNA polymerase), integrase (INT) and protease (PR), which are also incorporated within the virion [26]. The *env* (envelope) gene encodes the viral glycoproteins which become embedded in the host cell membrane, through which the virion buds to derive its outer covering [25,26]. The products of the other six ORFs are accessory proteins with gene regulatory (Tat and Rev) [27,28] or virulence-enhancing (Vif, Vpr, Vpu and Nef) functions [29,30]. Vif, Vpr and Nef are also found in the viral particle [26].

Figure 1-1 The HIV-1 virion

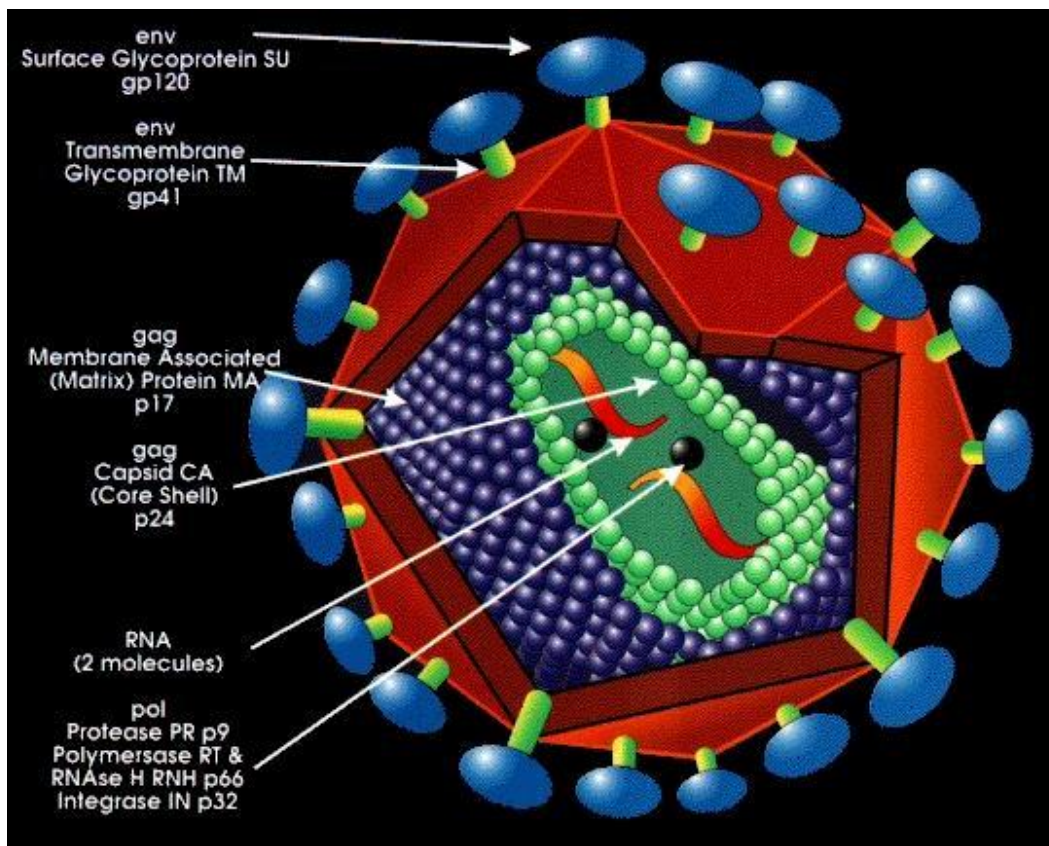


Figure reproduced with permission from evolutionarymodel.com. Accessed at http://www.evolutionarymodel.com/ERV_2.JPG on 2 June 2012.

Figure 1-2 The HIV-1 RNA genome

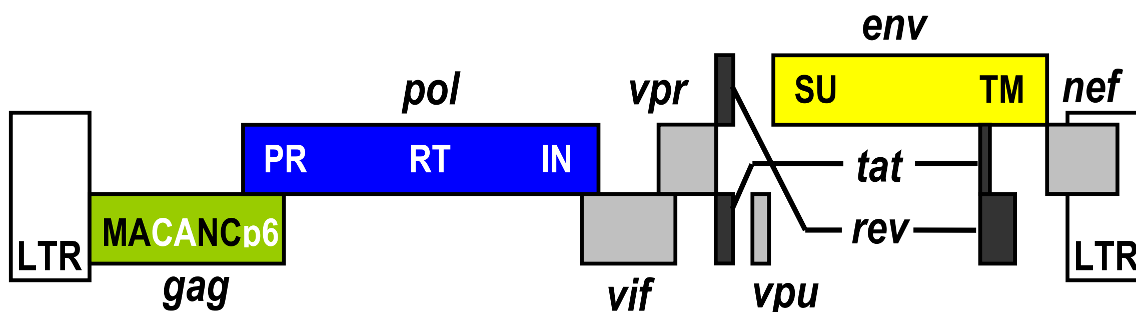


Table 1-1 HIV genes, proteins and their main functions

Gene	Protein	Function
Structural and enzymatic proteins [25, 26]		
Gag	p24 Capsid	Important for particle formation; encapsidates viral enzymes and genomic RNA.
	p17 Matrix	Forms a shell on inner side of membrane. Responsible for virion stability and maturation; role in uncoating and transport of pre-integration complex to the nucleus.
	p9 Nucleocapsid	Binds single stranded genomic RNA and ensures it is packaged into virions.
Pol	p6 Proline-rich protein	Involved in virion assembly and release.
	p10 Protease	Mediates cleavage of Gag and Gag/Pol polyproteins.
	p31 Integrase (IN)	Mediates integration of reverse transcribed HIV-1 DNA (provirus) into the host cell DNA.
	p66/p51 Reverse transcriptase (RT)	Has DNA polymerase and RNAse H activity, copies viral RNA into DNA.
Env	gp41 Transmembrane (TM)	Anchors envelope glycoprotein complex to host/virion surface membrane; mediates fusion of virus and target cell membranes.
	gp120 Surface (SU)	External glycoprotein which forms complexes with gp41; major determinant of cell tropism and target of most neutralizing antibodies; binds CD4 and chemokine co-receptors on target cells.
Regulatory and virulence-enhancing proteins		
Tat	p14 Transcriptional activator	Transcriptional and post transcriptional regulator of HIV-1 gene expression; enhances expression of viral genes [28].
Rev	p19 Regulator of virion expression	Post transcriptional regulator of HIV-1 gene expression; promotes nuclear export, stability and translation of mRNA transcripts containing the Rev responsive element [27].
Nef	p27 Negative regulatory factor	Promotes activation of infected cells to help establish persistent infection; downregulates expression of surface major histocompatibility complex (MHC) and CD4 molecules to assist infected cells in evading the host immune response [29].
Vpr	p14 Viral protein R	Helps regulate nuclear import of the HIV-1 pre-integration complex, and is required for virus replication in non-dividing cells such as macrophages. Vpr also induces G2 cell cycle arrest and apoptosis in proliferating cells [29].
Vpu	p16 Viral protein U	Binds to BST2/tetherin on host cells to facilitate virion budding and release [30].
Vif	p23 Viral infectivity factor	Binds the cytidine deaminase enzyme APOBEC 3G and targets it for cellular degradation. APOBEC 3G mutates viral nucleic acids and prevents ongoing replication [31].

Figure 1-3 HIV-1 Viral Life-Cycle

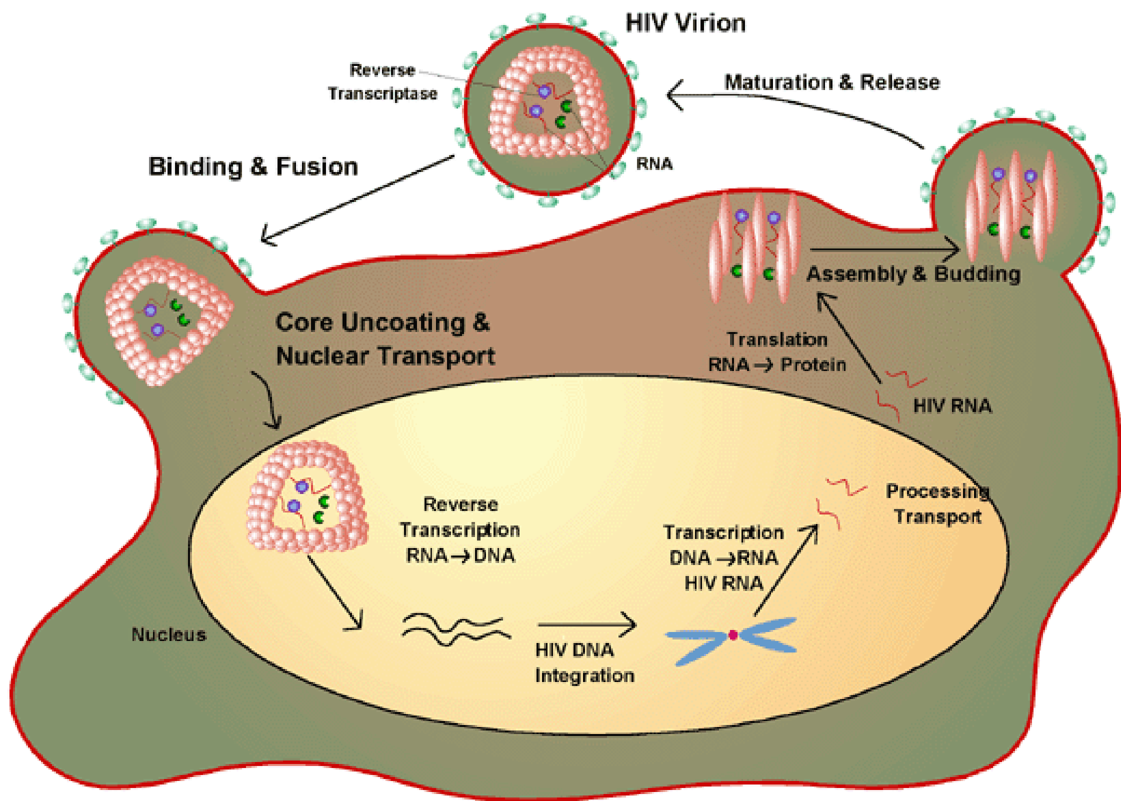


Figure adapted from Boyer R, 2002 [32]

The HIV-1 lifecycle can be divided into stages as depicted in Figure 1-3. These include: 1) Viral attachment to host cells; 2) Fusion of viral and host cell membranes; 3) Viral uncoating; 4) Reverse transcription of viral RNA to DNA; 5) Integration of viral DNA (provirus) into the host cell genome; 6) Transcription of provirus by host cell RNA polymerase II; 7) Translation of RNA transcript into viral proteins; 8) Virion assembly; and 8) Budding and maturation of infectious virus. Each of these stages represents a potential opportunity for intervention. Currently licensed ARV drugs for HIV-1 treatment fall into four main classes. Examples from each class and their mode of action are given in Table 1-2 [9].

A newly produced replication-competent HIV-1 virion can cause at least 3 types of infection event: 1) It may productively infect a target cell, e.g. an activated T Cell, which then rapidly produces a large quantity of virus prior to cell death; 2) It may infect a long-lived cell, e.g. a macrophage, with capacity to produce virions at a slower rate but over a longer duration; 3) It may become integrated as a 'latent' provirus into a non-activated

Table 1-2 Current licensed antiretroviral agents

Drug Class	Target/Mechanism of Action	Examples	Licensed dosage form
Entry inhibitors			
CCR5 inhibitors	Small molecule. Binds to small pocket on CCR5 trans-membrane domain to Inhibit gp120 - CCR5 interaction.	Maraviroc (Celsentri® (ViiV)	Oral
Fusion inhibitors	Bio-mimetic peptide. Inhibits gp41-mediated cell - viral membrane fusion.	Enfuvirtide (T20, Fuzeon®, Roche)	Subcut
Reverse transcriptase inhibitors			
Nucleoside RT inhibitors (NRTIs) and nucleotide RT inhibitors (NtRTIs)	Nucleoside/nucleotide analogues. Act intracellularly as competitive substrate inhibitors. Incorporated into the newly synthesized viral DNA strand as 'faulty' nucleotides causing chain-termination.	Zidovudine (AZT, Retrovir®, ViiV); Lamivudine (3TC, Epivir®, ViiV); Emtricitabine (FTC, Emtriva, Gilead); Abacavir (ABC, Ziagen®, ViiV); Tenofovir (the only NtRTI) (TDF, Viread®, Gilead) <u>Combinations include</u>: Truvada® (TDF and FTC); Kivexa® (ABC and 3TC); Combivir® (AZT and 3TC); Atripla® (Truvada plus Efavirenz); Eviplera® (Truvada plus Rilpivirine)	Oral
Non-nucleoside RT inhibitors (NNRTIs)	Non-competitive inhibitors. Bind to the RT enzyme at a site distant from its active site to inhibit function.	Nevirapine (Viramune®, Boehringer Ingelheim); Efavirenz (Sustiva®, Bristol-Myers-Squibb); Etravirine (Intelence®, Tibotec); Rilpivirine (Edurant®,Tibotec)	Oral
Protease inhibitors (PIs)	Inhibit the HIV-1 protease and block virion assembly.	Ritonavir (Norvir®, Abbott)(used in low dose to boost levels of other PIs); Atazavavir (Reyataz®, Bristol-Myers Squibb); Darunavir (Prezista®, Tibotec); Kaletra® (lopinavir plus ritonavir, Abbott)	Oral
Integrase strand – transfer inhibitors (INSTIs)	Inhibit the 'strand transfer' step of integration, in which the 3' ends of viral DNA are covalently ligated to host cell DNA.	Raltegravir (Isentress®, Merck)	Oral

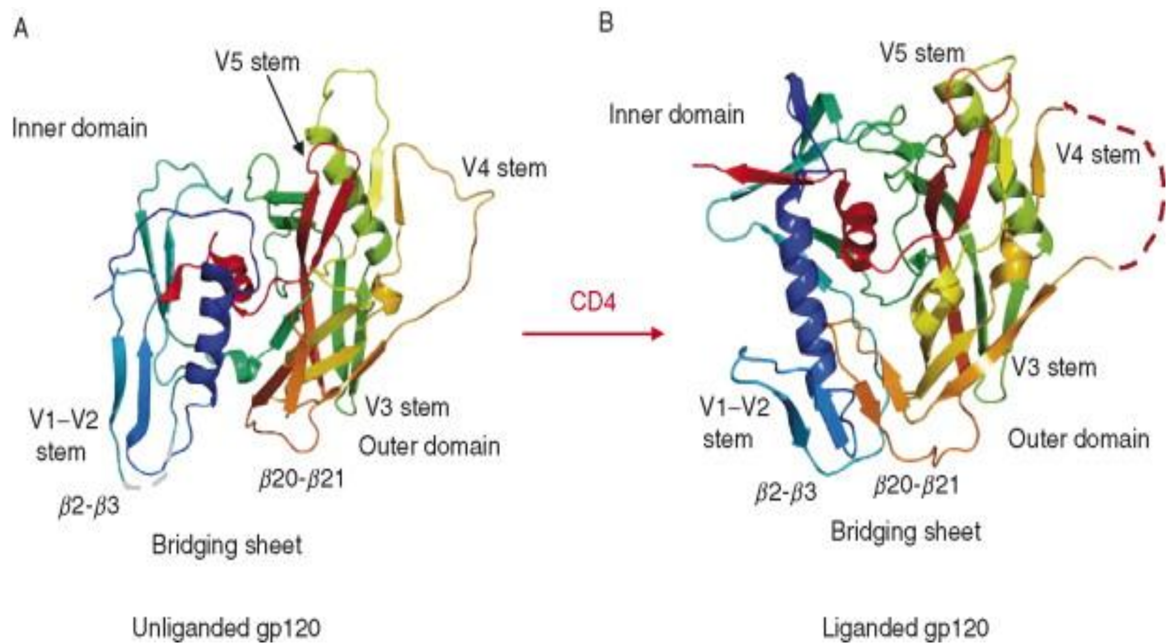
cell, e.g. a memory T Cell, with potential for production of viral progeny on future reactivation. The establishment of latent reservoirs clearly reduces any ability to eradicate infection [33]. Thus, strategies to prevent initiation of HIV-1 infection must primarily act before or during the integration stage.

1.2.2 Structure of HIV-1 envelope glycoproteins

The glycoproteins on the surface of HIV-1 play a key role in virus-cell binding and fusion and determine its cellular tropism. In addition, they are the main targets of the host neutralizing antibody response (see section 1.4.4) [34,35]. The *env* gene encodes a 160 kilodalton (kDa) polyprotein, gp160, which is cleaved intracellularly to produce two smaller proteins, gp 120 and gp41. The heavily glycosylated gp120 is positioned on the surface of the viral particle, whereas the relatively hydrophobic gp41 is a transmembrane protein embedded in the lipid-bilayer of the viral envelope (see Figure 1-1). Each gp120 molecule binds, non-covalently, to one of gp41 and, in their native state, form trimers with two other gp120-41 heterodimers. Cryoelectron tomographic studies suggest that each Env 'spike' consists of a globular 'head' formed from three gp120 molecules on a 'stalk' made up of three, obliquely projecting gp41 proteins, similar to a tripod. As opposed to being evenly spaced, these spikes form clusters and there may be as few as 14±7 spikes per virion [36].

Gp120 contains five regions of high nucleotide and amino acid heterogeneity (designated V1-V5), and five more conserved areas (C1-C5). Crystal structures of gp120 show that it is folded into three major domains (see Figure 1-4). The inner domain, formed mainly by regions C4-C5, is the interface of interaction with gp41. The outer domain, comprised of the variable regions, forms the external portion, most accessible to antibodies, and contains potential sites for glycosylation. Linking these major domains is a bridging sheet [34,35]. Of note, *Env* gp120 gene sequences can vary by > 25 % between different HIV-1 clades. In contrast, being largely hidden within the viral membrane, the entire sequence and structure of gp41 is relatively conserved [34].

Figure 1-4 Schematic Representation of the crystal structure of gp120 both unbound and bound to CD4



A. **Structure of gp120 in its native state (from the perspective of CD4)**
B. **Conformational changes induced in gp120 on binding CD4.**

Figure reproduced from Prabakaran P *et al*. 2007 [34].

1.2.3 HIV-1- target cell tropism and entry

Virus-cell attachment and fusion of viral and cell membranes occurs in a number of stages. Initially, gp120 binds to the receptor CD4; a molecule present on the surface of a number of cell types, including 'helper' T cells, macrophages and dendritic cells (DCs)[37]. The CD4 binding site (CD4bs) is a deeply recessed region on gp120, formed by the interface between the inner and outer domains and the bridging sheet, and is partly shielded by the V1/V2 loops within a long cavity [34].

Binding to CD4 triggers conformational changes in gp120 leading to the formation of a co-receptor binding site (consisting of a four stranded bridging sheet shaped from C4 and the V1/V2 stem and part of the V3 loop) [38]. Gp120/CD4 complex-co-receptor binding causes further conformational rearrangements in Env, resulting in the insertion of the hydrophobic amino-terminus of gp41 (the 'fusion peptide') into the host cell membrane. Gp41 then dissociates from gp120 and undergoes further re-folding, to produce a 6-helix bundle. This results in a complete fusion of cell and viral membranes, in which the fusion

peptide and the transmembrane segment of gp41 lie parallel on a contiguous bilayer [35]. Recent evidence suggests that complete virus-cell fusion does not occur at the cell surface but instead, following CD4 and co-receptor mediated endocytosis, takes place in endosomes [39]. Virion contents are subsequently released into the cytoplasm in close proximity to the nucleus. Although the interaction between a single Env spike and CD4 plus co-receptor may be sufficient to enable HIV to enter cells, this has not been fully determined, and it is possible that the binding of multiple spikes to multiple receptors may be required.

By far the most commonly used co-receptors are CC-chemokine receptor type 5 (CCR5) or CX-chemokine receptor type 4 (CXCR4). Most HIV-1 isolates utilize one of these in preference to the other, determined by the nature and distribution of charged amino acids in the V3 loop. Viruses binding to CCR5 are referred to as being R5 tropic, in contrast, CXCR4 using viruses are X4 tropic [40,41]. These chemokine receptors are expressed by a range of cell types, which, if also expressing CD4, constitute potential target cells for HIV-1. Both CXCR4 and CCR5 are found on activated and memory (but not 'naive') T cells and DCs, whereas 'naive' T cells (those which have not yet been exposed to cognate antigen) express only CXCR4 and macrophages only CCR5 [41,42,43,44]. In general, R5 tropic viruses predominate in early HIV infection, with X4 using isolates emerging with disease progression in some individuals [45,46,47]. However, the exact triggers of the R5 to X4 switch and its relationship to increasing HIV replication and immunosuppression are not fully understood.

1.2.4 Reverse transcription and genetic heterogeneity

Before the HIV-1 RNA genome can be integrated into the host chromosome it must first be reverse-transcribed into double-stranded DNA. Both RNA-dependent and DNA-dependent polymerization steps are catalyzed by RT, with RNA-DNA hybrids cleaved by its RNase H domain [26]. Reverse-transcription and the subsequent copying of the integrated provirus by host RNA polymerase II are both error prone processes, promoting frequent mutations and recombinations in the HIV-1 genome [48]. In the absence of inhibitors, HIV-1 replicates at a high rate and, on average, one mutation will be incorporated into each virion produced [23]. Thus, HIV-1 exists as a mixture of genetically heterogeneous quasi-species, even within a single individual.

1.3 Basic anatomy and physiology of the human female genital tract

The female genital tract (FGT) is primarily designed for the purpose of reproduction and the structure, lining epithelia and secretions of its constituent organs reflect their various roles in this process [49]. The lower FGT is comprised of the vagina and ectocervix whereas the upper tract consists of the endocervix, uterus, oviducts (Fallopian tubes) and ovaries (see Figure 1-5) [49,50]. Hormonal effects on these tissues have important implications both in the evaluation of a topical vaginal product and in influencing susceptibility to HIV transmission and are discussed in sections 1.3.1 and 1.3.2.

The vagina is the part of the FGT into which seminal fluid and spermatozoa are ejaculated during sexual intercourse. It is also the conduit for menstrual blood and for the delivery of the neonate. The human vagina is a fibro-muscular tube, approximately 9cm long, leading from an exterior orifice, the introitus, to the uterine cervix. The axes of the upper and lower vagina differ, giving it a slight S-shape: whilst the lower vagina in relation to a standing woman is vertical and posterior, the upper part (from the pelvic diaphragm to the cervix) lies more horizontal, with the final portion curving towards the hollow of the sacrum (see Figure 1-6) [51]. The relaxed vagina is a collapsed, potential space. However, the mucosal tissue covering its walls forms a series of transverse folds, known as rugae, most prominent in the lower third of the vagina, allowing considerable distension during penile penetration and childbirth [49].

The vaginal mucosa is composed of lamina propria, or stroma, covered by a largely nonkeratinized, pluristratified squamous epithelium. The lamina propria consists of fibrous connective tissue richly supplied by small blood and lymphatic vessels. The vaginal epithelium is about 150 to 200µm thick with 30 to 40 layers of cells. There are 5 different identifiable cell-types, namely the basal (adjacent to the lamina propria), parabasal, intermediate, transitional, and superficial (adjacent to the lumen) layers, reflecting increasing maturity and differentiation [52]. This provides mechanical protection against friction and trauma during sexual intercourse. The vagina is kept moist and lubricated through the presence of transudated fluid from the vaginal epithelium, secretions from Bartholin's and Skene's glands, located near the introitus and mucus and tubal fluids, produced by the epithelium of the upper FGT [49]. The constitution of vaginal fluid is

variable; it consists largely of water (90 – 95%) with smaller amounts of inorganic and organic salts, urea, carbohydrates, glycerol, mucins, fatty acids, albumin, immunoglobulins, enzymes, leukocytes, and epithelial debris [53]. During puberty the vagina becomes colonized by bacteria, the most important of which are Lactobacilli (see section 1.4.1). These are so-named because they produce lactic acid, which acidifies the vaginal secretions.

The cervix is the lowest, narrowest portion of the uterus, forming a cylindrical body at the upper end of the vagina. It can be divided into two parts; ectocervix and endocervix. The ectocervix protrudes into the upper part of the vagina, dividing it into recesses, known as the anterior, posterior and lateral fornices. It is lined with mucosa which is contiguous with and similar to that of the vagina. The opening of the ectocervix, known as the external os, leads to the endocervical canal, which links the vagina with the uterine cavity [49]. The size and shape of the external os and the ectocervix vary widely with age, hormonal state, and whether the woman has delivered a baby vaginally. In women who have not had a vaginal birth the external os appears as a small, circular opening. In women who have had a vaginal birth, the ectocervix appears bulkier and the external os appears wider, more slit-like and gaping [50]. In contrast to the ectocervix, the endocervix is lined with a single layer of columnar epithelium. The junction between the ectocervix and endocervix, known as the transformation zone, varies in location depending on the age and hormonal status of the woman [52]. In the presence of high levels of oestrogen e.g. during puberty, pregnancy or exposure to high dose exogenous sources, the columnar epithelium of the endocervix moves out of the endocervical canal, becoming visible on examination as a red area known as cervical ectopy. Once exposed to the harsh acidic environment in the vagina, it subsequently undergoes metaplasia, transforming to pink squamous epithelium [49].

The uterus is a hollow, pear-shaped structure which opens, at either side, into two Fallopian tubes at its upper end. The uterine epithelium, known as endometrium, is highly hormone responsive, and is shed periodically during menstruation. The Fallopian tubes act as a conduit between the uterus and the ovaries and are the site where oocytes, released by the ovaries, are potentially fertilized by spermatozoa. They possess a ciliated epithelium, which secretes tubal fluid and facilitates the transport of fertilized oocytes to the endometrium where they can undergo implantation [50].

The adult ovaries are oval organs which contain numerous oocytes within follicles at various stages of development. Dormant, primordial oocytes and follicles are present from birth but from puberty are gradually recruited into the maturation process known as folliculogenesis. Folliculogenesis takes around 375 days coinciding with thirteen menstrual cycles. The process begins with the recruitment of primordial follicles, continues through primary, secondary (pre-antral) and tertiary (antral) developmental stages and ends when a mature oocyte is released from the pre-ovulatory follicle in a process called ovulation [49].

Figure 1-5 Diagramatic representation of the human female genital tract

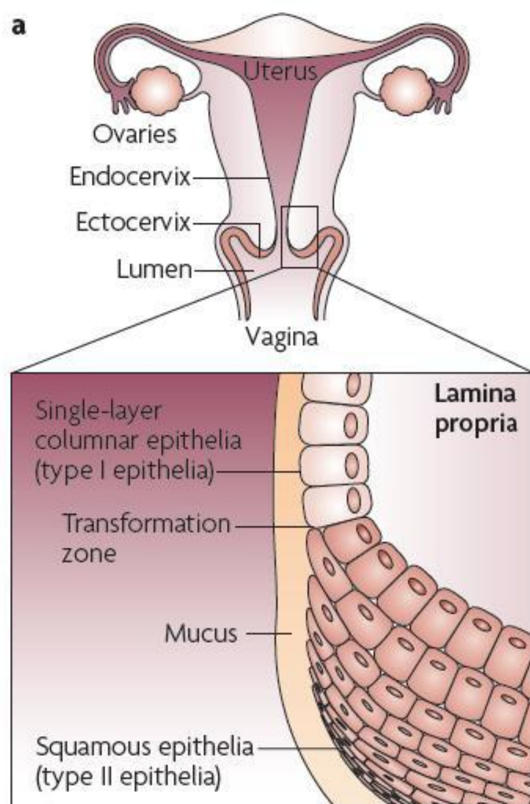


Figure reproduced from Iwasaki A, Nat Rev Immunol 2010 [54].

Figure 1-6 Sagittal view of the human female pelvis

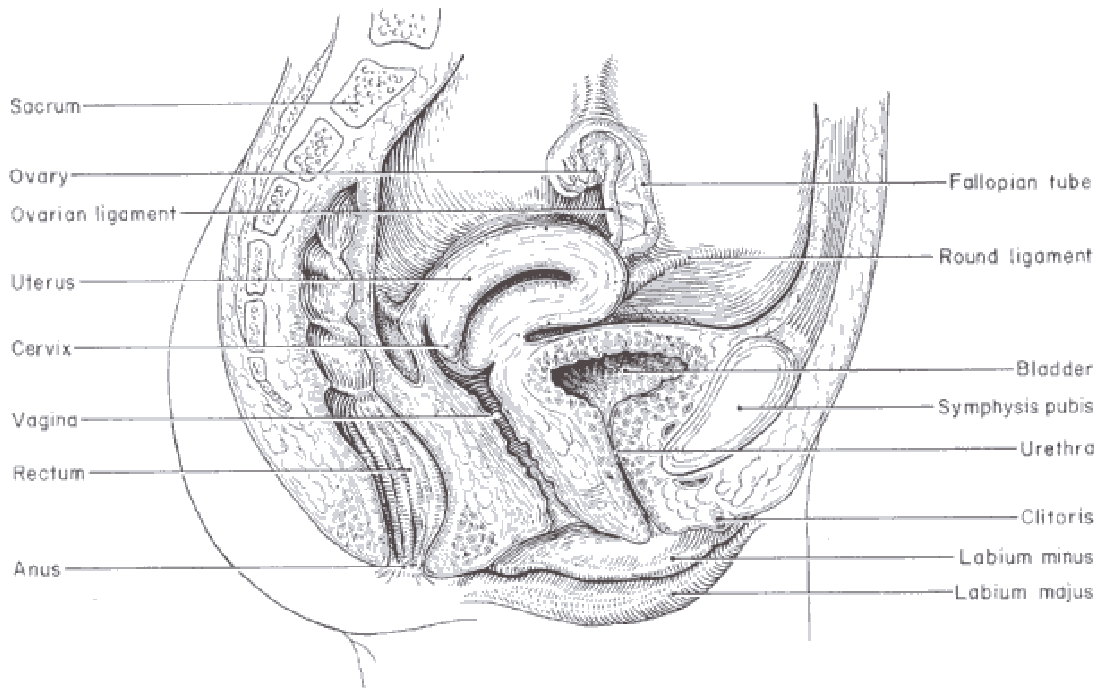


Figure reproduced from http//:www.qldscienceteachers.com. Accessed 4 June 2012.

1.3.1 The human menstrual cycle

Beginning during puberty (at menarche) until the 6th decade (menopause), women undergo a series of cyclical hormonal and physiological changes that are essential for reproduction [49]. Each of these cycles represents the period required for the selection and final maturation of tertiary (antral or Graafian) follicles, the emergence of a single dominant pre-ovulatory follicle, the process of ovulation, and the maintenance of the endometrium, for potential inplantation, by the corpus luteum (derived from the ruptured follicle). The menstrual cycle is separately described for both the ovary and endometrium and is generally divided into pre- and post- ovulatory stages. The first half of the cycle, prior to ovulation, corresponds to the follicular phase of the ovary and menstruation and the proliferative phase in the endometrium. The second half, post-ovulation, reflects the luteal phase of the ovary and the secretory phase in the endometrium. An average menstrual cycle lasts 28 days, beginning with the first day of menstrual bleeding. However the length of a woman's cycle will typically vary, with some shorter and some longer cycles. A cycle length of between 21 and 35 days is considered normal and regular. The menstrual period is variable, but usually lasts between 4 and 6 days. The duration of the follicular phase also varies between and within individual

women, but in a 28 day cycle is around 14 days (including menstrual period). In contrast, the length of the luteal phase tends to be approximately 14 days regardless of cycle length.

The menstrual cycle is under the control of the endocrine system [49]. Hormones participate in a complex system of positive and negative feedback to regulate the final stages of folliculogenesis and prepare the FGT for conception (see Figure 1-7). Gonadotrophin-releasing hormone (GnRH), produced by the hypothalamus, triggers the secretion of the gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the pituitary which, in turn, act on the ovary to stimulate follicle development and, eventually, induce ovulation. Estradiol, an estrogen, is produced in increasing amounts by the developing follicles, with levels peaking at around day 12 of the cycle, 24 to 36 hours before ovulation. Post ovulation, estradiol levels decline and the ruptured follicle becomes a yellow corpus luteum. The corpus luteum secretes high levels of progesterone, a progestagen, and moderate levels of estradiol which prepare the endometrium for implantation of a fertilized oocyte and establishment of pregnancy. In the absence of a fertilized oocyte, the corpus luteum degenerates after approximately 10 days and the consequent fall in progesterone and estradiol concentrations leads to menstruation and the start of a new menstrual cycle.

During menstruation, the vascular endometrium is shed down to a basal layer (the stratum basalis) with resultant blood and tissue lost through the vagina. Subsequently, in response to the increasing estradiol concentrations from the developing follicles, the endometrium undergoes proliferation, developing a layer known as the stratum functionalis containing numerous glandular structures and arterioles [52]. High mid-cycle estradiol also impacts on the endocervix, where crypts produce mucus which is thin and watery, facilitating passage of sperm into the uterus. Post-ovulation, epithelial proliferation stops, but the endometrial glands secrete glycogen and become more tortuous. In addition, under the influence of high progesterone, endocervical mucus becomes viscous and thick, impeding sperm migration [49].

The characteristics of the ectocervical and vaginal mucosae are also influenced by the menstrual cycle, but the changes are more subtle than in the uterus. During the first half of the cycle, estradiol induces the proliferation of the ectocervical-vaginal epithelium, the

thickness of which peaks at approximately mid-cycle. In the secretory phase, progesterone opposes those actions, but, although there is increased sloughing of the superficial epithelial layers during this period, any change in overall epithelial thickness is small [55,56,57]. Estrogens also increase vaginal blood flow, which, together with alterations in epithelial permeability, facilitates the transudation of fluid [49]. Although quite variable between women, average daily production of vaginal fluid is estimated to be around 6 mL, increasing at midcycle and decreasing around menstruation [53].

Figure 1-7 Human female menstrual cycle

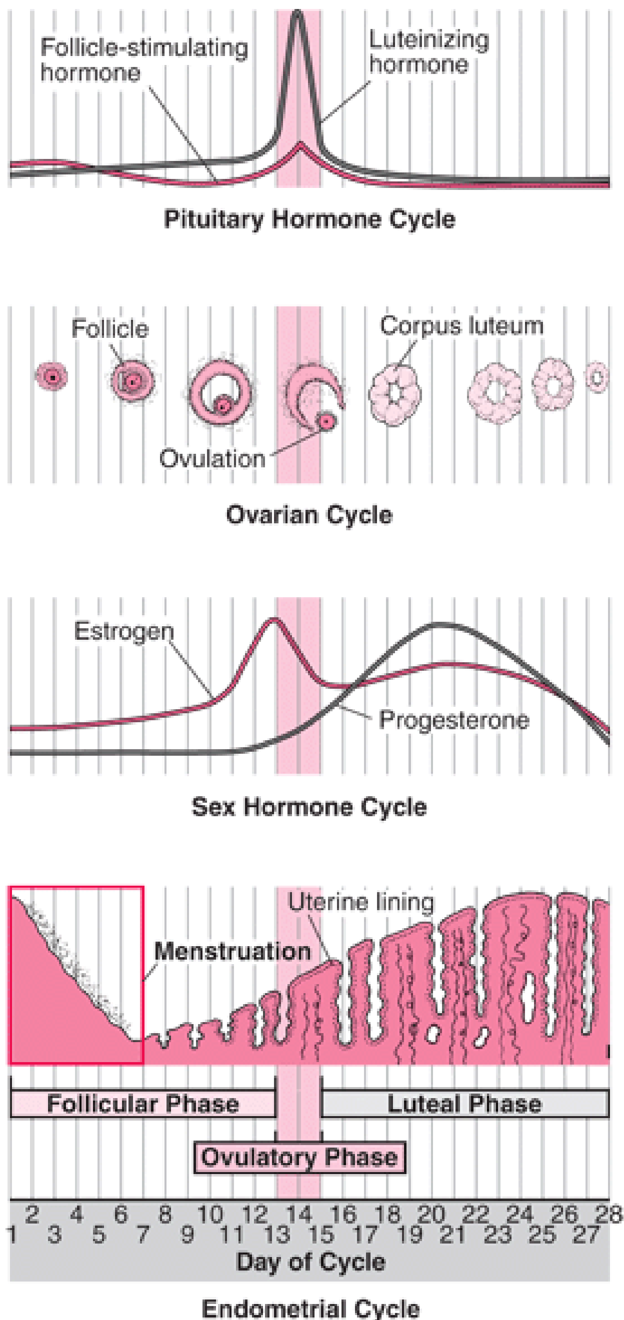


Figure reproduced from http://www.merckmanuals.com/media/ professional/figures/GYN_menstrual_cycle_PE.gif. Accessed 4 June 2012.

1.3.2 Hormonal contraception

Exogenous hormones are currently used as contraception by over 200 million women globally. The most common methods are combined oral contraceptive pills (COCP), with over 100 million users, and intramuscular (im) depot medroxyprogesterone acetate (DMPA, Depo-Provera®, Pfizer), with over 50 million users [58]. Most progestagen-only methods provide a continuous level of systemic progestagen sufficient to suppress ovulation (in most women) through inhibiting the release of GnRH. These include DMPA [59], the levonorgestrel-releasing sub-dermal implant (Implanon®, Nexplanon®, Organon) [60,61] and high dose desogestrol-containing progestagen only pills (Cerazette®, Organon) [62]. The intra-uterine system (IUS, Mirena®, Bayer), which releases a lower-dose of levonorgestrel directly into the uterus, has, in contrast, been shown to inhibit ovulation in only 15 % of cycles and does not significantly reduce the production of endogenous oestradiol in the majority of women [63,64]. Unlike progestagen-only contraceptives, most COCPs are taken as a fixed combination of a progestagen with a low-dose of synthetic oestrogen for 21 days. This is followed by a seven day break during which hormone levels fall to a level which causes endometrial epithelial shedding but maintains contraceptive efficacy. The contraceptive action of COCPs is largely mediated by the relatively high levels of progestagen (which inhibit ovulation), with the low dose oestrogen helping to stabilise the endometrium and prevent break-through bleeding [65,66].

Exogenous progestagens have similar effects to natural progesterone. In addition to any inhibition of ovulation, they exert their contraceptive effects through increasing the viscosity of cervical mucus and by thinning the endometrium: impeding sperm passage and implantation respectively [59]. In contrast to what is seen in macaques (where there is considerable progestagen-induced cervico-vaginal epithelial thinning even during the normal cycle) [67,68], there is no evidence that even the highest doses of progestagens (in DMPA) produce significant thinning of the human cervicovaginal epithelium [69,70,71]. However, the potential for hormonal contraceptives to enhance women's susceptibility to acquiring HIV-1 in other ways remains a controversial and on-going area of research (see section 1.5.1).

1.4 Defenses against HIV-1 infection in the FGT

The mucosal immune system of the FGT is unique as it has evolved to perform conflicting functions. Under the influence of estradiol and progesterone, it manages both to tolerate the passage of allogeneic sperm and the development of a hemi-allogeneic fetus, whilst also providing protection from STIs and other pathogens [72]. Although fully integrated, immune defenses are often broken down into two arms: innate and adaptive. The innate immune system is characterised by cells, mechanisms and molecules that are available immediately or are triggered rapidly to counteract invading pathogens and do not require a prolonged period of induction. In contrast, adaptive immunity takes days to weeks to develop, but provides a more complex and specific response [73]. In the context of HIV transmission, the innate defenses form the first line barrier and are largely responsible for preventing viral acquisition, replication, and dissemination in the FGT [54]. In contrast, adaptive immunity develops too late to inhibit initial infection but plays an important role in controlling subsequent viraemia. However, the creation of immunological memory, following a primary adaptive response to a specific pathogen, enables faster, enhanced responses to secondary encounters with the same pathogen, and forms the rationale behind vaccination [73].

1.4.1 Initial barriers to HIV-1 infection

HIV is present in semen both as free virions and as integrated provirus within infected macrophages and T cells (measured as HIV RNA and DNA respectively). Although both forms have been shown experimentally to be capable of transmitting infection *in-vitro* [74,75,76,77], and in animal models [78,79,80,81], it is currently unclear whether one or both is operative naturally *in-vivo.* In the absence of genital tract inflammation or infection, the quantity of free virus in human seminal fluid usually far exceeds the number of infected cells [82,83], but it has been suggested that much free virus may be replication incompetent [77]. Reliable non-human primate (NHP) models of cell-associated HIV transmission have proven more difficult to establish than those for cell-free virion transmission [80,81], hence the latter has been more widely studied (see section 1.5.2). However, once the epithelium has been breeched and target cells encountered, cell-associated virus may establish and disseminate infection more efficiently than transmitted free virions [75,76,79,80,81].

The initial defense against both cell-free and cell associated HIV-1, once deposited in the vaginal lumen, are FGT secretions. In addition to diluting the viral inoculum, these contain a number of chemical and biological factors, derived from the mucosa and commensal flora, that have broad activity against a range of pathogens. Such factors include mucus, antimicrobial peptides, lactic acid and hydrogen peroxide [84].

Mucus is a complex and dynamic gel: consisting of 90 to 98 % water, small quantities of salts and lipids and variable amounts of mucins (MUC glycoproteins) (2-5%) [85,86]. In the FGT, mucins are primarily produced and exocytosed by goblet cells located in the columnar epithelium of the endocervix [85]. Hydration and expansion then occurs, extracellularly, through contact with transudated fluid [85,86]. Hydrated mucus forms a gel layer against the mucosa which performs important physiological functions. These include lubrication, and physical protection of the epithelium, during sexual intercourse, and regulation of water-balance and sperm transport. It also acts as a mesh, trapping and carrying cell debris and micro-organisms; thus restricting access to the mucosa and the upper FGT [87]. Acidified cervico-vaginal mucus, obtained from healthy women with normal vaginal flora, has been shown to trap HIV-1 virions and slow their diffusion by 1000 fold compared to water [88]. In addition, certain purified mucins, including MUC5B, the most abundant in cervico-vaginal mucus, are capable, *in–vitro*, of preventing HIV infection of $CD4^{+}$ cells [89]. Although not fully understood, charge-dependant interactions between fucosylated glycans on mucin and carbohydrate moieties on gp120 may lead to binding and aggregation of HIV virions; similar to that seen with *Candida albicans* and other mucosal pathogens [88,89,90].

Several broadly-acting antimicrobial peptides are present in FGT luminal secretions, e.g. lactoferrin [91], alpha (α) and beta (β) defensins [92,93], the cathelicidin LL-37 [94], secretory leucocyte protease inhibitor-1 (SLP1) [95], and the serine protease inhibitor trappin-2/elafin [96]. These are expressed constitutively by epithelial cells (ECs) and immune cells and act through multiple, complex mechanisms to directly inactivate virions and/or interfere with HIV-1 binding and replication in target cells [97,98]. As with other FGT mucosal defenses, there is evidence that production of anti-microbials may be influenced by reproductive hormones, with some differences between sites in the upper and lower FGT in keeping with their differing roles [99].

Another component of the first-line defence against mucosal pathogens is the commensal bacterial flora. These organisms are not inhibited by antimicrobial peptides and have co-evolved to enhance mucosal barriers [100]. The healthy vagina is colonized by high concentrations of *Lactobacillus* species (10^8 colony forming units/ml) [101]. Glycogen, produced by the vaginal epithelial cells, provides metabolic fuel for the generation of lactic acid by lactobacilli, which, in turn, reduces the pH of the vagina to less than 4.5. This low pH, together with additional anti-microbial agents produced by lactobacilli, such as hydrogen peroxide, prevents the potentially pathogenic overgrowth of other commensals, such as *Candida albicans* and *Garderella vaginalis* [102,103]. Such acidic conditions have also been shown to inhibit T cell activation and proliferation; thus may reduce the number of potential target cells for HIV [104]. Hydrogen-peroxide producing lactobacilli have been shown to be directly virucidal against HIV-1 *in-vitro* and are themselves being studied as a potential biomicrobicide [105,106,107,108].

In mucosal tissues, the primary physical barrier between the host and the external environment is provided by the epithelium. As described in section 1.3, the entire luminal surface of the FGT is covered by epithelial cells (ECs), with simple, columnar epithelium lining the upper FGT (endocervix, endometrium, Fallopian tubes, ovaries) and stratified squamous epithelium lining the lower FGT (vagina and ectocervix). In most epithelia, the passage of polar molecules and micro-organisms between cells (paracellularly) is restricted by the presence of tight-junctions. These consist of a seal formed by intermembrane extensions of transmembrane proteins, such as occludins, claudins and junctional adhesion molecules (JAMs), across the intercellular space between the apical and basolateral membrane domains of adjacent ECs [109]. Not only do these provide a barrier to pathogen entry but they also polarize the cell, enabling the selective, directional transport of molecules between the mucosal lumen and the submucosa and/or blood stream.

1.4.2 Cytokines and chemokines and pathogen recognition

In addition to antimicrobial peptides, ECs and resident immune cells in the FGT stroma secrete a number of cytokines and chemokines, including: interleukins (IL) 6 and 8, Stromal cell derived factor (SDF)-1 (CX chemokine ligand 12)(CXCL-12)), macrophage inflammatory proteins (MIP) 1α, 1β, and 3α (CC-chemokine ligands (CCL) 3,4 and 20),

macrophage chemotactic protein (MCP)-1 and Regulated on Activation Normal T Cell Expressed and Secreted (RANTES) [84]. These are involved in a number of processes, from mediating endometrial proliferation, menstruation and implantation, to regulating cells of both the innate and adaptive immune systems [84,110]. Some of their actions may be important in protection from HIV. MIP- 1α and β and RANTES are the natural ligands for CCR5, whereas SDF-1 binds the alternative co-receptor CXCR4, thus they may block binding by HIV-1 and prevent it entering target cells. IL-6, IL-8 and MIP-3α promote the influx of type 1-interferon (Type 1 IFN) producing immune cells to the epithelium, the most important of which are plasmacytoid dendritic cells (pDCs), which play a key role in the defense against viruses [73].

Type 1 IFNs are pleiotropic cytokines that mediate antiviral effects through increasing transcription of multiple IFN-stimulated genes (ISGs) [111]. Many ISGs encode 'restriction factors'; proteins that act within infected cells to restrict viral replication. The three most well studied in relation to retroviruses are TRIM5α (tripartite motif 5-α), APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G) and tetherin (BST-2, CD317, or HM1.24) [30].

The secretion of antimicrobial peptides, chemokines and cytokines is upregulated in response to pathogens. This is triggered by the recognition of conserved pathogen-associated molecular patterns (PAMPs), derived from micro-organisms, by the pattern recognition receptors (PRRs) of immune cells [112]. Toll-like receptors (TLRs) are PRRs expressed on the surface and endosomal membranes of a wide variety of cells found in the FGT mucosa: including ECs, macrophages, neutrophils, myeloid (mDCs) and plasmacytoid dendritic cells, natural killer (NK) cells, B cells and T cells [72]. All 9 major TLRs identified in humans are expressed on the FGT epithelia, with vaginal and cervical EC lines expressing TLRs 1-3, 5 and 6, primary endocervical ECs TLRs 1-3 and 6, and primary uterine ECs expressing all TLR types (1-9) [113,114,115,116,117]; thus they can potentially respond to a wide-range of micro-organisms. The primary way HIV and other RNA viruses are recognized is via their nucleic acids. TLR3 and the cytosolic helicases RIG-1 and MDA5, all recognise double stranded RNA (dsRNA), whereas TLR7/8 and RIG-1 sense single stranded RNA (ssRNA) [112]. PAMP recognition initiates a signalling cascade, resulting in the activation of transcription factors, such as NF-κB, and an increase in the production of immune mediators.

1.4.3 Natural Killer Cells

NK cells are key players in the host defense against viruses, acting both as effector and regulatory cells. Through production of cytokines and chemokines (including IFN-γ, IL-12, MCP-1, MIP-1α/β and RANTES), and cell-cell interaction, they both inhibit viral entry into target cells and prime and recruit cells of the innate and adaptive immune system, in particular mDCs, pDCs, Th1 and $CD8^+$ T cells (see section 1.4.4). In addition, they mediate direct cytolysis of infected cells through secretion of granzyme or perforin [118]. NK cells express a variety of different surface receptors (NKRs) which either stimulate or inhibit their activity. An NK cell's response is determined by the balance of inhibitory and stimulatory signals it receives from other cells via its NKRs [119]. Killer cell immunoglobulin-like receptors (KIRs) are a range of NKRs which recognise major histocompatibility complex (MHC) type I on host cells [120]. In humans, MHC molecules are also known as the human-leucocyte antigens (HLA). Expression of MHC I is down-regulated in host cells infected with many viruses, including HIV-1, as a means of evading cytolysis by $CD8^+$ T cells [121]. Detection of 'missing MHC I' on such cells by inhibitory KIRs thus triggers their destruction by NK cells [120] as can recognition of any remaining MHC I (HLA) types by activating KIRs [119]. NK cells are activated in acute HIV-1 infection and can destroy infected cells *in-vitro*. However, efficiency of NK-mediated clearance of HIV-1-infected cells is variable between individuals and is related to the number and types of KIRs expressed on NK cells, specific for different HLA molecules [122]. For example, HLA-C expression is not downregulated by HIV-1 [123]; thus stimulatory NKRs that recognise HLA-C [124] or KIRs which recognise specific HLA-A or B allotypes are more likely to respond to HIV-1 infected cells. Combinations of HLA and KIR allotypes which promote NK activity towards infected cells, e.g. KIR3DS1 (activatory) and HLA-B Bω4-801, KIR3DL1 (inhibitory) and HLA B*57, KIR2DS2 (activatory) and HLA-C1 have been associated with lower VLs and delayed progression to AIDS in HIV-1 infected individuals [124,125,126]. In addition, there is evidence that some allele combinations (e.g. KIR2DL2 (inhibitory) without HLA-C1/C2, KIR3DL1 (inhibitory) and HLA B*57, KIR3DS1 (activatory) and HLA-B Bω4-801) may increase protection from initial HIV-1 infection [126,127,128].

1.4.4 Adaptive immunity

Adaptive immune responses are initiated following interactions between naive $CD4^+$, 'helper' T cells (Th cells) and antigen presenting cells (APCs), such as macrophages and mDCs. Phagocytosis of pathogens induces maturation of APCs and the subsequent presentation of antigen on type II MHC. Cognate antigen is then recognised by Th cells, which undergo stimulation and become activated [73]. Such encounters usually take place within organized lymphoid structures, containing large numbers of T cells, B-cells, and APCs in close proximity. Examples of such secondary lymphoid organs include lymph nodes, spleen and mucosa-associated lymphoid tissue (MALT). However, in contrast to other mucosal sites, such as the the nasal and GI tracts, which have well-defined lymphatic tissue (i.e. the nasal-associated lymphoid tissue (NALT), and gut-associated lymphoid tissue (GALT) respectively), the FGT tract lacks any specialised areas specific for antigen presentation. Instead, most priming encounters are thought to occur in the regional, draining lymph nodes [54]. Uniquely, lymphoid aggregates, consisting of a B cell core surrounded by $CD8^+$ T cells and macrophages, are found within the uterine mucosa. These develop during the menstrual cycle, becoming maximal during the secretory phase [129]. The function of these is poorly understood, but they are believed to be involved in the suppression of cell-mediated immune responses prior to conception and implantation [130].

Different types of adaptive response are elicited according to the nature of the pathogen. Broadly speaking, T-helper type 1 (Th1) responses are directed against intracellular microbes, e.g. viruses and some bacteria; Th2, against extracellular parasites, e.g. helminths; and Th17 against extracellular bacteria and fungi [73]. Unsurprisingly, therefore, adaptive defenses against HIV-1 are primarily Th1 mediated [54]. Th1 cells, characterised by the expression of CCR5 and the secretion of IFN-γ, stimulate largely cell-mediated responses [73]. However, in addition to follicular T-helper cells (Tfh), they also assist B-cells in producing (non-IgE) antibodies (see below).

1.4.4.1 Cytotoxic, $CD8^+$ T cell responses

IFN-γ enhances production of antimicrobial compounds by macrophages, promotes the generation of opsonizing and effector antibodies and activates cytotoxic, $CD8^+$ T cells (CTLs) [73]. The latter induce apoptosis of virus-infected cells expressing peptide-bound

MHC I, through perforin and granzyme-mediated cytolysis. CTLs have been proposed as playing a key role in the initial reduction of acute HIV-1 viraemia and in the maintenance of the VL set-point in chronic infection. Early anti-HIV/SIV CTLs, appearing from around 2 weeks post initial infection, have been temporally linked with the fall in plasma VL [131,132,133] and HLA-alleles, restricted to specific CTL epitopes, have been linked to lower VL set-points and delayed progression to AIDS [134]. In macaques, *in-vivo* depletion of $CD8^+$ T cells has been shown to abolish viral control in both acute and chronic SIV infection [135] and vaccines that induce SIV-specific $CD8^+$ T-cell responses have attenuated later SIV infection [136]. Recently, a comprehensive modelling study, based on serial analysis of both genomic sequences of initial, transmitted viral isolates and T Cell isolates in 4 acutely, HIV-1 infected individuals, has lent support to the importance of CTL responses in reducing initial viraemia, and has provided valuable clarification and insight into the emergence and evolution of viral CTL escape mutants [137]. Despite being rapidly evaded, due to targeting variable regions of Env, most individual initial CTL responses were potent, with each accounting for between 15 and 35 % of the total elimination of virus-infected cells. CTL responses elicited against more conserved HIV-1 components, e.g. Nef, Pol or Gag, where mutations confer a reduction in viral fitness, take at least one, and often several months to emerge and are generally sub-dominant [138, 139,140,141]. Although broader and more chronically effective at controlling viral replication (after initial suppression) than earlier responses, whether these CTLs would be as efficient at controlling initial, high viraemia is unknown [137]. Ongoing studies looking at the impact of early conserved CTL epitope responses in more detail, e.g. in individuals with the favourable HLA B*5701 allotype, will have importance for vaccine design.

1.4.4.2 Antibodies

Antibodies are glycoproteins, belonging to the immunoglobulin (Ig) super-family, which enhance and complement cell-mediated defenses. They are produced by mature B-cells, known as plasma cells, following recognition and internalisation of their cognate antigen (via an Ig receptor molecule expressed on their surface), presentation as peptide-MHC II complexes and stimulation from peptide-MHCII-specific Tfh cells [73]. Each Y-shaped molecule is formed of an identical pair of heavy and light chains, with each chain containing a constant and a variable part (see Figure 1-8). The ‘fragment of antigen binding’ or Fab domain is composed of one constant and one variable domain of each of

both a light and a heavy chain. The variable portions of each Fab contain an area known as the complementarity-determining region (CDR), which determines the antigen specificity of the antibody molecule. The CDR possesses a unique amino acid sequence and structure (known as the idiotope) which binds specific regions (epitopes) of a cognate antigen molecule. In contrast, the Fc (fragment, crystalizable) portion, formed by the constant regions of the heavy chains, binds to Fc receptors (FcR) on immune cells and complement proteins to mediate non-antigen-dependent effector functions [73,142].

Figure 1-8 Typical structure of an antibody

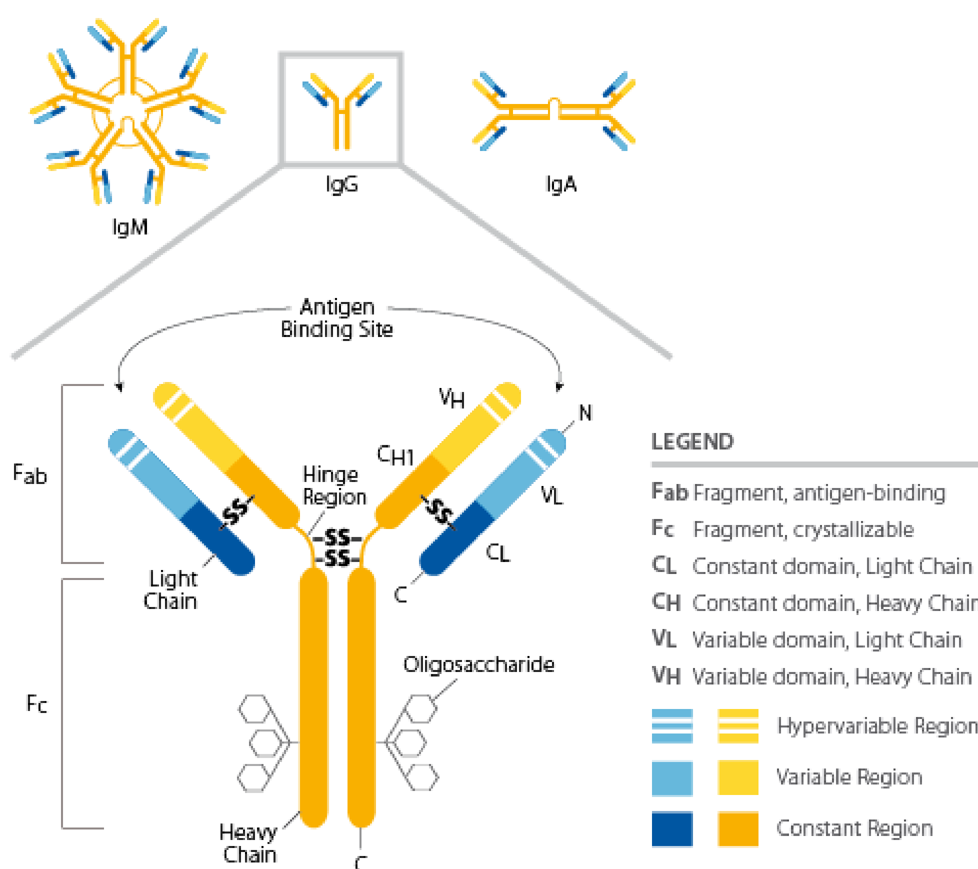


Figure reproduced from http://www.ebioscience.com/knowledgecenter/antigen/immunoglobulin/structure.htm. Accessed 25 May 2012.

In humans, there are 2 types of immunoglobulin light chain (κ and λ) and 5 main types of heavy chain (α, δ, ε, γ, and μ); the constant region of the latter determining the class or isotype of the resulting antibody (IgA, IgD, IgE, IgG and IgM), its molecular mass, oligomerisation, and nature of its Fc effector functions (see Table 1-3). Exposure of the B cell to specific cytokines determines which class or sub-class of antibody is produced with the aim of eliciting effector functions that are appropriate to the antigenic challenge and location [73,142].

Table 1-3 Human immunoglobulin classes and major characteristics

	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE
Heavy chain	γ1	γ2	γ3	γ4	μ	α1	α2	δ	ε
No. of constant domains	3	3	3	3	4	3	3	3	4
Light chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Molecular weight (kDa) (approx)	150	150	170	150	970	160	160	175	190
Susceptibility to proteolysis	++	+/-	++++	+					
Oligimerisation (number of monomers in typical molecule)	1	1	1	1	5	1/2	1/2	1	1
Serum concentration (gL–1) (approx)	7	3	0.5	0.5	1.5	3	0.5	0.05	5×10^{-4}
Half-life (days) (approx)	21	21	7	21	5	6	6	3	3
Placental transfer	+	+	+	+	-	-	-	-	-
Binding to macrophages and other phagocytic cells via									
- Fcγ RI (ADCC, uptake, phagocytosis)	+++	-	+++	+	-	-	-	-	-
- Fcγ RIIa, RIIc (uptake, phagocytosis) **Fcγ RIIb** (inhibitory)	++ +++	+++ ++	+/-	-	-	-	-	-	-
- Fcγ RIIIa (ADCC, uptake, phagocytosis) - **Fcγ RIIIb** (neutrophil/ eosinophil activation)	++	-	++	-	-	-	-	-	-
Binding to basophils and mast cells (histamine granule release)	-	-	-	-	-	-	-	-	+++
Complement activation (classical pathway via C1q)	++	+	+++	-	+++				

References: [73, 142,143,147]

Normally, over 80% of antibodies present in the body are of the IgG class and it is the most prevalent isotype in serum and non-mucosal tissues. In contrast, IgA is the predominant class in most mucosal tissues, including the GI and respiratory tracts [73]. Intriguingly, this is not the case in the FGT, where IgG is most abundant occurring at approximately two to four times the concentration of IgA, and six times that of IgM [144,145].

IgG antibodies are the most important class in terms of anti-HIV-1 immunity and can contribute in several ways [146,147]. Firstly, they can neutralize free virions directly, e.g. through binding to a critical portion of Env to prevent binding to CD4 and infecting target cells; such antibodies are called neutralizing antibodies (NAbs). Secondly, even if non-neutralizing (non-NAbs), they can bind to and coat virions to mediate opsonization and phagocytosis by macrophages, neutrophils and mDCs (via their surface FcγRIIa molecules). Thirdly, they can trigger complement-mediated lysis of free virions and infected cells, following specific antibody-antigen binding and complement activation. Lastly, they can destroy infected cells and/or limit viral spread, through triggering antibody-dependent cellular cytotoxicity (ADCC) or antibody- dependent cell-mediated virus inhibition (ADCVI) [148]. These are mainly effected by NK cells (through binding to FcγRIIIa) and to a lesser extent neutrophils, macrophages and mDCs (via FcγRIIIa and FcγRI) [143,147].

Vigorous antibody responses to HIV-1 are generated at all stages of infection, with antibodies detectable in plasma within a few weeks of acquisition [149]. However, initial antibodies are non-neutralizing: being directed at sites on gp120 and gp41 that are present in monomeric forms (shed from virions/infected cells), but not functional trimeric Env [150]. The Env spike displays a number of features which aid HIV in evading the adaptive immune system (see section 1.2.2). Firstly, the sugar moieties, which cover the most accessible, external, upward facing regions of gp120, are derived from the host cell and are, in general, poorly recognised by antibodies, forming a 'glycan shield'[151]. Secondly, conserved regions are 'protected' from antibodies by conformational masking or steric occlusion, e.g. interfaces are housed within deep or narrow pockets (CD4bs) or shielded by highly variable, flexible loops (gp120/41) [38,152,153]. Thirdly, sparsely distributed clusters of targets are less likely to allow binding of two epitopes

simultaneously than regular, dense arrays, reducing the avidity of bivalent IgG NAbs [36,154] Finally, many regions of gp120, including N-linked glycosylation sites, can vary widely without detriment to CD4/co-receptor binding, hence facilitating the generation of escape mutants when errors are produced by RT [34,35,151].

Antibodies that neutralize transmitted founder isolates are not detected until several months after HIV-1 acquisition [151,155] and it generally takes years before robust neutralizing activity is established [156]. However, even at this stage, the antibody response is continuously overcome by viral mutation and selective pressure, as is reflected by the lack of control of HIV-1 replication by autologous NAbs [155]. That said, 10 to 30 % of chronically-HIV-1 infected individuals have sera which is capable of broadly-neutralizing a range of cross-clade viral isolates [156,157,158,159]. Of these, fewer than 10% are 'elite neutralizers': neutralizing more than one HIV-1 strain within a clade group, across at least four different clades, at serum IC_{50} (median antibody concentration required to inhibit HIV activity by 50%) titres of 1/300 [158]. Such neutralizing capacity can be generated through two separate mechanisms, which can co-exist: the presence of a wide-range of antibodies targeting different HIV-1 epitopes (polyclonal) [160,161], or the production of a single (monoclonal) or limited (oligoclonal) number of potent, broadly NAbs against specific conserved regions of Env [162]. Identifying individuals with the latter responses has enabled the isolation and characterization of specific NAbs with the potential for use in preventative strategies (see sections 1.6.8 and 6.2.6). In addition, this approach has allowed new conserved epitopes to be determined in the Env trimer that could be used as immunogens in future vaccines [163].

Antibodies are likely to have the best chance of successfully neutralizing HIV-1 the earlier in infection they are present [164]. If pre-existing (e.g. due to passive administration or prior vaccination) they would have the potential to enhance innate protective effects and act on small viral inocula near the site of entry to prevent establishment of infection. Evidence that infection is established with a limited number of founder variants lends support to this concept [165,166], as does data correlating *in-vitro* anti-HIV-1 activity with anti-gp160 IgG levels in female genital tract secretions (obtained from healthy HIV-1 infected women)[167].

1.5 HIV-1 transmission via the FGT

1.5.1 Factors influencing efficiency of of HIV-1 transmission

Compared to viruses spread via the respiratory and/or faecal-oral route or others affecting the genital tract mucosa, such as herpes simplex virus (HSV) or human papillomavirus (HPV), sexual transmission of HIV-1 is relatively inefficient [168]. In general, being the receptive rather than the insertive partner carries at least a 2-fold greater risk, with probabilities of transmission varying with the nature of the sexual act. Estimates from meta-analysis of cohort studies of HIV-1 transmission, suggest that transmissions to women from HIV-1 infected male partners, who are not taking ARVs, may occur in fewer than 1 in 1000 episodes of unprotected vaginal intercourse (URVI), particularly in the context of high-income countries and stable relationships, with per-contact risks being up to 10 fold higher in low-income settings [169]. In contrast, the average risk of acquiring HIV following unprotected receptive anal intercourse (URAI) is estimated to be greater, at around 1.4%, with no significant difference in per act risks of URAI for heterosexuals and MSM [170,171]. However, although anal intercourse is practiced by heterosexuals as well as MSM world-wide, and should not be ignored in the development of prevention strategies [172,173,174,175], the extent to which it is contributing to the generalized HIV epidemics of southern Africa is yet to be fully determined [176,177].

Although the above estimates reflect the general inefficiency of HIV-1 transmission across mucosal barriers, and the relatively low rates of acquisition per act from individuals in the chronic, asymptomatic phase of HIV-1 infection, they are not representative of all scenarios. A number of biological co-factors are known to facilitate HIV transmission, by increasing the quantity of infectious virus in donor secretions, impairing mucosal defences and/or increasing target cell availability in the recipient. Those co-factors with greatest relevance to women (and associated increase in relative risk (RR) of HIV transmission (if known)) are summarised in Table 1-4. In the presence of one or more of these, HIV could be transmitted as frequently as once every 10 URVI acts or every 3 URAI acts [169,178].

Primary, or acute HIV infection, (PHI) represents the first 6 months post acquisition of infection and is characterised by very high HIV VLs in plasma and genital tract secretions, the former often being > 1 million RNA copies/ml in the first few weeks, before gradually declining to a chronic set-point [179,180]. Analyses of data from heterosexual HIV-1-serodiscordant couples, obtained, in the pre-HAART era, from a population cohort in Rakai, Uganda, identified donor HIV-1 plasma VL as being the most important determinant in predicting transmission [181,182]. However, onward transmission in PHI is higher than would be predicted based on VL alone and may reflect better adaptation of recently acquired viral isolates to initiate infection [183], and/or the influence of other factors, such as concomitant STIs. Estimates from the Rakai cohort suggest that individuals with PHI are at least 8 to 10 times, and may be up to 26 times more infectious than those in the chronic, asymptomatic phase of the disease [184,185]. The majority of recently infected individuals will experience only a brief, non-specific seroconversion illness and be generally well, and unaware of their HIV status. This has profound public health consequences, the severity of which will be influenced by the phase of the HIV epidemic and rates of partner change within a population. In mathematical models, PHI accounts for a greater proportion of transmissions in early phase epidemics, where a higher proportion of HIV positive individuals are newly infected, and in populations where there are high rates of partner change [186]. That said, in a recent study conducted in Lilongwe, Malawi, which utilized both behavioural and biological data, 38 % of incident HIV cases were attributed to sexual transmissions from partners who had been infected within the previous 5 months [187]. Malawi, like most of sub-Saharan Africa, has a long-standing epidemic, thus these findings may be representative for much of the region.

Further evidence that both host and viral factors play a role in determining likelihood of HIV-1 transmission comes from studying viruses isolated from newly HIV-1 infected individuals. Use of the technique of single- genome amplification (SGA), together with a mathematical model of viral evolution, has revealed that approximately 80 % of heterosexually acquired infections are initiated by a single virion [165,166,188,189, 190,191]. In contrast, this is only true of about 60% of infections in MSM and 40% in IDUs, the latter being associated with as many as 16 transmitted virions [192,193,194]. In heterosexuals, transmission of multiple variants (up to 5) is more likely in the presence of inflammatory STIs [195].

It has long been recognised that the vast majority of founder viruses are R5 tropic [165,166,192,196,197], explaining why individuals who lack CCR5 expression, due to homozygosity for the Δ32 mutation, are virtually resistant to acquiring HIV-1 [198,199]. However, recent research supports selection even within CCR5-using isolates. Comparison of viruses found in newly infected individuals with those in the donor show that, in 90% of cases, the most closely related donor variant comprises < 5% of their total quasispecies [195]. Although there is some evidence emerging of differences in the length or charge distribution of the gp120 variable loops, amino acid changes in the signal peptide and cytoplasmic domains of Env and/or reduced Env glycosylation, clear 'transmission signatures' of founder isolates are yet to be defined [200,201,202]. Elucidating the sites and mechanisms responsible for CCR5 restriction and the viral 'genetic bottle-neck' may provide clues on how to enhance barriers against HIV transmission [203]. In addition, identification of unique genetic or phenotypic characteristics in transmitted viruses could facilitate the development of vaccines by highlighting important targets.

Table 1-4 Biological co-factors which facilitate HIV-1 transmission

Co-factor	RR of HIV-1 acquisition if co-factor present in recipient	RR of HIV-1 transmission if co-factor present in donor	Biological Explanation
Plasma HIV-1 VL	-	RR =2.5-3.0 per log_{10} copies/ml increment (no/very low risk of transmission if VL < 1500 copies/ml)[181,182,184,204].	Risk of transmission proportional to quantity of infectious virus in the inoculum (indirect measure).
Genital tract HIV-1 VL	-	RR = 1.7 per log_{10} copies/ml increment [205].	As above but direct measure.
Acute HIV-1 infection (within 1st 6/12)	-	RR= 8-10 but may be up to 26 [184,185].	Very high plasma and genital VL (> 1 million copies/ml); early CCR5 tropic viral strains potentially better adapted to initiating infection [202,203].
HIV-1 subtype C (accounts for > 50% of infections world-wide).	-	unknown	Predominant CCR5 usage even in late stages, prolonged high-viraemic phase, 3 rather than the usual 1 or 2 binding sites for NF-κB on its LTR [206,207,208].
STIs - HSV-2 - Gonorrhoea - Chlamydia - Candidiasis - TV - BV	RR= 3-7 (highest in acute, symptomatic infection) [209,210,211]. RR=1.5-3 [211,212,213]. RR=1.5-2 [14,211,214,215,216].	Around a 2-3 fold increase in HIV detection in genital tract secretions in presence of STI [217]. RR= 3 in presence of BV [218].	Increased genital tract VL. Possibly increased cellular /blood-blood transmission. (i) Generation of a clinical or sub-clinical pro-inflammatory response through the activation of TLRs; (ii) Increased recruitment of target cells; (iii) Disruption of the cervico-vaginal epithelium and (iv) Perturbation of the normal protective vaginal microflora, pH and/or antimicrobial peptide activity [219,220].
Hormonal status -Adolescence -Menopause -Day 14-23 of cycle - Hormonal contraceptive use -Pregnancy	Unknown but likely ↑ RR= 4-8 [221,222]. Possibly ↑ [99,223]. DMPA RR= 2 ?COCP [15,224] RR= 2 [225,226,227].	Possibly ↑ [15]. RR= 2 [224]. RR=2 [227].	-cervical ectopy, trauma during early coitus -reduced lubrication/increased mucosal friability -Progestagen-dominant environment -controversial:? epithelial thinning, ↓lactobacilli, innate immune effects (↓antimicrobial peptides, ↑ target cells), cell-mediated immune suppression,↑ HIV replication/shedding [15,99,228].
Trauma	Unknown but likely ↑	Unknown but likely ↑	Disruption of the cervico-vaginal epithelium

1.5.2 Early events in HIV-1 transmission and dissemination

Given the practical and ethical difficulties of studying acutely HIV-1 infected people, most of what we know regarding the initial stages of HIV-1 infection and dissemination has been ascertained using Simian Immunodeficiency Virus (SIV) macaque NHP models of vaginal HIV-1 transmission [79,229,230,231]. These have traditionally been high dose vaginal challenge studies in which rhesus macaques (or other susceptible macaque species, e.g. cynomolgus, pig-tailed) are atraumatically exposed once, or twice in the same day, with two 10^5 TCID50 (50% tissue culture infectious dose) inocula containing billions of virions [230,231]. Although convenient in that they provide reliable access to tissues infected *in-vivo*, within a known time-scale, they may only approximate to what happens naturally in women, where HIV-1 infection generally occurs after much lower viral exposures, even from donors with the highest seminal VLs [232]. Recently, low-dose SIV challenge models have been developed, where animals receive repeated physiologically-congruent viral inoculae over several weeks, to initiate infections with only 1 or 2 viral variants [233,234,235]. HIV-1 strains encoding Vif proteins derived from SIV/HIV-2 isolates have also been developed which can establish productive infection in NHPs [236]. In addition, improvements in culture systems for studying intact human cervical or vaginal mucosal tissues (known as explants) have facilitated the study of complex HIV-cellular interactions *ex-vivo* [74,237]. By more closely recapitulating events in natural HIV-1 transmission, it is hoped such models will generate reliable insights and provide a more realistic system for evaluating the pre-clinical efficacy of prevention methodologies in humans [238].

Knowledge to date regarding the initial steps in the establishment of HIV-1 infection via the FGT mucosa is summarised in Figure 1-9. In male-to-female transmission, epithelial penetration and initiation of viral replication has been shown to be possible in the vaginal, ectocervical, endocervical and endometrial mucosa [239,240]. However, it is unclear which sites predominate in natural infection. Given its relatively huge surface area, the ectocervical-vaginal mucosa has been proposed as the likely site for most HIV-1 transmission [15,239,240]. Although its multi-layered, squamous epithelium generally presents a much thicker barrier against trauma and pathogen entry than the columnar epithelium of the upper tract, tight-junctions are restricted to its lower 2/3, in the basal, parabasal and intermediate layers [241]. The epithelium is also penetrated by numerous

stromal papillae, which may be covered by only 1 or 2 layers of cells [242]. While containing fewer HIV-1 target cells than the TZ, in the absence of inflammation [242], the vagina and ectocervix are the regions most commonly affected by coital trauma [243] and are the main sites of several genital infections known to facilitate HIV-1 acquisition, including Herpes Simplex Virus (HSV), bacterial vaginosis (BV), *Trichomonas vaginalis* (TV) and *Candida* species [220]. HIV-1 infection occurs in women with congenitally absent uteri [244] and SIV can be transmitted to macaques following hysterectomy [245]. In addition, contraceptive diaphragms, which shield the cervix and upper FGT but not the vagina, were shown to be ineffective at reducing rates of HIV-1 acquisition in a large randomised trial. That said, it is noted that there was a significantly lower rate of reported condom use in the intervention arm (54% of visits) compared with the control arm (85 %) [246].

Several possible mechanisms may be involved in the translocation of HIV-1 virions across the FGT epithelium and these may vary at different sites. HIV-1 enters the body most easily at sites where the mucosal barrier has been disrupted e.g. where there is an area of microtrauma resulting from sexual intercourse, or ulceration from infection, and it can gain direct access to the submucosa [243,247]. However, free virus could also penetrate between the squamous cells of the superficial layers of the intact vaginal and ectocervical epithelium, before being restricted by the tight-junctions of deeper layers. In addition, the interdigitating dendrites of Langerhan's cells and mDCs can cross several epithelial layers thus may bind HIV virions close to the the vaginal/ectocervical lumen [240,248].

Tight-junctions, together with adjacent, supporting, adherens junctions and desmosomes, are coupled to the cytoskeleton. They can mediate changes in permeability, measured *in-vitro* as transepithelial resistance (TER), in response to cytokines, hormones and other stimuli [249]. Reduction in vaginal and endocervical TER mid-cycle, in response to high levels of estradiol, increases the transudation of fluid into the FGT lumen and helps to reduce the viscosity of ovulatory mucus [250]. Although designed to assist the passage of sperm into the uterus, this may also facilitate pathogen entry, both through the epithelium and into the upper FGT. Interestingly, Nazli *et al.* have shown that direct exposure of ECs to gp120 can cause a 30 to 60 % decrease in TER, which is associated with a reduction in the number of junctional proteins [251].

There is also *in-vitro* evidence that HIV-1 can transcytose ECs without infecting them productively [252,253,254,255,256,257]. Although trancytosis of virions through squamous ECs has been suggested, it most classically occurs *in-vivo* through single-celled, columnar epithelia [258]. Some ECs lining the FGT have been shown to express CD4, CCR5 and CXCR4, which may enable HIV to enter. Although there is little evidence that CD4, CCR5 or CXCR4 are expressed on the superficial and mid-zone layers of the vaginal and ectocervical epithelium that would be most easily accessible, $CD4^+$ $CCR5^+$ ECs are brought closer to the lumen within the stromal papillae [259]. There is some evidence that expression of these receptors may be influenced by hormones. Recognised as being important for implantation, apical expression of CCR5, CXCR4 and CD4 by uterine ECs has been found to be low during the 1st half of the cycle, peak around ovulation and decline (CCR5) or plateau (CD4, CXCR4) thereafter [250]. Similar, but less pronounced, changes in receptor levels have been seen in parabasal and basal ECs in the ectocervix.

In the absence of classical receptors, HIV entry into ECs may be facilitated by cell surface glycosphingolipids, such as sulphated lactosylceramide expressed by vaginal ECs and galactosylceramide expressed by ectocervical ECs, as well as transmembrane heparin sulphate molecules, e.g. syndecans [255,256,260,261]. In addition, interaction with the DMBT (deleted in malignant brain tumours)-1 gene encoded glycoprotein gp340, found in mucosal secretions, has been shown to promote HIV-1 transcytosis in FGT-derived EC lines and primary endocervical epithelium [262,263]. It has also been proposed that through binding to molecules on ECs, e.g. the human mannose receptor, HIV-1 per se may trigger an increase in the production of matrix metalloproteinases and/or inflammatory cytokines, impairing the integrity of the FGT epithelium and facilitating migration of virus and/or target immune cells across the mucosa [264]. The extent to which these mechanisms play a role in HIV transmission *in-vivo* has yet to be determined.

Semen and its constituents have complex effects on the immunologic milleu of the FGT, which facilitate conception and may potentially enhance HIV transmission. Seminal fluid is alkaline (pH 7.2-8.0), so neutralizes the acidic pH of the vagina. It also reduces the activity of SLPI, defensins, and other antimicrobial proteins [265]. Semen contains several cytokines and chemokines, including IL-7, IL-8, SDF-1, MCP-1 [266], enhances the production of further inflammatory cytokines, e.g. IL6 and MCP-1, by the FGT epithelium [267] and promotes the migration of leukocytes, including macrophages and mDCs into

the cervico-vaginal epithelium and endometrial stroma [268]. It may also directly increase HIV infectivity through the action of prostatic acidic phosphatase, which forms amyloid fibrils that capture HIV particles and facilitate their subsequent attachment to target cells [269].

Being sentinels to invading pathogens, mDCs and Langerhans cells are among the first cells to encounter HIV within the FGT epithelium [239]. mDCs possess receptors on their surface known as C-type lectins which recognise polysaccharide moieties on pathogens not commonly found on mammalian cells [270]. Langerhans cells express Langerin (CD207) whereas other mDCs express dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (CD209). Both Langerin and DC-SIGN bind HIV gp120 with high affinity causing it to be taken up into Birbeck granules and endosomes respectively where it is protected from destruction [271]. Research using *ex-vivo* vaginal explant tissue has demonstrated the rapid uptake of HIV-1 virions into both CD4 $^{+}$ T cells and mDCs [272]. However, whereas HIV enters T cells via CD4 and CCR5 mediated binding and fusion, leading to productive infection, virions remain intact within DC endosomes for up to 3 days, inducing maturation and migration to the deeper submucosa or regional lymph nodes [272,273]. It thus appears that, in addition to directly infecting available target cells, HIV may be initially captured by mucosal mDCs, and harboured as infectious virus for later transfer to CD4^{+} T cells.

Cell-cell propagation, or trans-infection, occurs via a cytoskeletal-based virological synapse (VS), which, in the case of mDC to CD4^{+} T cell transmission, is enhanced by DC-SIGN mediated signalling [274,275,276,277]. Within an hour of HIV binding DC-SIGN, and undergoing endocytosis, extensions form over the entire mDC cell membrane [277]. Subsequently, HIV is transferred to microvillar extensions from T cells which make contact with virions beneath the mDC cell surface [278]. Such transfer is thought to be dependent on HIV-CD4/co-receptor interactions [274,279]. Similar cell-cell trans-infection also takes place between CD4 $^{+}$T cells, and has been shown to be around 10 times more efficient than cell-free viral spread [279].

Increasing evidence points to the interactions of HIV-1 per se with the inate immune system as being critical to tipping the balance between aborting and promoting infection. An example is its subversion of the type 1 IFN response. RNA viruses are taken up by

pDCs into endosomes, where they are recognised by TLR7 and trigger type 1 IFN production. However, HIV-1 is abberantly trafficked to early endosomes resulting in induction of a partially matured pDC phenotype, which secretes IFN-1, MIP1α and 1β and other chemotactic factors persistently [280]. This amplifies the pool of locally available $CD4^+$ T cells, facilitates cell-cell spread and enables the rapid expansion of the 'founder population' necessary for dissemination to lymph nodes and secondary lymphoid organs to generate a systemic infection [281].

Over 90% of detectable productively infected cells in the initial and locally expanding foci in the FGT stroma are $CD4^+$ T cells [282,283,284]. Yet, rather than being activated, they are predominately of a 'resting' or 'memory' phenotype, with residual rather than high levels of CCR5 expression. Recent research suggests that a subset of mucosal memory $CD4^+CCR5^+$ Th17 cells, expressing the chemokine receptors CCR6 and CXCR3 with and without integrin α4β7, may be particularly permissive to HIV-1 infection and replication [285,286,287,288] and that Env glycans may play a role in determining specificity of initial target cells and selective transmission [289]. α4β7, CXCR3 and CCR6 are homing receptors, controlling cell migration into the GALT. Preferential infection of these cells may explain how HIV-1 disseminates from the site of entry to the GI tract, and why the latter becomes an early focus for HIV-1 replication and $CD4^+$ T cell depletion [283].

The 'eclipse' period, before widespread viral dissemination takes place, ranges from 4 days to several weeks, and is lengthened by reducing viral exposures [234]. Typically, in humans, and following low dose SIV challenge, HIV/SIV RNA becomes detectable in plasma (at concentrations > 50 copies/ml) from around 7 to 10 days post infection with levels peaking around the end of the second week [229,234,290]. However, although the adaptive immune system begins to control replication by 1 month post acquisition, reflected in a gradual reduction in plasma and tissue VL, this is too weak and too late to eradicate infection [229,137]. By this stage, a large reservoir of virus has already been established in lymphatic tissues, with ongoing replication and $CD4^+$ T cell depletion. In addition, a potential pool of proviruses have become integrated into the DNA of resting T cells [291,292].

It is clear that there is just a brief window of opportunity to block the establishment of HIV-1 infection. To be successful, prevention measures must act at the time, or within a

few days, of HIV-1 exposure, to prevent the infection and expansion of founder populations [229]. Recent research efforts have focused on improving understanding of these initial key steps, to elucidate vulnerabilities in host defenses that may be exploited by HIV, and identify new potential strategies for prevention.

Figure 1-9 Initial steps in the establishment of HIV-1 infection in the FGT mucosa

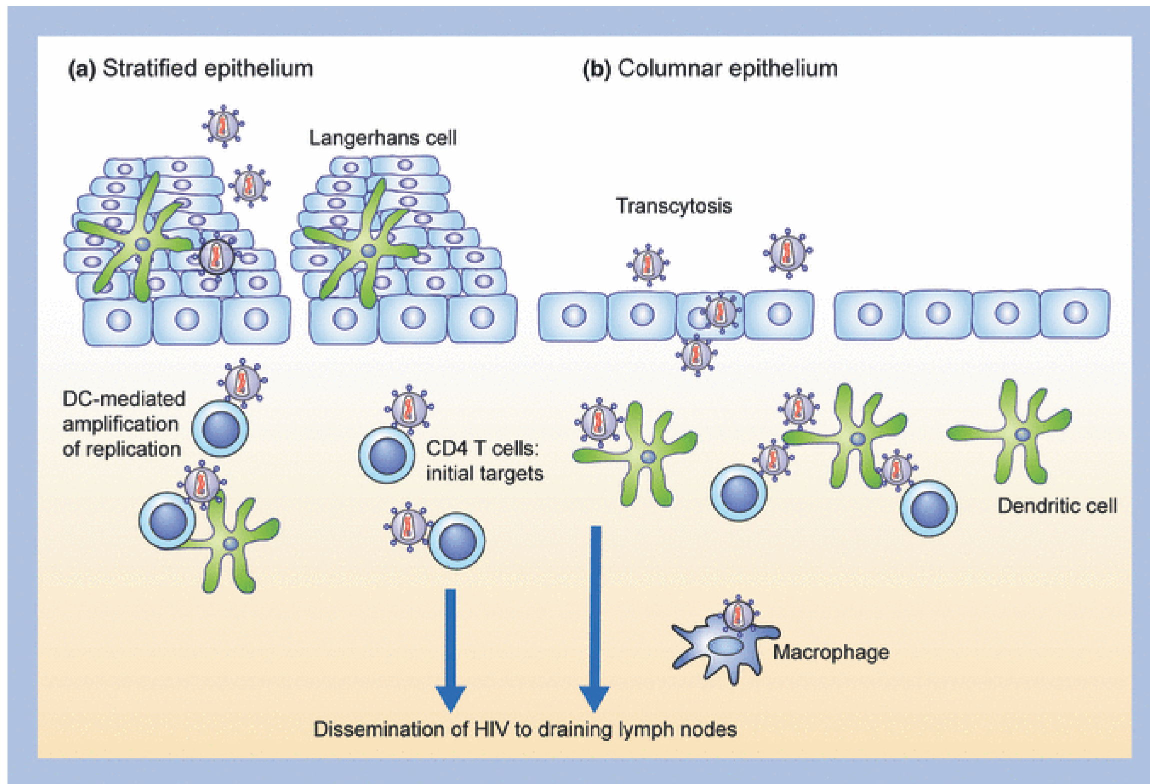


Figure reproduced from Kelly CG and Shattock RJ, J Intern Med 2011 [293].

1.6 Strategies for Preventing Sexual Transmission of HIV-1

At a population level, sexual transmission of HIV-1, like that of other STIs, can be described using the formula: $R_o = ßcD + ßc\Gamma$

Where: R_o = the basic reproductive rate and will determine the risk of spread of new infectious cases; ß represents the efficiency of transmission, c the number of new sexual partners per unit time, D the duration of infectiousness of the index case and Γ the average partnership duration [294]. If $R_o > 1$ new, secondary cases of HIV will occur, and the epidemic will grow. Conversely, any strategy that reduces Ro to ≤ 1 will lead to a slowing of epidemic spread. As can be seen from the formula, reducing any of the 4 factors ß, c, D, or Γ will decrease the spread of HIV, however, minimising ß and/or c will potentially have the greatest impact.

Transmission probability per partnership ($ß_p$) is related to the transmission probability per act ($ß_a$) and the number of unprotected acts (N_a): $ß_p = 1-(1- ß_a)^{Na}$ [294].

A number of different approaches are being taken to reduce sexual transmission of HIV-1 (see Table 1-5) [17,295]. The aim of biomedical prevention methods is largely to reduce the efficiency of HIV transmission and, in the case of identifying and providing ARVS to HIV seropositive individuals, the duration of infectiousness. In contrast, behavioural interventions are mostly concerned with reducing numbers of sexual partners and, in particular, concurrency (multiple, overlapping sexual partners) and age-disparate/ transactional sex, which have been proposed to be important in influencing the size and speed of growth of an HIV epidemic within a population [296,297,298]. Although important, and likely to be utilized in combination with successful biomedical strategies, behavioural interventions and those of a structural nature, e.g. implementing changes to the legal or economic framework and/or healthcare infrastructure of societies, are beyond the scope of this thesis but have been reviewed elsewhere [17,299,300].

Table 1-5 Strategies for preventing sexual transmission of HIV-1

Uninfected individuals to reduce own susceptibility		
Time window for initiating activity	**Behavioural**	**Biomedical**
Pre-exposure, lasts months/years	Delay coitarche	Prophylactic Vaccine (acquired immunity)
	Reduce number/ concurrency of partners	Medical male circumcision
	Reduce inter-generational partnerships	Treatment of STIs
		Long-acting/slow-release ARV technologies e.g. Intravaginal rings (IVRs), intramuscular depots.
Prior to and during coitus	Correct and consistent use of condoms	Oral ARVs (PrEP)
		Topical ARVs and/or other microbicides
Post coitus		Oral ARVs (PEP, ?PrEP)
		? Topical ARVs and/or other microbicides
HIV-1 infected individuals to reduce onward transmission		
Time window for activity	**Behavioural**	**Biomedical**
Years/lifelong	Knowledge of HIV positive serostatus	HAART
Years/lifelong	Reduce number/ concurrency of partners	Therapeutic vaccination
Prior to and during coitus	Correct and consistent use of condoms	
Structural Interventions		
Economic	**Legal (Laws and Policies)**	**Healthcare Infrastructural**
Conditional cash transfers	Protecting sex workers, tackling gender-based inequality and violence	Improved access to HIV screening, prevention and treatment

1.6.1 Treatment of HIV infected individuals to prevent transmission

Given its proven ability to substantially reduce plasma HIV viral load (VL) [9], and the correlation between VL and risk of sexual and vertical HIV transmission [181,182,184,204,301,302], great emphasis is currently being placed on using HAART as a prevention tool. The provision of ARVs to HIV infected pregnant women and their neonates has reduced mother to child transmission rates from around 20 % without intervention to below 2 % [303,304]. Likewise, observational studies in both developing and Western countries have demonstrated that initiation of HAART by HIV infected individuals within sero-discordant heterosexual relationships can dramatically reduce the incidence of HIV infection [305,306,307,308]. In a cohort analysis of a recent randomised controlled trial (RCT) involving 3381 HIV-1 and HSV-2 sero-discordant heterosexual couples, starting ARVs reduced the risk of HIV transmission from infected individuals to uninfected partners by 92 % [309]. A phase III RCT (HPTN 052) was established (at sites in 9 countries; 5 in Africa, Brazil, India, Thailand and USA) specifically to evaluate the benefits, with regards to reducing heterosexual HIV transmission, of earlier HAART initiation at CD4 counts between 350 and 550 compared with at CD4 counts < 250 or AIDS-related illness. This has recently been halted prematurely as it was clear there was a 96 % reduction in phylogenetically linked transmissions between sero-discordant couples if the HIV positive partner was taking ARVs [310]. Although, it remains to be determined how well these benefits will translate outside of a trial setting, where adherence counselling, condoms and STI treatment were also provided on a regular basis, the use of ART as ‘couples prevention’ could have a significant public health impact; in some regions up to 50% of HIV transmissions occur within HIV discordant relationships lasting more than 1 year [311,312,313].

Such is the faith in the power of suppressive HAART to prevent onward transmission that, the Swiss Federal AIDS Commission issued a declaration stating “if an HIV positive (heterosexual) individual has had a VL < 40 copies/ml for at least 6 months, remains adherent to HAART, free of sexually transmitted infections and under regular follow up by their HIV physician, then they should be considered to be ‘not sexually infectious’” [314]. However, this has proven to be controversial. Although it is acknowledged that there is evidence in heterosexuals for a significantly reduced risk of onward HIV transmission if an individual maintains an undetectable plasma VL on HAART, there is as yet no conclusive

evidence to prove that it eliminates the risk of transmission. Of some concern is the fact that it has been shown that whilst there is a correlation between HIV VL measured in plasma and that found in cervico-vaginal secretions and semen, it does not always follow that an individual with an undetectable plasma VL will also have an undetectable VL in their genital secretions. Numerous studies have reported discordance between blood and seminal VLs, with between 5 and 48 % of men with fully suppressed blood plasma HIV VLs continuing to have detectable levels in seminal plasma, even in the absence of STIs [315,316,317,318]. Such shedding can occur intermittently; thus a single undetectable measurement of genital tract HIV VL does not preclude transmission from that individual. In HIV infected women, discrepancies in VL between the genital and systemic compartments may be even more common than in men, with cervico-vaginal shedding being influenced by additional factors such as reproductive hormones and the vaginal flora [319]. Degree of inhibition of HIV-1 replication in the genital tract could potentially be influenced by choice of oral ARVs used, with protease inhibitors generally penetrating the genital tract less well than other classes of agent [320]. Recovery of different viral phenotypes and genotypes from blood and genital tract secretions, and determination of localised replication within the genital tract, suggest that differential compartment alisation of HIV-1 infection can occur [321,322]. Thus, poor genital tract penetration by certain agents could give rise to distinct drug-resistant HIV-1 variants from those found in plasma, with potential for onward transmission. As yet, no study has conclusively shown that ARV drug regimens which contain several agents which concentrate in the genital tract are any more likely to maintain undetectable genital VLs than regimens that penetrate poorly. That said, most research to date has looked at HAART combinations including at least 1 drug which is likely to penetrate the genital tract well [320]. Genital penetration of specific agents may be of greater importance if used individually as HIV treatment or as pre-exposure prophylaxis (PrEP) by HIV-1 uninfected individuals (see section 1.6.2).

Another factor which needs to be taken into account when prescribing ARVs is the balance of risk to benefit for the individual. There is now clear evidence from cohort analyses and randomized controlled trials that starting HAART in asymptomatic HIV positive individuals before their CD4 count falls below 350 offers a benefit in terms of reducing morbidity and mortality from AIDS and non AIDS conditions [323,324,325,326,327]. Significant reductions in morbidity have also been demonstrated

through starting ARVs at CD4 counts between 350 and 500, although there is no clear evidence of reductions in mortality [310,325,328,329,330]. There is currently conflicting evidence from cohorts regarding the benefits:risks to individuals of starting at higher CD4 counts [325,327,328,329]. A randomized controlled trial (START) has been set up to establish differences in outcome between initiating treatment at CD4 counts of 500 or above compared with at 350 [331]. Even if it is shown that there is an undisputed advantage to initiating HAART in all asymptomatic HIV-1 infected individuals, there will undoubtedly be a significant sub-set of HIV-1 infected people who will wish to delay taking ARVs until they are absolutely essential for their immediate health. Many ARVs are associated with significant side effects which can greatly diminish an individual's quality of life [9]. There is also the potential for long term toxicities and sequelae which, in view of the relatively short period of clinical experience of using some agents, are as yet not fully determined. In addition, there is the burden of needing to take treatment for life: as interruptions following initiation are detrimental to the individual [330] and may increase the risk of onward transmission. Worryingly, due to the rapid, error prone replicative ability of HIV, poor adherence can quickly result in the development of ARV resistance mutations, which render drugs less effective at controlling VL and can be transmitted to subsequent individuals [23]. Thus, people on HAART require frequent monitoring and follow up by healthcare professionals.

In addition to using HAART to prevent transmissions between individual couples, modelers have attempted to estimate the potential impact on populations of establishing universal annual HIV testing programmes coupled with immediate initiation of HAART in those found to be HIV infected:- the 'Universal Test and Treat' approach. Although receiving criticism for some of their assumptions (for example that all transmission of HIV is heterosexual and treatment coverage quickly reaches 90% with few drop-outs), Granich *et al*. predicted that in generalized HIV epidemics such as that in South Africa it would be possible to reduce HIV incidence to fewer than 1 case per thousand per year within 10 years [332]. However, despite the potential for significant long-term financial and health benefits [333], there will be enormous economic, organizational and infrastructural challenges in trying to implement increased testing and ARV provision in the low and middle-income countries where it is needed most, particularly given that only 54 % of individuals requiring HAART for their immediate health are currently receiving them [4]. PopART (HPTN 071), a three-arm, cluster randomised trial of 1) a combined intervention

(community-wide house-to-house voluntary testing for HIV, the offer of medical circumcision to men who test HIV-negative and immediate initiation of ARVs for all individuals testing HIV-positive) compared with; 2) an intervention where ARV initiation is restricted to those meeting current national guidelines; and 3) standard of care, has recently been initiated in 24 communities in Zambia and South Africa [334]. Final results are expected in 2017.

Even if shown to be feasible and effective, through the results of PopART and other studies, there are likely to be considerable delays to the widespead roll-out of ‘Test and Treat’ programmes in low and middle income countries and ongoing restrictions on the availability of ARVs. Thus, many of the poorest, most vulnerable people will continue to remain unprotected. As is currently evident in some resource-rich regions, where policies for targeted, routine HIV testing and early ART initiation are already being implemented, e.g. Australia and San Francisco, USA, a significant proportion of HIV infected individuals continue to remain undiagnosed or decline testing or HAART [335,336]. These include individuals with acute HIV infection who are at highest risk of transmitting the virus [185,186]. Thus, even if the average per individual infectiousness is reduced, there are still likely to be significant numbers of new HIV-1 infections occurring at the population level each year [337,338]. Although there are differences between these relatively concentrated, largely MSM focused epidemics, and more generalized heterosexual ones, such ‘natural experiments’ have highlighted a number of logistical barriers which may limit the effectiveness of the ‘Test and Treat’ approach on a population level [335]. Despite the undeniable potential of HAART for prevention, it seems unlikely that it will be possible to simply ‘treat ourselves out of the epidemic’ [339,340]. Thus, there remains a need to develop additional, complementary HIV prevention strategies.

1.6.2 Interventions to reduce individuals’ susceptibility to HIV

Thus far, the majority of HIV prevention interventions have been aimed at reducing susceptibility in people at risk of acquiring infection. The ideal scenario would be the development of a cheap, easy to deliver intervention capable of providing long-lasting, high-level protection to both men and women following a single application. To this end, a prophylactic vaccine, providing sterilizing immunity against all routes of transmission, remains the ultimate goal. However, development of an effective prophylactic HIV

vaccine has presented an enormous challenge, not least because of the huge diversity of HIV isolates and the difficulty in eliciting broadly cross-neutralizing antibodies [341, 342,343, 344,345]. A shift in focus to the generation of $CD8^+$ T Cell responses, with the aim of attenuating HIV replication in those who become infected and reduce onward transmission, has also been met with major disappointments [346,347,348]. Although the results of the RV144 trial (of a replication-defective canarypox vector (ALVAC) 'prime' containing an AE_01 recombinant gp120 membrane anchored insert followed by a clade B/E recombinant gp120 protein (AIDSVAX B/E) 'boost') in 16,402 heterosexuals in Thailand, are encouraging, the efficacy shown was modest (31 %) with apparent greatest protection in those at lowest baseline risk [349]. The characteristics of protection, with a trend to decreased efficacy after 6 months and the lack of any reduction in HIV VL in those vaccinees who became infected, were suggestive of a short-lived, antibody-mediated response [343]. Plasma IgG antibodies against gp120 V1/V2 have subsequenctly been found to correlate significantly with the HIV-1 infection rate in RV144 vaccine recipients. Presence of these antibodies conferred a 43% reduction in HIV-1 incidence. Individuals with high titres had a 71% lower rate of HIV-1 acquisition than those with medium- or low-level responses [350]. However, although able to bind some vaccine-specific HIV-1 subtype E epitopes, these antibodies are incapable of neutralizing most primary isolates to any meaningful degree [351]. Since no mucosal samples were collected in this trial, it is unclear what responses were elicited in the genital tract.

Despite recent insights and a more rational approach to immunogen design we are still likely many years away from developing a vaccine able to induce strong and sustained immune responses capable of preventing infection with a diverse range of viral subtypes [163,352]. Thus, other approaches have been taken to protect individuals from HIV. Currently, the most effective, one-off, HIV prevention intervention is medical male circumcision (MMC), which was demonstrated in three randomised trials to reduce HIV incidence among African heterosexual men by around 60 % [353,354,355]. The benefits of this procedure have been shown to be long-lasting and large-scale implementation programmes are already underway in some countries [356]. However, any benefit of MMC in decreasing women's risk of HIV acquisition is indirect and is dependent on their male partners having undergone the procedure [357]. In addition, circumcision does not reduce HIV transmission from men who become HIV positive to their uninfected female partners [358].

The strong association between bacterial and viral STIs and both the transmission and acquisition of HIV-1 have led to calls for STI treatment to be an essential part of HIV prevention strategies. Enhanced syndromic treatment of curable STIs achieved a reduction in HIV incidence of almost 40% in a large community study in Mwanza, Tanzania in the early 1990s [359]. However, such an impact has failed to be reproduced in subsequent trials, conducted in regions with more generalized epidemics [360,361,362]. Identified as a key driver of some HIV epidemics, HSV-2 has been particularly targeted for intervention. Unfortunately, in several large RCTs in discordant couples, providing acyclovir for people with HSV-2 infection did not protect them from acquiring HIV and likewise treating HSV in those with HIV-1/HSV-2 dual infection failed to prevent onward HIV transmission [363,364,365]. There has been much speculation as to why these disappointing findings arose. Significant reductions in genital ulcers [363,365], and plasma HIV-1 VL (through a direct effect of acyclovir on HIV-1 RT) [365] were achieved in some studies suggesting that adherence was good. However, it has been suggested that the dose of acyclovir used in these trials (400mg bd) was inadequate and that 800mg bd or valacyclovir 500mg bd may have achieved greater HSV2 +/- HIV-1 viral suppression [366]. Interestingly, increased populations of both 'resting' and 'active' T cells are detectable in the genital mucosa of HSV-2 infected individuals even in the absence of visible inflammation or ulceration [367,368]. Thus, subtle changes may persist which enhance HIV susceptibility even when HSV is well-controlled.

Correct and consistent use of penile latex condoms has been shown, in observational studies, to reduce the probability of HIV-1 acquisition by heterosexual men and women by around 80% whilst also providing variable degrees of protection against other STIs [369,370]. In addition, although none have yet been demonstrated to reduce HIV-1 incidence, a number of behavioural interventions, in HIV positives, MSM, adolescents and women, have been found, in meta-analyses, to significantly decrease HIV transmission risk behaviours [371,372,373,374]. However, even if they wish to use them, approaches such as 'ABC' (*A*bstinence, *B*e faithful, use *C*ondoms) are outside of the control of many women in resource-poor settings, who lack power and influence over their partners' behaviour [375].

At present, the only HIV prevention method that is ostensibly marketed as being under the complete control of women is the female condom. Although there is some evidence

to suggest that these are effective at preventing STIs, including HIV, they are not widely used, having been subject to issues with cost, acceptability and lack of availability [376,377,378]. They also invariably require some co-operation from men, and can be noisy and difficult to use. Although a new, lower cost version (the FC2) was approved by the US Food and Drug Administration (FDA) in 2009, it is still 10 times more expensive than the male condom making provision difficult in the developing world [379]. In general, there is little robust evidence of the effectiveness of initiatives to promote use of male or female condoms [380] and neither are a viable option for women who wish to become pregnant.

1.6.3 Potential Role of Microbicides in HIV prevention

Microbicides are topical agents that are designed to be applied by the receptive partner to protect against sexual transmission of HIV. They are particularly seen as having potential as a key, female-controlled defence against HIV acquisition [381,382]. It is envisaged that microbicides could provide women with a simple way to protect themselves, independent of whether their partner agrees to use condoms, undergoes circumcision, is HIV tested or initiates HAART. They could also be used in combination with these other strategies to increase protection. Although primarily intended for vaginal use by women living in areas with high HIV prevalence, it is increasingly recognized that products should also be developed for use by women and MSM for anal intercourse [383,384]. Although a dual-use product would offer greatest convenience, given the marked differences in the mucosal environments of the vagina and rectum, specific, separate formulations may be required for each compartment [385,386,387].

A microbicide is designed to be used in advance of sexual intercourse. Topical application should, in theory, enable high, protective doses of an effective agent to be administered directly to the site of action and have a lower propensity for systemic toxicity and inadequate genital tract compartmentalization than oral dosing. However, these advantages are balanced by a higher potential for local adverse effects, which may impact on womens' willingness to use the product, or even enhance the risk of HIV infection.

An ideal product would be well tolerated, effective against multiple HIV subtypes, inexpensive to produce for use in developing countries, stable to storage in the absence of a cold chain, provide long-duration protection for use independent of time of coitus, be

durable in the presence of vaginal secretions and semen and, not least, be acceptable to potential users [388,389].

1.6.4 Microbicide Development

Like other biomedical interventions, potential microbicides are identified and evaluated pre-clinically, *in-vitro* and in animal models, before proceeding to trials in humans. At present, neither the FDA nor any other regulatory authorities, including the UK Medicines and Healthcare products Regulatory Agency (MHRA), have issued specific guidance for the development of topical anti-HIV microbicides; however, detailed and extensive recommendations for pre-clinical assessment were devised by the International Working Group for Microbicides (IWGM) in 2004 [390]. Essential laboratory mile-stones, adapted from FDA requirements for systemic antimicrobials, include definition of the mechanism of action of the active pharmaceutical ingredient (API), efficacy and toxicity testing in cells or explants relevant to the site of transmission, range-of action testing against a wide diversity of HIV subtypes and isolates, and evaluation of the APIs ability to both prevent infection with, and generate/select for, HIV strains which are resistant to the agent and other products [391].

Should an API be selected, it will be formulated as a gel, or other administration form, which will be then be tested for its safety, drug-release and distribution abilities (physic-chemical properties), pharmacokinetics (PK), pharmacodynamics (PD) and efficacy, in cellular, tissue and animal models [392]. The latter have classically involved rodents for initial toxicity testing, followed rabbits for assessment of vaginal irritation [393,394], and finally, NHPs for evaluation of safety, PK, PD and efficacy. Challenge studies are generally performed in macaques, using SIV or chimeric simian/human immunodeficiency virus (SHIV) isolates (SIV virions that contain genes derived from HIV-1 and, therefore, express HIV-1 proteins e.g *env*-containing SHIVs express gp120/41 on their surface) as a surrogate for HIV-1 infection [79,230]. Although no defined cut-offs exist (reflecting, in part, variability, in the virulence and applicability of different challenge isolates, and use of DMPA, between studies), a viable candidate would generally be expected to protect against at least 70%, and ideally 90%, of high dose (300 TID_{50}) virus exposures to warrant progression to clinical trials [392].

Preliminary trials in humans (phase I and IIa; involving approximately 20 to 100 participants) are largely concerned with evaluating toxicity and/or pharmacokinetics with subsequent larger trials (phase IIb and III, involving hundreds to thousands of participants) assessing extended safety and efficacy. Acceptibility evaluations can be conducted within or in parallel to clinical trials [391]. Reflecting the complexities and expense involved in the evaluation, formulation and manufacture of potential microbicides, of over 100 candidates identified thus far, only 8 have progressed to human efficacy trials [391, 392].

1.6.5 Early Microbicide Candidates

Initial research was focused on developing microbicides from established vaginal products or excipients that had demonstrated anti-HIV activity *in-vitro*. These were compounds with non-specific mechanisms of action against a range of micro-organisms +/- sperm, and included surfactants, polyanions, and vaginal pH buffering agents (see Table 1-6). Six of these early candidates were tested in efficacy trials with disappointing results [395]. The first product to be tested, the surfactant nonoxynol-9 (N-9), was widely available as a spermicide and lubricant. Results of initial trials of N9-containing microbicides (a vaginal sponge in Kenya [396] and film in Cameroon [397]) in HIV-negative female sex workers were inconclusive. However, in a more definitive trial, involving female sex workers in Benin, Cote D'Ivoire, South Africa and Thailand, an association was found between frequent use of an N9-based gel (> 3 per day) and an increased risk of HIV acquisition [398]. A suggestion of an increased number of HIV seroconversions was also seen in the active arm compared with the placebo arm in a phase III efficacy study of another surfactant C31G (Savvy®, Cellegy Pharmaceuticals, Quakertown, Pennsylvania, USA). This result was not conclusive as both this study, in Nigeria, and another study in Ghana were halted because of a lower than expected HIV incidence rate in the study population [399,400]. With hindsight, the mechanism of action of surfactants, disruption of cellular and microbial lipid membranes, should have perhaps led to earlier questioning of their safety. N-9 has subsequently been shown to be toxic to the genital mucosa, with associated epithelial disruption, inflammation, immune cell recruitment and alterations to the vaginal flora, which may help explain the clinical observations [401,402].

Table 1-6 1st Generation HIV-1 Microbicides

Category of Microbicide	Mechanism of Action	Examples in Class	Stage of Development
Surfactants	Non-specific disruption of phospholipid membranes on cells and micro-organisms	Nonoxynol- 9 (N9)	No longer under consideration due to safety concerns [398,401,402].
		Savvy® (C31G)	2 Phase III trials halted due to low HIV incidence in study population [399,400].
		Sodium lauryl sulfate (Invisible condom®)	Phase II safety study completed [403].
Negatively charged (Anionic) Polyanions	Interact with HIV gp 120 (envelope), CD4 and CXCR4 to inhibit viral attachment and entry into cells	Carageenan (Carraguard®)	Completed phase III trial [410].Safe, but not efficacious.
		Cellulose sulfate (Ushercell®)	2 phase III trials [411,412] halted early due to possibility of increased HIV incidence in one study [411].
		Naphthalene sulfonate (PRO 2000®)	Phase IIb and III trials completed [415,416]. Safe, but not efficacious.
		Cellulose acetate phthalate	Phase I trial halted due to heavy vaginal discharge (osmotic effect) [404].
		Dendrimers (Vivagel®)	Phase I trials completed [405,406].
Acidifying agents	Act to maintain acidic vaginal pH by buffering effects of alkaline semen	Carbopol 974P (Buffergel®)	Lack of efficacy in phase IIb trial [415].
		Acidiform (Amphora®)	Phase I and II/IIb studies completed (with diaphragm)[407,408].

The next group of compounds to be tested were polyanions. These bind to positively charged regions on HIV gp120 to inhibit binding to target cells [409]. Carraguard® (R515, Population Council, New York, USA) a gel containing the seaweed derived polysaccharide carageenan, was shown to be safe, but not efficacious when applied by 3011 women over a 2 year period. However, the reliability of the findings have been questioned since self-reported adherence, from which estimates of total sex acts were derived, was much higher than that calculated from testing of returned applicators, the latter of which suggested that gel had been used in only 42.1 % of sex acts [410]. Two phase III efficacy studies of another polyanion cellulose sulfate (Ushercell®, Polydex Pharmaceuticals, Toronto, Ontario, Canada and Topical Prevention of Conception and Disease [TOPCAD], Chicago, Ilinois, USA) were halted prematurely after an interim analysis of one suggested a higher HIV incidence among participants in the cellulose sulfate arm than in the placebo arm. On final analysis, the HIV incidence was 5.29/100 woman-years in those receiving cellulose sulphate compared with 3.33/100 woman years in placebo users; a difference which did not reach statistical significance (hazard ratio 1.61 95 % confidence interval (CI) (0.86-3.01)) [411,412]. Subsequently, it has been demonstrated, *in-vitro*, that cellulose sulphate generates a sustained drop in TER, reducing the integrity of the genital epithelial barrier. It also induces activation of NF-κB, which may promote HIV-1 replication [413]. Subversion of TLR mediated pathways may underlie this and may also affect levels of other cytokines [414].

Initial findings with PRO 2000® (naphthalene sulfonate, Indevus Pharmaceuticals, Lexington, MA, USA), the final polyanion to undergo efficacy trials, were encouraging. HPTN 035 was a 4 arm phase IIb trial in which 3087 women were randomized 1:1:1:1 to receive PRO 2000, BufferGel® (Carbopol 974P, Reprotect, Baltimore, MD, USA)- a gelling agent which acts to preserve low vaginal pH), placebo gel or no gel. No effect of BufferGel on HIV acquisition was detected (hazard ratio 1.1, 95 % CI 0.8-1.6); however, PRO 2000 (0.5 %) gel was 30 % effective at reducing HIV incidence compared with placebo gel (hazard ratio 0.7, 95 % CI 0.5-1.1) and 33% (hazard ratio 0.7, 95 % CI 0.4-1.1) compared with no gel [415]. However, since this positive effect was not statistically significant, it may possibly have occurred by chance. The Microbicides Development Programme (MDP) recruited over 9,000 women from different population groups (the general population in South Africa and Zambia), those at high risk in Tanzania and Ugandan HIV serodiscordant couples) to the MDP301 trial. This phase III trial was designed to compare two

concentrations of PRO 2000 (0.5 % and 2 %) against placebo; however, the 2 % arm was stopped for futility following a Data and Safety Monitoring Committee review in February 2008. The final results, released in December 2009, indicated that whilst both concentrations of PRO 2000 were safe neither was any better than placebo at preventing HIV acquisition (hazard ratio for 0.5 % gel 1.05, 95 % CI 0.82-1.34; for 2 % gel 1.21, 95 % CI 0.88-1.68)[416].

These initial failures called into question whether the concept of microbicides for HIV prevention was viable and highlighted deficiencies in the processes for evaluating potential candidates [417,418]. In the face of these challenges, and with enhancements in our understanding of mucosal HIV transmission, new models, assays and biomarkers have been developed to provide better, and earlier insights into the likely safety and efficacy of the next generation of microbicides [392] (discussed further in section 6.2.2).

1.6.6 Antiretroviral microbicides and oral pre-exposure prophylaxis

Although some agents with similar mechanisms of action to 1st generation microbicide candidates remain in the development pipeline, including the dendrimer SPL7013 (VivaGel®, Starapharma, Australia) [405,406], the focus has shifted towards more potent molecules, which specifically inhibit a particular stage of the HIV life-cycle (see Table 1-7). Currently, the leading microbicide candidates are ARVs, although more novel, non-ARV agents are also being evaluated (see section 1.6.7) [293]. As well as topical application, use of oral ARVs as pre-exposure prophylaxis (PrEP) is also being explored, given that the latter are already provided to HIV negative individuals in Western healthcare settings as post-exposure prophylaxis (PEP) following high-risk occupational (needle-stick) or sexual exposures [419].

The most advanced candidates for both topical and oral PrEP are reverse transcriptase inhibitors. Proof of concept for microbicides was demonstrated following the results, in 2010, of a phase IIb safety and efficacy trial of 1% tenofovir gel (Gilead, Foster City, USA). This study (CAPRISA 004) was conducted over a 30 month period in 889 women, recruited in both an urban and a rural clinic in KwaZulu Natal, South Africa. Women were asked to apply a dose of tenofovir gel within 12 hours before vaginal intercourse with a second dose immediately afterwards with no more than 2 doses in 24 hours (the BAT24 regimen). The trial was end-point driven, with participants due to be followed until at

least 92 HIV infections were observed, providing 90% power to detect a 50 % effect. When the trial was terminated, there were 38 HIV infections in the tenofovir arm and 60 in the placebo arm with incidence rates of 5.6 and 9.1 per 100 woman years respectively. Overall, the intervention was found to reduce HIV transmissions by 39% (rate ratio 0.61 95 % CI 0.40-0.96) [420]. HIV infection trends show that the tenofovir gel effect was evident soon after initiation of gel use and decreased over time. The HIV incidence rate in the tenofovir gel arm was 50% (P=0.007) lower compared to the placebo arm after 12 months and only 40 % lower (P=0.013) after 24 months follow up. This apparent reducing efficacy likely resulted from the effect of a declining number of reported coital acts (7.2 acts/month in 1st 6 months to 3.1 acts/month in months 18 to 24) as the study went on and a declining HIV incidence rate in the placebo arm over time whilst that in the tenofovir arm remained stable. As might be predicted and reflecting the robustness of the data, efficacy was greatest in those with the highest adherence (> 80 % gel adherence, 54 % lower HIV incidence) and least in those with the lowest adherence (< 50 % gel adherence, 28 % lower HIV incidence). This was also reflected in pharmacodynamic data. There was a clear correlation seen between cervico-vaginal fluid (CVF) levels of tenofovir and intracellular levels of the active metabolite tenofovir–diphosphate (measured at first study visit post infection, in 34/38 women in tenofovir arm who seroconverted, or random study visit in 303 women who remained HIV negative) and these in turn predicted rates of HIV seroconversion. In those women who remained HIV negative throughout the trial in the tenofovir arm, 96% had detectable concentrations in CVF, compared with 45 % of those who seroconverted [421]. HIV incidence in women with CVF levels > 1000ng/ml (n=82) were significantly lower than those in the placebo group (2·4 vs 9·1 per 100 person-years; incidence rate ratio (IRR)=0·26, 95% CI 0·05–0·80, p=0·01) whereas those with with CFV levels < 1000ng/ml (n = 253) were similar (7·8 vs 9·1 per 100 women-years; IRR=0·86; 95% CI 0·54–1·35, p=0·51) [422].

In safety analyses, there was no statistical difference between the overall numbers of adverse events reported in the tenofovir arm compared to in the placebo arm (3.55 and 3.44 per woman year P=0.265) and no safety concerns in pregnancy, although gel use was discontinued in those women found to be pregnant. One theoretical concern with using ARVs for PrEP is that users who become HIV positive whilst still exposed to the drug could develop resistance mutations. One advantage of using topical microbicides over oral PrEP

is that systemic levels of drug are likely to be much lower which may make the development of resistance less likely.

No NRTI resistance mutations, including those particularly associated with tenofovir use (K65R, K70E), have been found using standard population sequencing in plasma samples from women in the tenofovir arm with breakthough HIV infection. Although low levels of resistance mutations, in particular K65R, were detected in women from both study arms using deep 454 pyrosequencing, frequencies were not increased in those exposed to tenofovir gel suggesting it had no appreciable effect [423] . However, one participant was also found to have very low levels (~ 1%) of the K65R mutation in cervicovaginal fluid using sensitive proviral DNA PCR assays. This woman had the highest vaginal aspirate tenofovir level of any participant who seroconverted (213,000 ng/mL), with an estimated infection duration of 13 days, and a CVL viral load of 740 copies. This level of K65R falls within a frequency that has been reported to be natural quasispecies error for subtype C in the absence of drug; thus, although it has raised concern, it might also simply reflect background levels of mutation and not tenofovir-induced resistance [424,425]. Of note, samples for resistance testing were performed on average 20 weeks after the estimated date of HIV seroconversion when many participants were no longer exposed to tenofovir. There therefore remains a small chance that some other mutations may have been archived and may emerge at a later date if the individual is re-exposed to the drug.

An additional beneficial finding from a sub-study within CAPRISA 004 looking at women who were HSV-2 serologically negative at enrolment, was that women in the 1% tenofovir arm displayed a 51 % reduction in their incidence of HSV2 (95 % CI: 22-70%) [420]. This finding was a surprise bonus, and is linked to the anti-HSV activity exhibited by tenofovir (which is structurally related to the drug Cidofovir used to treat HSV and Cytomegalovirus) at high concentrations [426]. As for HIV, there was a correlation between tenofovir levels in cervico-vaginal fluid and incidence of HSV2 [421]. Although numbers were too small for a formal statistical analysis, tenofovir appeared to protect women from HIV-1 independent of their baseline HSV status; thus, it is thought unlikely that its HIV protective effect was mediated through the prevention of HSV2 acquisition.

Initial results of the iPrEX study, a Phase III trial of oral PrEP in 2,499 sexually active MSMs and transgendered women who have sex with men, were also released in 2010. The

strategy showed promise, with 43.8 % fewer HIV infections reported in participants randomised to daily oral Truvada (tenofovir plus emtricitabine, Gilead) than those who in the placebo arm (95% CI 15.4 to 62.6%; P=0.005) [427]. Detectable levels of the study drugs in participants' blood strongly correlated with the prophylactic effect, suggesting efficacy was directly related to adherence. In the final intention-to–treat (ITT) analysis, Truvada PrEP was shown to be 42 % effective overall. However, modelling, based on detected drug levels, suggested a 92 % reduction in HIV infection was achieved with high adherence to daily dosing [428].

Findings from four other important PrEP studies, all in heterosexuals, were announced in 2011. The TDF-2 Study was a Phase IIb trial of daily oral Truvada vs placebo, conducted in 1,200 HIV uninfected men (54.7%) and women (45.3%) in Botswana. Nine of 601 participants became HIV-1-infected in the Truvada group and 24 of 599 in the placebo group. Overall protective efficacy of Truvada was 62.2% (95% CI 21.5 to 83.4, p=0.0133). Although not designed and powered for such, ITT analysis by gender suggested the intervention was protective in men (p=0.026) but not in women (p=0.107) [429]. The Partners PrEP study was a 3 arm phase III trial comparing daily use of oral tenofovir alone, oral Truvada, and placebo in preventing HIV transmission in serodiscordant couples. 4,758 couples were enrolled in Uganda and Kenya, between July 2008 and November 2010, with study follow-up planned until 2012. However, following an adhoc review of the data up until May 2011, the DSMC concluded that there was clear evidence that both tenofovir and Truvada were effective at preventing transmission and that individuals in the placebo arm should be offered active product. In the final published results, 82 participants acquired HIV-1 after enrolment, n= 52 placebo arm, n= 17 tenofovir arm, and n= 13 Truvada arm. Overall, compared to placebo, there were 67 % fewer infections in the tenofovir arm (95 % CI 0.44- 0.81) and 77% fewer in the Truvada arm (95 % CI 0.55- 0.87). There was no significant difference in the efficacy of tenofovir and truvada. Both tenofovir and Truvada significantly reduced risk of HIV acquisition in both men and women [430].

In contrast with the findings from CAPRISA 004, suspected ARV-induced resistance mutations have been detected in several PrEP trial participants who were subsequently found to already have been recently HIV-1 infected (HIV-1 antigen (p24)/antibody negative/RNA positive) at baseline. In the iPREX trial 2 such particpants randomised to the

Truvada arm were subsequently found to have mutations associated with resistance to emtricitabine; 1 was confirmed to have become detectable during the study, i.e. the HIV-1 genotype was wild-type at baseline, but M184I positive at 4 weeks; in the other patient, the baseline RNA sample was too low to perform resistance testing [431]. In the TDF-2 study, 1 acutely HIV-1 infected participant who received Truvada developed K65R, M184V and A62V RT mutations at high levels (approximately 100%), having previously had a wild-type genotype [429]. In the Partners PrEP trial, 2 of 8 participants with acute HIV-1 at enrolment developed mutations on treatment, 1 in the tenofovir arm developed K65R and 1 in the Truvada arm developed M184V [430]. However, although resistance induced by taking the study ARVs is suspected it cannot be proven and there is a possibility that the mutations may have been present at low level as archived transmitted resistance which was then selected out under drug pressure. In particular the presence of A62V, a broad-spectrum NNRTI mutation, is supportive of this theory. Evidence of transmitted drug resistance was found in patients in the placebo arms of both iPREX (1 individual with M184V, T215Y and K103N at baseline; the latter being an NNRTI mutation) and potentially in TDF-2 (1 participant had a K65R mutation intermittently and at very low levels (<1%) after seroconversion, although the mutation was not detected in the blood sample that had been obtained closest to the estimated date of seroconversion and may just reflect natural Subtype C variation [425]). Resistance appears to be uncommon in individuals seroconverting after randomisation, a minority of whom had detectable drug levels, although further analyses are awaited. These findings, and the small but significant decreases in bone mineral density induced through tenofovir use in 2 of the studies (in populations with lower than average bone mineral density at baseline) [430,432] are potentially worrying and could have long term consequences. What is certain is that if PrEP is rolled out widely as a prevention methodology, every effort should be made to identify those individuals at risk of being in the serological detection ‘window period’ for acute infection, and PrEP deferred, and/or triple drug PEP offered as appropriate, until HIV infection can be definitively excluded.

Interestingly, the efficacy of oral tenofovir and Truvada as PrEP in the above studies is in contrast to findings from two others. The FEM-PrEP trial, in which daily oral Truvada was compared to placebo in high risk African women, was terminated early in April 2011 having enrolled 1,951 participants and accrued 75 % of the planned study endpoints (56 HIV infections, 68 in final analysis). With virtually equal numbers of HIV transmissions

occurring in both arms of the study, the DSMC decided that it was futile to continue as it was highly unlikely to be able to demonstrate effectiveness of the intervention [433]. Despite a requirement for all women to be using a reliable method of contraception (66 % were using injectables and 30 % were using oral contraceptives at enrolment) there was an overall pregnancy rate of 9 %. This in itself is not unusual, since conception is a common occurrence in trials involving young African women. Interestingly, among study participants assigned to the Truvada arm, observed pregnancy rates were higher than among women in the placebo arm. This is unexpected and inconsistent with known drug interactions involving tenofovir or emtricitabine and contraceptive hormones. Results suggest that, despite intensive counselling, drug adherence within the trial was generally poor. Among women assigned to Truvada, drug was detectable in plasma in < 30% of infected cases and < 40% of uninfected controls matched on time of infection [434]. This contrasts with adherence levels reported from the Partners PrEP study where drug level analyses indicated adherence rates of > 80 % in uninfected controls in both treatment arms. Adherence was found to be much lower in iPREX, where tenofovir was detected in only 44% of uninfected controls. Evidence from pharmacokinetic studies suggests that active tenofovir and emtricitabine metabolites concentrate in greater quantities in the rectum than in vaginal tissues following oral dosing [422]. Thus, tenofovir and Truvada may be more forgiving of poor adherence in protecting against rectal compared with vaginal HIV exposures.

The Vaginal and Oral Interventions to Control the Epidemic (VOICE) trial was designed to directly compare safety, efficacy and acceptability of topical and oral ARVs for HIV prevention in women [435]. Participants were randomly assigned to one of five study groups; three tablet groups: tenofovir, Truvada, or oral placebo; and two gel groups: tenofovir 1% gel or placebo gel. Interventions were to be used once daily at any time. VOICE was designed to enroll approximately 5,000 women, about 1,000 in each group. Enrolment of 5,029 women was completed in June 2011 with follow up expected to continue until mid-2012. However, similar to the FEM-PrEP trial, two study arms, first oral tenofovir, then tenofovir gel, have been stopped early due to a lack of difference in HIV incidence between the intervention and placebo arms [436].

On the basis of the findings from iPREX, Partners PrEP and TDF-2 Trials, the FDA has very recently granted a change of license for Truvada to be used as PrEP. As a condition of this

license, Gilead Sciences is required to "collect viral isolates from individuals who acquire HIV while taking Truvada and to evaluate these isolates for the presence of resistance. Additionally, the company is required to collect data on pregnancy outcomes for women who become pregnant while taking Truvada for PrEP and to conduct a trial to evaluate drug adherence and its relationship to adverse events, risk of seroconversion, and resistance development in seroconverters. Gilead has committed to provide national drug utilization data in order to better characterize individuals who utilize Truvada for a PrEP indication and to develop an adherence questionnaire that will assist prescribers in identifying individuals at risk for low compliance" [437].

Following the promising results of CAPRISA 004, the FDA indicated that it would review data from VOICE as the second pivotal trial to support possible fast-track licensure of tenofovir gel, and a deal was brokered between CONRAD (the HIV and women's health research organization who manufactured and supplied the 1% tenofovir gel for CAPRISA 004) and the South African Government's Technology Innovation Agency to grant the latter the right to manufacture and distibute the gel in Africa if it were to be approved [438]. However, instead of affirming the efficacy of tenofovir gel, VOICE has provided clear evidence that gel use was not effective in the women in the study. Although disappointing, this information adds a new dimension to discussions about the future of tenofovir gel. Of particular interest is the potential for the different dosing regimens (pre and post coital dosing vs once every day) to have played a part in observed efficacy differences. A Phase III trial (Follow-on African Consortium for Tenofovir Studies (FACTS 001)), testing the same BAT24 regimen of tenofovir gel used in CAPRISA 004, began enrolling participants in October 2011 [439]. This study will involve approximately 2,200 women at up to nine sites in South Africa, with results expected in 2014. Unfortunately, plans for another trial, led by the MDP, that would have compared tenofovir 1% gel administered via the BAT24 regimen vs tenofovir 1% in a single pre-coital dose, have been put on hold due to funding constraints. This is a pity since the latter would potentially make for a more convenient and realistic 'real world' dosing regimen. However, a phase I study comparing the post-coital pharmacokinetics and pharmacodynamics of 1% tenofovir gel applied in one of 3 ways (once daily, in a BAT24 regimen or peri-coitally) is going ahead (ClinicalTrials.gov identifier: NCT01369303).

Two other ARVs which are currently being tested clinically for use in HIV-1 pre-exposure prevention strategies are the NNRTI Dapivirine (Tibotec BVBA, Mechelen, Belgium and International Partnership For Microbicides) and the CCR5 inhibitor Maraviroc (Pfizer, New York, USA). Dapivirine is a highly lipophilic molecule which was not suitable for oral dosing. It has been formulated both as a gel and as an intravaginal ring (IVR). The latter are capable of controlled release of agents over a prolonged period, enabling continuous, coitally-independent delivery of a high concentration of active drug [440]. Studies in rabbits and macaques of dapivirine vaginal gel demonstrated drug accumulation in the keratinized epithelial layer of the vagina with some penetration of the superficial cell layers. Dapivirine was detectable in tissue at 48 h post administration [441]. Dapivirine gel has subsequently been tested in women and found to be well-tolerated with low-level systemic absorption (< 1.1ng/ml) and a long terminal half-life (t ½) in both plasma (72-73 hours) and genital fluids (15-17 hours) [442,443,444]. The dapivine-loaded IVR has also been tested in a number of phase I and II pharmacokinetic, safety and acceptability studies [445,446], and a phase II study of long-term safety plus efficacy (The Ring Study) is on-going [447]. A phase III efficacy trial (ASPIRE) began recruitment in July 2012 [448].

As shown in Table 1-7, maraviroc is currently the only inhibitor of CCR5 licensed for HIV treatment. Its action early in the HIV lifecycle, favourable safety and pharmacokinetic profile and relatively limited use in current ARV therapeutic regimens make it an ideal candidate both as a microbicide and as oral PrEP [449]. A randomised, double-blind trial of oral maraviroc (300mg OD), vs maraviroc plus either emtricitabine or tenofovir, vs Truvada as daily PrEP in MSM (Novel Exploration of Therapeutics for PrEP (NEXT-PrEP), HPTN 069/ACTG 5305), has just been initiated [450]. A vaginally applied gel formulation of maraviroc, which effectively protected against infection with SHIV in a macaque model, is currently undergoing phase I trials [451]. Maraviroc is also being assessed, both alone and in combination with dapivirine, in an IVR [452]. A combined maraviroc /tenofovir film is also being developed.

Table 1-7 Specific anti-HIV-1 agents with potential for use as microbicides or oral PrEP

Category of Microbicide	Mechanism of Action	Examples in Class	Stage of Development of Most Advanced Candidate(s) in Class
Reverse transcriptase inhibitors	Inhibit HIV reverse transcriptase enzyme to block viral replication	Nucleoside/Nucleotide e.g. Tenofovir	Microbicide: 1 % tenofovir gel;-Phase IIb (CAPRISA 004)- overall 39% efficacy [420]. VOICE- gel arm halted by DSMC [435].
		Tenofovir +/- FTC (Truvada®)	Oral PrEP: Phase IIb/III iPREX, TDF-2, Partners PrEP:- Overall 42% (MSM) and 73% (heterosexual) efficacy [428,429,430]. FEM-PrEP, VOICE (tenofovir) Not protective [434,436].
		Non-nucleosides (NNRTIs) e.g. Dapivirine (TMC-120), UC-781, MIV-150	Microbicide: Dapivirine:-Phase I/II studies completed (gel and IVR). Phase II studies ongoing; Phase III study started (IVR) (ASPIRE) [448,452].
Entry inhibitors:	Prevent HIV-1 binding/entering cells		
CCR5 ligands	Bind to CCR5 to inhibit binding of co-receptor to gp120	Maraviroc	Oral PrEP: Study in MSM started [450] Microbicide: Phase 1 studies ongoing (gel and IVR (latter combined with Dapivirine)[452].
		PSC RANTES, 5P12 RANTES	Microbicide: Pre-clinical [456,457].
Fusion inhibitors	Prevent gp41 helical bundle formation and fusion	T20, BMS806	Microbicide: Pre-clinical[293].
Lectins	Bind to mannose moieties on gp120	Cyanovirin-N, Griffithsin	Microbicide: Pre-clinical [459,460,461,463], phase I trial in development (Griffithsin) (C Lacey, personal communication, 2012).
Monoclonal Antibodies	Bind to a range of different targets on gp 120 or 41, or to cellular receptors (e.g. CD4, CCR5, CXCR4)	2G12, 2F5, 4E10,b12, VRC01, PGV04, PGT NAbs	Microbicide: Pre-clinical and phase 1 [500]

Other classes of ARVs, which act during (integrase inhibitors) or post-integration (protease inhibitors), are also being considered for HIV-1 prevention. These could potentially contribute to eliminating the initial founder infection foci, especially if used in combination with an entry blocker or RT inhibitor. Given the demonstrated success of combination therapy in HIV treatment, multi-target ARV formulations are being evaluated as microbicides or PrEP [293]. Indeed, a dedicated European project (Combined Highly Active Antiretroviral Microbicides (CHAARM)) has been established to specifically develop new combination ARV-based microbicides [453].

1.6.7 Current non-ARV microbicides

Despite the undoubted potential of ARVs as PrEP, there remains a concern that intermittent dosing of oral agents could give rise to resistance mutations in users who acquire or who are already infected with HIV. This could theoretically occur even with the low levels of systemic absorption seen with topical agents (In CAPRISA 004, blood plasma tenofovir concentrations measured at a mean of 4.5 days of gel use were < 1ng/ml (0-0.8 ng/ml) in women using tenofovir gel, similar to levels seen with dapivirine) [420,421]. The efficacy of ARVs for PrEP may also be reduced in the presence of drug-resistant HIV strains in an infected partner. ARV-resistance has become a widespread problem with the increasing availability of HAART and relative lack of resources for detecting virological failure in developing countries [454,455]. Thus, there remains an impetus to develop microbicides based on classes of molecule which differ from those used in therapy.

Most non-ARV agents in the potential microbicide pipeline act through various mechanisms to inhibit HIV attachment and entry into cells. Many of these are peptides or proteins related to molecules involved in innate immune defenses [391]. PSC-RANTES, a synthetically produced analogue of the natural CCR5 ligand RANTES, displayed remarkable potency *in- vitro* and in macaque vaginal challenge studies [456], but was thought to be too costly to develop as a microbicide. It also acted as a CCR5 agonist, with potential for inducing inflammation. However, a fully recombinant compound, 5P12-RANTES, displays potent cross-clade anti-HIV-1 activity, without any signalling effects, and is compatible with cheaper, biosynthetic production [457].

Several non-mammalian lectins have been identified which, through binding mannose residues on gp120, inhibit both attachment of HIV-1 virions to target cells and onward

transfer via transcytosis [458]. The first of these, cyanovirin–N, is derived from cyanobacteria. It has broad and potent *in-vitro* anti-HIV-1 activity and has been shown to protect macaques from both vaginal and rectal SHIV challenge [459 ,460]. Scientists have expressed cyanovirin-N in Lactobacilli to produce a 'living-microbicide'; a concept which may hold promise for the future [461]. Unfortunately, cyanovirin-N has been found to stimulate lymphocyte proliferation and cytokine production, raising safety concerns [462]. Griffithsin, a red algal protein, is another candidate. It has even more potent HIV inhibitory activity than cyanovirin-N *in-vitro* and displays no mitogenic or inflammatory properties, either *in-vitro* or on rabbit vaginal irritation testing. In addition it has been expressed in the tobacco plant *Nicotiana Benthamiana*, with potential for large scale affordable production [463].

1.6.8 Broadly neutralizing monoclonal antibodies against HIV-1

The ability to induce protective neutralizing antibodies has long been considered an optimal goal of candidate HIV vaccines as they have been shown to offer protection against many other viral infections [146,464,465,466]. More recently, interest has developed in the potential of applying combinations of neutralizing monoclonal antibodies topically as a vaginal microbicide.

1.6.8.1 Definition of monoclonal antibodies (mAbs)

Antibodies may be characterized as 'polyclonal' or 'monoclonal', according to the nature of the B-lymphocyte population from which they are derived. Naturally produced sera is polyclonal, containing antibodies with a broad range of different antigen affinities and specificities, which have been secreted by a large number of genetically distinct B-cells. However, it is possible to combine individual Ig –producing B cells with myeloma cells to create hybridomas, which can replicate indefinitely in culture and be cloned. Antibodies produced in such systems are identical in their isotype, antigen affinity and specificity and are termed monoclonal [467].

There are currently more than 20 IgG mAbs approved for the treatment of a variety of conditions, including autoimmune and inflammatory disorders, transplant rejection and malignancies [468]. At present, only one mAb is licensed for preventing an infection in humans; Palivizumab (AstraZeneca, London, UK), a humanized murine IgG1 against

respiratory syncytial virus, which is administered intramuscularly (IM). Topical administration of mAbs has been shown to have the potential to prevent a number of mucosally-transmitted infections [469,470]. However, to date, this strategy is yet to be tested in humans.

1.6.8.2 b12, 2F5, 4E10 and 2G12

A number of antibodies have been identified in the sera of selected individuals with long-standing HIV-1 infection which are individually capable of neutralizing a broad range of isolates. These NAbs inhibit various stages of HIV attachment and fusion though binding to gp120, gp41, CD4 or co-receptors (see Figure 1-10). Among the earliest to be discovered (and produced as mAbs) and the best characterised are b12, 2G12, 2F5 and 4E10. Results of key research involving these (and other early anti-HIV mAbs, where relevant) is summarised below:

Figure 1-10 Binding sites of NAbs on gp120 and gp41

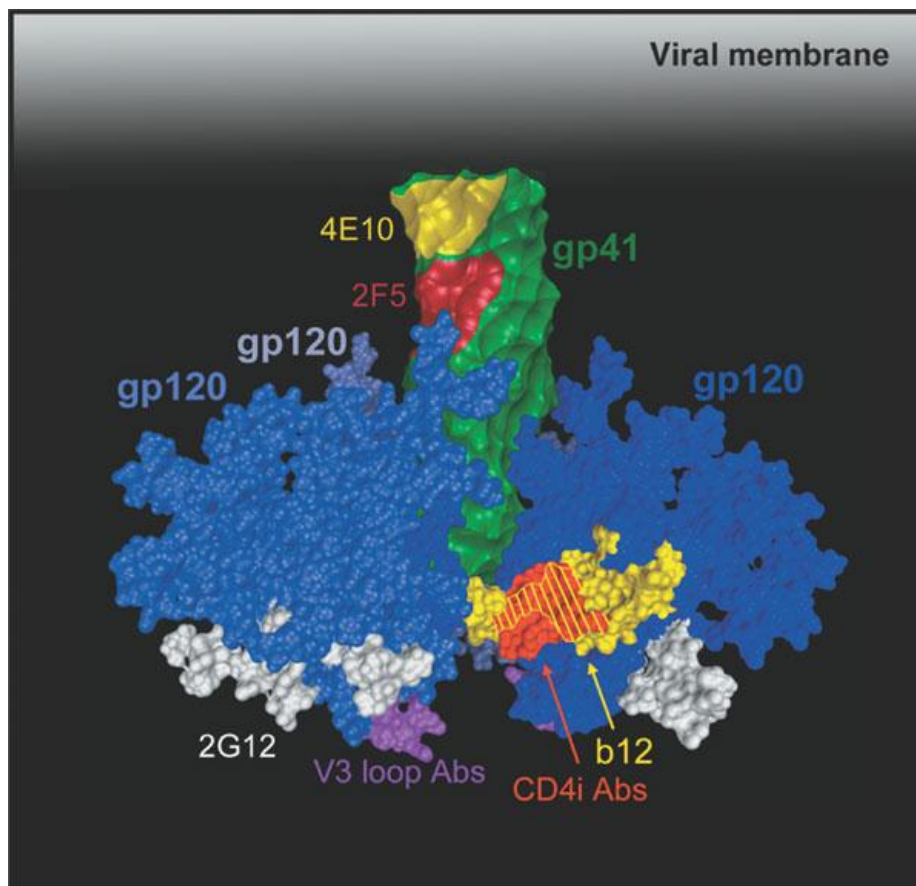


Figure reproduced from Burton *et al.* Nat Immunol 2004 [465].

1.6.8.3 *In-vitro* evaluation

b12 binds to the CD4 binding site (CD4bs) of gp120 [471,472]. In contrast to non-neutralizing CD4bs mAbs (e.g. b3, b6, F105, 15e, and F91), b12 is able to interact not only with monomeric gp120 but also with functional oligomeric gp120 at the virion surface [472].

2G12 recognises a conformational dependent epitope on gp120. Binding of 2G12 to monomeric gp120 is abolished by amino acid substitutions removing N-linked glycosylation in the C2, C3, V4 and C4 regions of gp120 [473]. Using crystal structures of Fab 2G12, it was demonstrated that this antibody binds to an unusually dense cluster of carbohydrate moieties on the "silent", outer face of gp120 by a previously unappreciated mechanism for high affinity recognition of carbohydrate [474]. 2G12 binds to the surface of native virions and has been demonstrated to inhibit gp120 binding to both CD4 and CCR5. It has been proposed that binding of 2G12 to its epitope (which includes glycan residues on opposite borders of the V3 loop) restricts conformational changes involving V3, which are induced through CD4 and CCR5 binding, and weakens the affinity of these receptors for their binding sites [475].

2F5 and 4E10 recognise neighbouring, conserved epitopes (amino acid sequences ELDKWA and NWFDIT respectively) on the portion of gp41 which is extracellular and adjacent to the viral membrane in the native virion (the membrane proximal external region (MPER)), with 4E10 binding closer to the C-terminus [476,477,478]. Peptides corresponding to this region are largely unstructured in aqueous solution, but, acquire α-helical secondary structure within lipid bilayers [479]. The MPER has 2 α- helices linked by a kink with the N-terminus more exposed to the external, aqueous medium and the C-terminal region inserted in a shallow position in the lipid membrane [480]. The mechanism by which 2F5 and 4E10 mediate neutralization *in-vitro* appears more complex than that for 2G12 and is incompletely understood. MPER antibodies have been shown to preferentially bind to the pre-hairpin intermediate that forms upon receptor engagement and it has been proposed that their binding interferes with the process of virus-cellular membrane fusion, preventing target cell entry [479, 481, 482, 483]. However, although this may play a role in their neutralization activity, it may not be their only mode of action (see section 6.2.3.2). It has been demonstrated that 2F5 and 4E10

can bind and interact with their epitopes, in their lipid-bound state, prior to receptor engagement and that this may be a requirement for successful neutralization [479].

Although all 4 mAbs were isolated from individuals infected with HIV-1 subtype B, the conserved nature of their epitopes enables them to bind HIV-1 strains from other clades. However, differences exist between the mAbs with regards to the breadth and potency of their *in-vitro* neutralization capabilities. The most broadly neutralizing are the MPER antibodies, in particular 4E10. In a cross-clade neutralization study, 4E10 neutralized 100% and 2F5 neutralized 67% of a diverse panel of 93 primary isolates compared to 50% for b12 and 41% for 2G12 [484]. Results from an even more comprehensive study showed that out of a panel of 162 viruses, 4E10 neutralized 159 isolates (98%), 2F5, 97 isolates (60%) b12, 57 isolates (35%) and 2G12, 52 isolates (32%) [485]. Similar neutralization breadth for 4E10, 2F5 and 2G12 was seen against 91 sexually transmitted HIV-1 isolates cloned from acutely infected patients [486].

It has been observed in the above studies that b12, 2G12 and 2F5 are individually less capable of neutralizing non-clade B subtypes than 4E10. However, triple or quadruple combinations of mAbs (2G12, 2F5 and F105, b12 +/- 4E10) have been shown to demonstrate synergistic effects against primary HIV clade C isolates (BW11, 1084i) as well as against clade B isolates [487,488,489]. Results suggest that the presence of two or more mAbs in combination reduces the levels of each antibody needing to be present in order to achieve the same level of viral neutralization.

1.6.8.4 Studies in animal models

Intravenous (IV) passive transfer of the above mAbs, in varying combinations, has protected macaques from infection following intravenous, oral, rectal and vaginal SHIV challenge [487,490,491,492,493,494,495,496, 497,498]. mAb concentrations for infusion were defined based on neutralization studies with primary isolates. Recently, Hessel *et al.* demonstrated clear protection by 2F5 and 4E10 used individually against rectal $SHIV_{Ba\text{-}L}$ challenge [499]. Macaque mucosal challenge studies involving mAbs 2F5, 4E10 and 2G12 +/- b12 are summarised in Table 1-8.

Table 1-8 Summary of mucosal SHIV challenge studies in macaques involving anti-HIV-1 neutralizing mAbs 2F5, 4E10 and 2G12 +/-b12

mAb combinations	**Dose (route) and time of mAb relative to challenge**	**SHIV challenge (dose) *+route***	**Monkeys protected/ treated**	**Reference**
HIVIG/2F5/2G12	400mg/kg HIVIG +15mg/kg each of 2F5, 2G12; -24hr (i.v)	SHIV89.6P (10-50 AID$_{50}$) *vaginal*	4/5	Mascola *et al.* 1999 [491], 2000 [492]
2F5/2G12	15mg/kg each mAb -24hr (i.v)	SHIV89.6P (10-50 AID$_{50}$) *vaginal*	2/5	As above
2G12	15mg/kg -24hr (i.v)	SHIV89.6P (10-50 AID$_{50}$) *vaginal*	2/4	As above
Total macaques protected from vaginal challenge by iv mAbs **Control animals (irrelevant IVIG)**			**8/14** **0/5**	
2G12+2F5+F105	10 mg/kg (i.v.)5d before birth, d0 (-1hr), +d8	SHIV-vpu^{+} (10 AID$_{50}$) *oral*	4/4	Baba *et al.* 2000 [487]
2G12+2F5+F105	10 mg/kg (i.v.) d0 (-1hr), +d8	SHIV-vpu^{+} (10 AID$_{50}$) *oral*	2/2	Hofmann *et al.* 2001[494],2002 [495]
2G12+2F5+F105	10 mg/kg (i.v.) PEP +1hr,+d8	SHIV-vpu^{+} (10 AID$_{50}$) *oral*	2/2	Hofmann *et al.* 2002[495]
2G12+2F5+b12	10 mg/kg (i.v.) d0 (- 1hr), +d8	SHIV89.6P (15 AID$_{50}$) *oral*	1/4	Hofmann *et al.* 2001[494]
2G12+2F5+4E10 +b12	30 mg/kg (i.v.) PEP: +1hr, +d8	SHIV89.6P (15 AID$_{50}$) *oral*	2/4	Ferrantelli *et al.* 2003[496]
2G12+2F5+4E10 +b12	30 mg/kg (i.v.) PEP: +1hr, +d8 +12hr, +d8	SHIV89.6P (15 AID$_{50}$) *oral*	3/4 +1/3	Ferrantelli *et al.* 2007[498]
2G12+2F5+4E10 +b12	30 mg/kg (i.v.) PEP[i]: +1hr, +d8	SHIV-1157ip (15 AID$_{50}$) *oral*	3/4	Xu *et al.* unpublished
2G12+2F5+4E10	40 mg/kg (i.m.) PEP: +1hr, +d8	SHIV89.6P (15 AID$_{50}$) *oral*	4/4	Ferrantelli *et al.* 2004[497]
Total protected macaques from oral challenge by iv mAbs **Control animals (untreated)**			**22/31** **0/31**	
2F5	50mg/kg (i.v) –d1, +1d	SHIVBa-L (2000 TCID$_{50}$) *rectal*	5+/6	Hessell *et al.* 2010 [499]
4E10	50mg/kg (i.v) –d1, +1d	SHIVBa-L (2000 TCID$_{50}$) *rectal*	6/6	As above
Total protected macaques from rectal challenge by iv mAbs **Control animals (2 untreated, 2 isotype control IgG)**			**11+/12** **4/4**	

PEP = Post-exposure prophylaxis; hr = hour(s); d = day(s); AID$_{50}$ = 50 % animal infectious doses; TCID$_{50}$ = 50 % tissue culture infectious doses; HIVIG= HIV immune globulin (polyclonal).

Proof of concept that vaginally-applied mAbs could prevent SHIV transmission was provided by Veasey *et al* [500]. Only 2 of 8 (25%) DMPA treated macaques became infected with SHIV $_{162P4}$ (following 300 TID_{50} vaginal challenge up to 2 hours later) after vaginal application of 5mg b12 in saline compared with 9 of 10 (90%) controls. However, results using a gel were less clear-cut. 1 of 2 animals became infected post application of 5mg b12 in a 2.5 % hydroxyethyl-cellulose (HEC) based gel, after a similar challenge, compared with all of 3 controls.

1.6.8.5 Studies in humans: passive immunotherapy in HIV infected individuals

In addition to the above animal studies, the mAbs 2F5, 2G12, and 4E10 have been used in passive immunotherapy studies in humans (see Table 1-9). 4 studies involving a total of 39 HIV-1 positive individuals have been conducted [501,502,503,504,505, 506]. Subjects received a dose of each mAb, ranging from 1 to 5g per infusion, at weekly intervals for between 4 and 16 weeks. Overall, the mAbs were shown to be safe, with no serious adverse events or thrombotic complications reported. In the latter 2 studies, where all participants had stored, pre- HAART viral isolates which were sensitive to neutralization by at least 2 of the mAbs (all 3 mAbs in the majority), some efficacy in temporarily delaying viral rebound after cessation of HAART was observed in 14/16 individuals who had initated HAART during acute/early HIV-1 infection, with 4 maintaining VL suppression to < 50 copies/ml during the entire infusion period. However, in keeping with the limited efficacy of natural broadly neutralizing sera in controlling HIV-1 in most chronically infected individuals (in view of their more varied quasi-species), the mAbs had less effect in those participants who had initated HAART later in the course of their HIV-1 infection, with only 2/8 showing any delay in VL rebound, and only 1 maintaining VL suppression until the end of the infusion period. Viral mutants emerged during rebound which were resistant to 2G12 in 19/22 participants, providing additional evidence that it produced a neutralizing effect causing a selective pressure [504, 505]. Interestingly, although no 2F5 or 4E10 resistant mutants were generated, one acutely infected individual who displayed prolonged VL suppression displayed 2G12 resistance pre-HAART suggesting that suppression of his virus during the study was mediated by the MPER mAbs [504]. It is not known whether resistance to 2G12 alone arose because it was more active in these studies than 2F5 and 4E10, e.g. because of pharmacokinetic or mechanistic differences, or whether perhaps resistance to 2G12 (due to loss of one or more glycosylation site(s)

within its epitope) is generated more easily, with less impairment of viral fitness, than changes to the MPER (reflected in the lower proportion of cross-clade isolates sensitive to neutralization by 2G12 than by 2F5, and in particular 4E10) [484]. Laboratory studies have shown that generating resistance to all 3 of 4E10, 2F5, 2G12 is difficult and is associated with reduced viral replication [507].

The results of the above pre-clinical and clinical studies suggest that, although limited in their ability to control HIV-1 infection in the long-term, if present at the time of infection (when the quasi-species is limited) combinations of HIV-1 NAbs may have the potential to prevent the establishment or expansion of the founder population.

Table 1-9 Summary of clinical trials using C2F5, C4E10 and C2G12 to date

	Study			
	1 Armbruster *et al.* 2002 [501]	**2 Armbruster *et al*. 2004 [503]**	**3 Trkola *et al.* 2005 [504]**	**4 Mehandru *et al.* 2006 [505]**
No. of patients	7	8	14	10
Male/Female	4/3	4/4	10/4	10/0
mAbs	C2F5, C2G12	C2F5,C2G12, C4E10	C2F5, C2G12, C4E10	C2F5, C2G12, C4E10
Dose of mAb per infusion (g)	1/1	1/0.5/1	1.3/1/1	1/1/1 (n = 6) 2/1/2 (n =4)
No. infusion days	8	4	13	16
Length of dosing	4 weeks	4 weeks	13 weeks	16 weeks
SAEs	0	0	0	0
Thrombotic complications	0	0	0	0
Possible ADRs				
None	7/7	8/8	7/14	7/10
Myalgia	0	0	5/14	3/10
Arthralgia	0	0	4/14	1/10
Skin rash	0	0	1/14	1/10

1.6.8.6 Antibody isotypes of the commercially produced mAbs

To generate the original hybridomas producing 2G12, 2F5 and 4E10 mAbs, B-lymphocytes isolated from asymptomatic HIV-infected subjects were fused with

heteromyeloma cell lines by a combined polyethylene glycol/electrofusion method [508]. 2G12 was initially produced as IgG1, whereas 2F5 and 4E10 were originally IgG3 antibodies. Subsequently, to enable safe mass production for use in research studies, and to maximise their plasma half-life, the mAbs were all expressed recombinantly in Chinese Hamster Ovary (CHO) cells as IgG1 (k)[509]. Clinical-grade mAbs, suitable for administration in humans, are produced commercially by this method by Polymun Scientific, Vienna; a company established by the immunologist who first discovered them. Such mAbs are given the prefix 'C' to distinguish them from mAbs produced in other systems.

1.6.8.7 Mabgel

A potential vaginal microbicide containing a combination of the 3 mAbs, C4E10, C2F5 and C2G12, in a hydroxyethylcellulose (HEC)-based matrix (Mabgel) was developed and evaluated by scientists working within the European Microbicides Project (EMPRO). EMPRO was a network of 24 partners consisting of academic institutions and small and medium sized enterprises from across Europe and Africa. It was funded for 5 years (2004-9) by the European Commission as part of its Sixth Framework Programme.

The culmination of the work of EMPRO was a Phase 1 clinical trial (MABGEL 1), which aimed to assess the pharmacokinetics and safety of Mabgel in healthy, HIV negative, female volunteers. Two doses of the Mabgel would be studied; high dose Mabgel, which was evaluated in preclinical studies, contained 20mg/g of each mAb, whereas the low-dose Mabgel contained 10mg/g of each mAb. A comparator placebo gel was also produced, which would be used to increase the validity of safety endpoint analyses. A full description of all 3 study gels is given in section 2.6.2.

The design, conduct, results and conclusions from the MABGEL1 study (and relevant pre-clinical evaluations) are the focus of the remainder of this thesis, which will also consider the potential of Mabgel as an effective vaginal microbicide and ideas for future research.

2. MABGEL1: CLINICAL TRIAL DESIGN AND METHODOLOGY

2.1 Overview of Clinical Trial Design and Conduct

The MABGEL 1 study was a single centre, Phase 1, randomised, double-blinded, prospective clinical trial with the overall aim to assess the pharmacokinetics and safety of the anti-HIV-1 monoclonal antibodies (mAbs) C2F5, C2G12, and C4E10 when administered together in a gel vehicle as a vaginal microbicide. The trial was conducted from 29th September 2009 until the 29th July 2010 on the HYMS Experimental Medicine Unit (HYMS EMU), York Hospital. It was the first study to be conducted in this unit, which opened in April 2009. Professor Charles Lacey was the Chief Investigator (CI).

2.1.1 Involvement of the author in trial design, conduct and data interpretation

The author of this thesis was the principal Study Physician (SP) and co-ordinating investigator, responsible for overseeing recruitment, dosing, sampling and evaluation of all study participants and liaison with laboratory investigators and third parties. In addition, she was involved in producing the trial protocol, patient information sheets and revised statistical analysis plan. Although the author performed the microscopy at screening and assisted the laboratory technician with initial processing of samples, she did not perform the main laboratory analyses. However, analysis and interpretation of the data arising from the pharmacokinetic assays and vaginal flora analyses was performed by the author, in addition to evaluation of the safety data, and she participated in the qualitatative analysis of transcripts from the interview sub-study.

2.2 Research Governance and Approvals

The trial was co-Sponsored by the University of York and York Teaching Hospital NHS Foundation Trust. Prior to study commencement, Clinical Trials Authorisation was sought and obtained from the MHRA and a favourable opinion was granted by Cambridgeshire 1

NHS Research Ethics Committee (REC). NHS permissions were obtained from the North and East Yorkshire Research and Development (R &D) Alliance.

All advertisements, patient information sheets, consent forms and instruction leaflets used in the study were pre-approved by the Sponsor and the REC.

The trial was conducted in accordance with the UK Clinical Trials Regulations and all study staff were trained in Good Clinical Practice (GCP). Monitoring and data management was performed by Covance Clinical Research Unit, Leeds in line with GCP requirements. All study gels were manufactured to EU Good Manufacturing Practice (GMP) standards by Polymun Scientific, Vienna.

The Trial was registered on the European and International Clinical Trials Databases- EudraCT (2008-000312-32), ISRCTN: 64808733.

2.3 Research Question and Rationale

2.3.1 Primary Study Objective: Assessment of local mAb pharmacokinetics following vaginal application

The primary objective of the study was to assess the pharmacokinetics of C2F5, C4E10 and C2G12 when applied vaginally, as this would be crucial in guiding future development of such mAb combinations as microbicides. Specifically, we wished to investigate how long the antibodies persisted in the lower FGT, and at what concentration, as this would provide an indication of the potential duration of efficacy and recommended dosing frequency of the mAbs. An ideal microbicide would have a long duration of activity to allow dosing independent from time of coitus [388]. At present, there is very limited published data relating to the pharmacokinetics of passively applied antibodies within the FGT. Studies in mice suggest that, excluding leakage, the residence t ½ of IgG applied to the vagina is 5 ± 2 hours [510]. In the only previous study in humans, the residence t ½ of polyclonal human anti-D IgG solution (RhoGAM®, Ortho-Clinical Diagnostics, Raritan, New Jersey, USA) applied directly to the posterior vaginal fornix via a catheter, was approximately 9 hours [511].

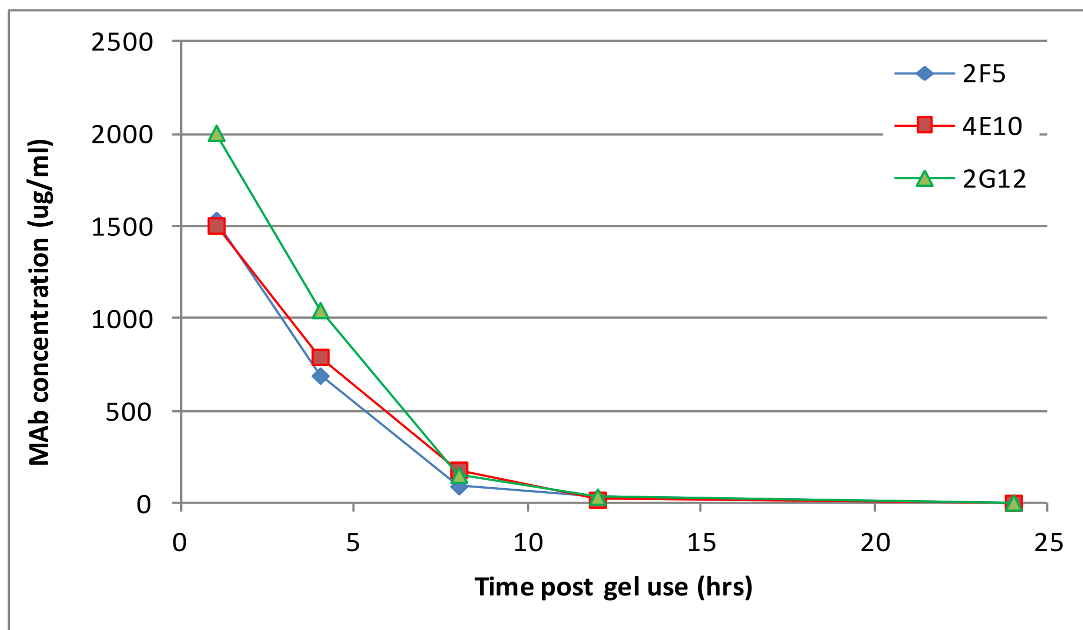
The pharmacokinetics of the mAbs applied as a single 2g vaginal application of 20mg/g Mabgel was investigated in six cynomolgus macaques by collaborators within the EMPRO consortium. All animals were sampled at baseline, then two animals were sampled at each of three time-points; 1, 4 and 12 hours post gel administration, using Weck-Cel ophthalmic sponges and cervico-vaginal lavage (CVL). mAb levels were 3.5 ± 2.3 fold higher in Weck-Cel samples than CVLs performed at the same time-points, with good correlation between both sampling methods (data not shown). In a separate experiment, following a 3 day wash-out period, a second dose of gel was applied to all six animals and samples obtained using Weck-Cels alone at baseline, 1, 4, 8, 12, 24, 48 and 72 hours. As shown in Table 2-1, concentrations of the 3 mAbs were maximal 1 hour after administration and were still detectable, in the nanogram range, at 72 hours. Concentrations up to 24 hours are displayed in Table 2-1. The investigators did not derive any estimates of t ½ for each mAb [Dereuddre-Bosquet *et al*. study THE0703, CEA animal facilities, Fontenay-aux-Roses, France, personal communication].

Table 2-1 Median, minimum and maximum mAb Concentrations detected using Weck-Cels in macaques (CEA study THE0703) following application of 20mg/g Mabgel

Time post gel use (hours)	C2F5(µg/ml)			C4E10 (µg/ml)			C2G12 (µg/ml)		
	Med	Min	Max	Med	Min	Max	Med	Min	Max
Pre dose	0.07	ND	0.69	ND	ND	ND	0.25	0.07	1.02
1	1535	944	2351	1505	1045	1969	2008	1689	3882
4	691	564	1317	793	713	1529	1046	812	2002
8	91	65	525	181	54	471	153	136	992
12	30	22	105	18	14	154	35	21	390
24	1.7	0.40	3.70	1.40	0.59	2.40	3.6	0.60	9.5
48	0.15	0.08	4.4	0.21	0.12	4.40	0.35	0.20	16
72	0.06	ND	1.6	0.30	0.06	1.60	0.06	ND	0.13

ND = not detectable

Figure 2-1 Median mAb concentrations in Weck-Cel secretion samples in macaques



In view of the invasive nature of sampling, there was a need to space out and limit the number of vaginal examinations performed on the human participants to the time-points most likely to provide the most valuable information. It was thus decided to perform sampling at baseline, then at 1, 8 and 24 hours after administration of the 1st dose of gel and perform additional samples at 12, and 36 hours post 12th dose of gel. Levels of mAbs detected at 48 and 72 hours in macaques were so low that it was decided to limit the sampling period to 36 hours post gel dose. The primary sampling method chosen was Weck-Cel ophthalmic sponges (Medtronic, Xomed), to be performed at all time-points, with CVLs providing a secondary measure at baseline and the final time-point per dose (24 hours post 1st dose, 36 hours post 12th dose). In addition, participants would be asked to perform self-taken samples, using an aspirator, at the same time-points as CVLs. Sampling is discussed further in section 2.4.1 in relation to the vaginal sampling sub-study. The schedule of study visits and procedures is displayed in Table 2-2.

2.3.2 Primary Study Objective: Determination of evidence of systemic absorption of the mAbs following vaginal application

In addition to local pharmacokinetics, we wished to establish whether there was any evidence of systemic absorption of the mAbs following vaginal application. Our

hypothesis was that the mAbs would not be systemically absorbed because of the thickness of the vaginal mucosa and their relatively large molecular weight. In support of this, no systemic absorption was detected (at limit 15ng/ml) after repeated topical application to rabbits in toxicology studies [Polymun Scientific, Mabgel Investigator Brochure (IB)]. In macaques, very low serum levels of the mAbs C4E10 and C2F5, but not C2G12, were detected following vaginal application [Dereuddre-Bosquet *et al*. study THE0703, CEA animal facilities, Fontenay-aux-Roses, France, personal communication]. These levels were very close to the quantification limit of the assay and in all but one animal were not significantly higher than baseline (pre-dose) levels suggesting that they may represent spurious background detection by the assay rather than a true '*in-vivo*' result. However, in a single animal, a significant increase (around 100µg) was detected at 12 and 72 hours post dose; thus, leaving open the possibility that some minor systemic absorption may have occurred.

It is noteworthy, that the primates, but not the rabbits, had all been pre-treated with DMPA to synchronise their menstrual cycles. This has the secondary effect of thinning the vaginal mucosa and may have facilitated absorption into the blood-stream. It was therefore decided that it was necessary to investigate whether there was any evidence of systemic absorption in healthy women and blood samples would be obtained at baseline, 8 hours post 1st dose and 12 hours post 12th dose. The latter two time-points were felt to cover the time-period where peak absorption was most likely to occur.

2.3.3 Secondary Study Objective: Assessment of the local and systemic safety of the mAbs when applied vaginally

This trial was designed to assess both the pharmacokinetics and the safety of the mAb combination when applied vaginally. However, given that the antibodies had already been administered systemically to HIV infected men and women without any serious adverse events [501,502,504,505,506], and gels with similar compositions and formulations as the gel vehicle and placebo had been evaluated previously in microbicide safety and efficacy studies [512,513,398,399,415], it was expected that the IMP would be safe when applied vaginally. Repeat dose toxicity evaluation in rabbits, and application in macaques, in the CEA studies, suggested the Mabgel was generally well tolerated

[Polymun Scientific, Mabgel IB]. Thus, assessment of the local and systemic safety of Mabgel was a secondary objective of the study.

One concern that had been raised previously was the potential auto-reactivity of the mAbs. Although the generation of individual broadly-NAbs during chronic HIV-1 infection is more common than previously appreciated, antibodies against the MPER region are relatively rare [162,514,515162], with most NAbs targeting gp120 [156,157,158,160,161]. It has been suggested, therefore, that they may arise as a result of atypical B cell induction pathways and potentially bind host ('self') epitopes [515,516,517]. Initially it was shown that both 2F5 and 4E10, but not 2G12, displayed auto-reactivity *in-vitro* through binding to various phospholipids, histones and other auto-antigens [516]. Further analyses and experiments in relation to the human clinical trials were thus performed to investigate the possibility of *in-vivo* autoreactivity and possible thromboembolic disorders associated with 4E10, 2F5 and 2G12. During the last trial the study design was modified to collect prospective data from the last four high dose patients. Very mild (<1.25 x ULN) prolongation of the activated partial thromboplastin time (APTT) 30 minutes post-infusion was observed which rapidly remitted, but there was no effect on the prothrombin time (PT). *In-vitro* experiments indicated that the effect on coagulation profile was mediated by 4E10. Further analyses have indicated that 4E10, but not 2F5 or 2G12, shows low-level cross-reactivity with cardiolipin, and that infusion of 4E10 resulted in transient low anti-cardiolipin antibody titres [506,518]. Of interest similar transient rises in anti-cardiolipin antibodies are a recognised feature of some viral infections, including HIV-1, and are rarely pathogenic, in contrast with the 'classical' anticardiolipin associated with autoimmune diseases such as systemic lupus erythematosus [519,520,521,522]. It was concluded that there was little evidence that the mAbs conferred an increased risk of thrombosis or other autoimmune phenomena, but that monitoring of both coagulation parameters and anti-cardiolipin antibodies (aCL) should be carried out in any further clinical trials [506].

Table 2-2 Study Schedule		Screening cycle	Dosing cycle										Follow-up cycle	
Visit number		**1**	**t/c**	**2 (1st dose)**		**3**	**4**	**t/c**	**5**	**t/c**	**6**	**7**	**t/c**	**8**
Day of menstrual cycle			1	7-13	7-13	7-13	8-14	8-16	11-19	14-22	19-25	20-26	1	7-21
Study day relative to first dose		-42 to -14	-6 to -12	1	1	1	2	2-4	5-7	8-10	13	14		
Time of visit (pre/post dose)				pre-1st	1hr post-1st	8 hrs post-1st	24 hrs post-1st				12 hrs post-12th	36 hrs post-12th		
Informed Consent		x												
Randomisation				x										
Medical & other history		x												x
Physical examination		x												x
Cervico-Vaginal examination		x		x	x	x	x		x		x	x		x
Colposcopy		x		x	x	x	x		x		x	x		
GU infection screen	CT cervical NAAT	x												
	GC cervical culture	x												
	Vaginal pH	x										x		x
	Gram stain cervix	x												
	Gram stain vagina	x										x		x
	Wet mount	x												
	Candida culture			x										
	HIV antibody test	x												
	Syphilis serology	x												
Laboratory parameters	Pregnancy test	X		x										x
	Urinalysis	x												
	FBC and Biochemistry	x									x			x
	Hepatitis B/C serology	x												
	APTT/aCL	x		x		x					x			x
Pharmacokinetic parameters (for MAbs)	Serum sample			x		x					x			
	Vaginal self-sample	x					x					x		
	Vaginal Weck-Cel			x	x	x	x				x	x		
	CVL supernatant			x			x					x		
Vaginal swab for vaginal flora substudy		x										x		x
Side-effect, compliance check								x	x	x	x			
Qualitative interview														x

2.4 Sub-Studies

In addition to the main study aims and objectives outlined above, some additional exploratory objectives were identified and investigated by means of 3 sub-studies conducted alongside the main study.

2.4.1 Investigation of the reliability of the Weck-Cel technique for collection of vaginal secretions (for antibody quantification) post gel application and comparison of participant self-sampling to sampling by clinicians

A range of techniques have been used to sample genital tract secretions in HIV prevention trials, each with their own advantages and disadvantages [523,524]. Two of the most widely utilised methods for collecting secretions for the analysis of antibodies or cytokines are Weck-Cel Ophthalmic sponges (Weck-Cels) and CVL. Weck-Cels have the advantage over CVL in that they enable the collection of neat secretions, and provided that the collected secretion volume is measured and a known volume of buffer is used during processing, allow accurate quantification of mucosal immune factors [525,526,527]. However, correction for 'dilution factors' is not performed universally and has not been formally evaluated on samples obtained following the application of a gel.

CVL involves the introduction of a known volume of saline (normal or phosphate-buffered) into the vagina but results in a range of different recovered volumes due to variability in clinician technique, absorption and amount and consistency of secretions already present in the vagina [528,529]. Lithium chloride can be added as a tracer to CVLs to help quantify the dilution factor in recovered fluid but its analysis is time-consuming and requires the use of specialized equipment such as a flame atomic absorption spectrophotometer, hence is not routinely performed [523,528]. In addition, since introduction of saline washes the vaginal mucosa, CVL is unsuitable for obtaining serial pharmacokinetic samples of topically applied agents such as microbicides. Both techniques require the insertion of a speculum by a clinician, which, whilst enabling

greater standardization and concurrent clinical evaluation, restricts sampling to clinic visits and can be poorly tolerated by participants.

Several collection devices have been developed to allow patients and trial participants to sample their own genital tract secretions. Some, such as tampons, swabs and self-lavages, have been shown to produce reliability and validity for the detection of infections that is equal to or, in the case of gonorrhoea and chlamydia, better than that of clinician taken samples, and are deemed acceptable by most users [530,531,532,533,534,535]. Others, such as aspirators and vaginal cups, have the potential to allow the collection of up to 10 times greater volumes of neat secretions than current methods, including Weck-Cels [536,537].

A volumetric vaginal aspirator was developed (Rovumeter; Recipe Pharmaceuticals, Munich, Germany) and has been shown to produce adequate, reproducible volumes of vaginal secretions for analysis in studies measuring ARV drug levels [538,539]. These studies were conducted by Professor Angela Kashuba and co-investigators at the Center for AIDS Research, University of North Carolina, Durham, North Carolina, USA and involved samples being collected both by clinicians (via speculum) and participants (self-collection) at different time-points using the aspirator. However, the device has not been validated by comparison with Weck-Cel or CVL sampling.

Thus, the objectives of this sub-study were to:

1) Quantify the degree of variability in weights/volumes of secretion samples obtained using Weck-Cels and explore the relationship between sample weight/volume and detected 2F5, 4E10 and 2G12 mAb concentrations

2) Compare the concentrations of mAbs measured in clinician-obtained CVL and Weck-Cel samples with those in participant self-taken aspirate samples and;

3) Ascertain whether self-sampling with the aspirator was 'user-friendly' and acceptable to study participants. NB: this was performed as part of the interview sub-study (see section 2.4.3 and Chapter 5).

Ascertaining that Weck-Cel sampling was reliable in our study would support its continued use in microbicide trials for obtaining mucosal secretions (for analyses of both natural and topically applied immune mediators) post gel application. If self-sampling using the aspirators was found to be effective and acceptable, then it could broaden their use in subsequent studies, allowing sampling outside of clinic visits and fewer invasive examinations.

2.4.2 Investigation of the impact of Mabgel on vaginal flora

As described in section 1.4.1, commensal vaginal bacteria, in particular lactobacilli, are an important defense against pathogens, including HIV-1. Various different species of lactobacilli have been isolated from the human vagina, the most dominant being the hydrogen peroxide-producing species *L. crispatus* and *L. jenseni* [540,541]. Other common species include *L. gasseri, L. iners* and *L. vaginalis* [542,543].

Bacterial vaginosis (BV) is a perturbation of the normal vaginal flora characterized by an overgrowth of mostly anaerobic bacteria and a decreased prevalence of *Lactobacillus* species. It is symptomatic in half of affected women, causing a white homogenous discharge with a 'fishy' smelling odour [544]. Although associated with a number of different bacteria and mycoplasma, *Gardnerella vaginalis*, *Atopobium vaginae* and *Mobiluncus curtisii* are considered to be the most characteristic organisms for BV [545]. In contrast to lactobacilli, BV associated bacteria produce amines as opposed to lactic acid, resulting in an increased vaginal pH, and secrete a number of immunomodulatory and pro-inflammatory substances, including sialidases, proteases, lipopolysaccharides, lipoteichoic acids and peptidoglycans [546,547,548].

BV can cause sequelae such as post-procedural pelvic inflammatory disease (PID), post-partum endometritis, and pre-term delivery [549,550,551]. It has been associated with increased incidence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection, and related PID [552], and a greater risk of both HIV-1 acquisition [14,211,214,215,216] and transmission [218].

It is, therefore, important to assess any effects on the vaginal flora when determining the local safety of a microbicide [553]. To date, this has been performed primarily

through the serial evaluation of vaginal pH, microscopy (using Nugent scoring) and culture, before, during and after product use [554,555]. However, studies using molecular, culture-independent techniques have shown that the flora in many human microbial environments, including the vagina, are much more complex than previously appreciated and that conventional culture techniques may only detect a small fraction of the microbes in the environment [556]. In addition, there remains incomplete understanding of the failure of early microbicide candidates and it is possible that even subtle perturbations of the vaginal flora may have an impact on HIV acquisition, particularly in areas of high HIV prevalence [557]. Since it is now possible to identify and quantify individual organisms in the vaginal flora using the polymerase chain reaction (quantitative (q) PCR) [558,559,560] it was felt prescient to utilize such techniques in the assessment of Mabgel.

Dr Tania Crucitti and Dr Vicky Jespers (Institute for Tropical Medicine, Antwerp, Belgium) are colleagues from within the EMPRO Consortium who have been developing qPCR techniques for the analysis of vaginal flora in microbicide trials and agreed to perform the laboratory analyses for this sub-study.

The working hypothesis for the sub-study was that there would be no difference in the vaginal flora at the species level between participants in the placebo and Mabgel arms throughout the trial. A single additional vaginal swab and a vaginal secretion smear (slide) for microscopy were taken in all participants (i) before dosing (Screening Visit), (ii) at the end of dosing (Visit 7), and (iii) during the next menstrual cycle (Visit 8), to assess any changes in the vaginal flora caused by the use of the study gels. The slides were Gram-stained and interpreted according to the standard methodology described by Nugent *et al*. [561], whereas the swabs were processed for assessment of *Lactobacillus* species by qPCR. Methodology is described in more detail in section 2.11.4. Further analysis of the swab samples for BV associated organisms is planned, but due to logistical and time constraints, this will be performed in conjunction with samples from another microbicide trial and reported separately.

2.4.3 Investigation of participants' experience of taking part in the clinical trial

The third sub-study was designed to evaluate participants' experience of taking part in the MABGEL 1 trial. This took the form of a qualitative interview conducted at the final follow up visit by a member of the research team who had no other involvement with the trial. This sub-study is discussed and its findings presented in Chapter 5.

2.5 Selection of Study Participants

2.5.1 Number and Source of Study Participants

As this was the first intra-vaginal administration of the mAbs in humans, there was no information on which to base power calculations. We aimed to recruit and randomise 30 healthy HIV negative female volunteers (10 per arm) as this seemed reasonable and achievable given the nature of the study. Women were recruited from York and surrounding areas via advertisements placed at the University of York, York Teaching Hospitals NHS Foundation Trust, and in local media (websites, newspapers, magazines, radio interviews). The advert mentioned the need for healthy volunteers, the age and gender of volunteers required, and the main purpose of the study. It also explained that participants would be 'reimbursed for their time and expenses'; however, the actual amount of money available was not stated in the advert. For details of financial reimbursement to participants see section 2.10.

2.5.2 Informed Consent and Screening Procedures

Women aged between 18 and 45 who responded to the advertisements by email or telephone were sent detailed written information about the study (the 'full' participant information sheet (PIS)). In view of the relatively intimate and complex nature of the study, an abridged, 'brief' version of the PIS containing a summary of key facts about the study and the procedures involved was also provided. Both versions were also available to download from the York HIV Research Group website, details of which were provided

on the adverts. Having been given at least 24 hours to read the study information, women who remained interested in taking part were invited to attend for screening.

At the screening visit, following an in-depth discussion about the trial and an opportunity to ask questions, written informed consent was obtained from potential participants by the SP. This took place prior to commencing any trial-related procedures. Following consent, details of socio-demographic characteristics and a thorough medical history were taken. A physical examination was performed including an evaluation of the lower genital tract by colposcopy (see section 2.8.3). An assessment of vaginal pH, a wet mount, and a slide for Gram stain and microscopy were performed as were cervical samples for *Neisseria gonorrhoea* (GC) (culture) and *Chlamydia trachomatis* (CT) (nucleic acid amplification test).

A urine sample was provided for urinalysis (blood, protein, leucocytes, nitrites and glucose) and pregnancy testing. Blood tests were performed for baseline haematological (full blood count (FBC), coagulation profile (PT, APTT)), biochemical (urea and electrolytes (U and Es), liver function tests (LFTs)), and serological (HIV, hepatitis C virus (HCV), hepatitis B virus (HBV), Syphillis, anti-cardiolipin (aCL)) testing. Permission was obtained from participants to inform their general practitioner (GP) regarding their involvement in the study and to seek verification from their GP of their medical suitability via a short questionnaire. Participants were informed of any infections or other abnormalities detected on screening and offered treatment in the York Sexual Health Clinic or referral to their GP as deemed appropriate.

2.5.3 Eligibility

Eligibility was determined by the SP, in accordance with the inclusion and exclusion criteria for the study, on receipt of results from screening investigations and GP response.

Participants were eligible for the study if they were HIV negative, at low risk of acquiring HIV and using a reliable method of contraception as defined in the study protocol (combined oral contraceptive (COCP), desogestrel-containing progesterone only oral contraceptive (POP) (Cerazette®, Organon), etornogestrol inplant (Implanon®, Organon), depot medroxyprogesterone acetate (DMPA) injections (Depo-Provera®, Pfizer), intra-

uterine contraceptive device (IUCD) or levornogestrel-releasing system (IUS) (Mirena®, Bayer) or condoms with every coital act). They had to be in good health, as determined by medical history, clinical examination, and GP report, with no clinically significant abnormalities on baseline blood tests. In view of the mild transient prolongation of the APTT seen previously when high doses of the mAbs were given intravenously [506], any history of thrombotic or other coagulation disorders, or baseline aCL IgG antibody positivity was considered a bar to study entry. Also for safety reasons, women who were pregnant, within 12 weeks postpartum, or breast feeding and those who had participated in another clinical trial within the previous 2 months were also unable to take part.

To minimise the risk of cervico-vaginal bleeding during the dosing phase of the study, only women who had a regular menstrual cycle (>26 days) or had been amenorrhoeic for at least 3 months (as a result of contraceptives) were included. Women who gave a history of post-coital or inter-menstrual bleeding, previous or current genital ulceration, or had another condition that in the opinion of the SP and/or CI might interfere with the evaluation of study objectives, were considered ineligible. Also excluded were those who had received treatment for cervical-intraepithelial neoplasia (CIN) or undergone gynaecological instrumentation of the cervix within the past three months. Women with untreated urinary tract infections, GC, CT, trichomonas or BV were also ineligible (although participants were eligible after successful treatment and re-screening). To minimise the risk of interfering with gel pharmacokinetics or creating any trauma that could be falsely attributed to gel use, participants had to be willing to abstain from using tampons or any genital preparations, other than the study gel, during the period of gel administration until after the final pharmacokinetic sampling visit (36 hours after the last dose). They also had to agree to abstain from any receptive oral or vaginal intercourse from 48 hours before using the first dose of gel until after the final pharmacokinetic sampling visit.

Participants were allowed to be taking concomitant medications at study entry provided, in the opinion of the SP and/or CI, they had no significant current medical problems that would render them ineligible.

2.6 Study Interventions

2.6.1 Randomisation and blinding

Participants who provided informed consent and were still eligible after screening were invited to attend in their next menstrual cycle for the first dose of the vaginal gel. Participants enrolled into the dosing phase of the study were allocated consecutive subject numbers (MAB001 onwards) at their first dosing visit. The subject numbers were recorded on the participant log in the trial master file. This subject number was used to identify the participant throughout the trial and was used on Case Report Forms (CRFs), samples for external analysis and study documentation. Note: participants were also allocated a separate number, the screening identification number (SIN), for the purposes of performing investigations at the screening visit only. In this report, to avoid confusion, participants will be referred to by their subject number only.

Randomisation was carried out by the York Trials Unit, University of York. Subject numbers 01 to 30 (MAB001 to MAB030) were randomised to Mabgel (high dose), Mabgel (low dose) or placebo using blocked randomisation of mixed block sizes. Randomisation was in the ratio of 1:1:1. The randomisation program was generated using Microsoft Visual Basic and a copy of the generated data stored in Microsoft SQL Server. The randomisation list was forwarded to the Qualified Person (QP) at Polymun Scientific for the purposes of labelling the study gel. However, laboratory personnel carrying out subsequent analyses, including Dr Vcelar, did not have access to this list.

An emergency unblinding website was established by the York Trials Unit so that individual participants could be unblinded in the event of a serious adverse event (SAE) where knowing the treatment group of the participant might be of benefit in terms of treating them or protecting other participants. Access to the website was password protected. If accessed, the website was set-up to generate an email notification to the study Sponsors and CI and the password would be changed. Where possible, every effort was to be made to avoid unblinding any Clinical Investigators.

2.6.2 The Study Gels

The gels were manufactured by Polymun Scientific in accordance with Good Manufacturing Practice (GMP), supplied in 3ml luer lock syringes as a single batch and stored in a refrigerator (2 to 8 °C) prior to being dispensed. Syringes were individually labelled and each one was separately packaged. Twelve syringes were provided per participant in a re-sealable plastic bag.

The study gels consisted of 2.5ml of extractable volume of gel per dose containing 20mg/g (Mabgel high dose), 10mg/g (Mabgel low dose) or zero (placebo) of each of the mAbs in a 1.6 % HEC-based gel. Each of the formulations had the same quantitative composition of excipients and differed only in their antibody contents (Table 2-3). Each syringe of gel dispensed contained one dose and the gel was transferred into an Ortho® vaginal applicator (Janssen- Cilag) before use.

As the gel was labelled as needing storage at 2 to 8 °C it was a requirement that each participant should have access to a refrigerator for storage of the study gels to be eligible for the study and they were instructed to keep the gels in the refrigerator until shortly before administering each dose.

Table 2-3 Composition of Study Gels

	Composition (per 1g)		
	Mabgel (high dose)	**Mabgel (low dose)**	**Placebo**
mAbs	20 mg C2F5 20 mg C2G12 20 mg C4E10	10 mg of C2F5 10 mg of C2G12 10 mg C4E10	0
Hydroxyethylcellulose	16 mg	16 mg	16 mg
Glycerin	25 mg	25 mg	25 mg
Methylparaben	1.8 mg	1.8 mg	1.8 mg
Propylparaben	0.2 mg	0.2 mg	0.2 mg
Maltose	50 mg	51 mg	52 mg
Purified water	847 mg	876 mg	905 mg

2.6.3 Administration of the Study Gel (Gel Dosing)

Dosing with the randomised study gel began between days 7 and 13 of the menstrual cycle following the screening visit (2 to 6 weeks after screening). Women who were amenorrhoeic were assigned an arbitrary 28 day cycle to ensure visits occurred within the specified schedule (see Table 2-2). The first dose of the study gel was administered in the HYMS EMU under the supervision of the SP. Before being issued with the remaining 11 gels and Ortho® applicators, participants were given a demonstration by the SP on how to transfer the gel into the Ortho® applicator, and were witnessed performing a practice transfer using a 'dummy' gel. They were also provided with written instructions on storing, preparing and administering the gel. Provided there were no safety concerns, the participant proceeded to use the study gel at home on a daily basis for the following 11 days.

Due to scheduled evaluations at 1 and 8 hours post gel administration, the initial dose was applied in the morning. The 12th dose was applied at a pre-arranged time in the evening in accordance with when each participant was scheduled to attend for their 12 and 36 hour post 12th dose visits e.g. the gel was applied at 10pm if they were due to attend at 10am. Participants were instructed to apply other doses at night at bedtime as this was thought to be the most convenient time.

All participants were offered daily text message reminders from the research team as an aide memoire. They were advised to contact the EMU before their next scheduled visit if they encountered any problems with using the gel or had any queries. In addition they were provided with a contact card containing details of how to contact the research team outside of normal working hours. They were advised to always contact the team if they had an urgent problem or if an emergency situation arose and to inform any health care professionals treating them of their involvement in the study.

2.6.4 Measures of Compliance and Adherence

Compliance/adherence with using the gel was assessed by history, diary cards and by inspection of returned syringes. At each visit and in telephone calls, participants were asked whether they had remembered to use the gel and if they had encountered any

difficulties with applying it. Participants were issued with a diary card and asked to record the time and date each time they used the gel. All participants were asked to retain used syringes and return them and any unused syringes to the clinic at the 12 hours post 12th dose sampling visit. All returned syringes were inspected by the SP or Research Nurse and assessed to see whether they had been fully or partially emptied or not emptied at all. All used syringes were stored at the trial site until after the trial monitor had verified their return, and were subsequently disposed of as clinical waste.

Compliance with sexual abstinence and avoiding tampons and vaginal products was assessed by history at visits and telephone calls. Cervico-vaginal lavage samples were also assessed for the presence of spermatozoa prior to processing. In addition, women were asked regarding sexual activity during the study as part of the qualitative interview (see Chapter 5).

2.7 Pharmacokinetic Evaluations

As discussed in section 2.3 the primary objective of the study was to assess the local and systemic pharmacokinetics of C2F5, C2G12 and C4E10 when applied vaginally. In addition, one of the exploratory objectives was to compare participant self-taken samples with those taken by a physician. The time-points for sampling and hence outcome measures were chosen with the aim of providing an adequate number and spread of samples for analysis whilst minimising the number of invasive genital examinations and venepunctures required for each participant.

2.7.1 Pharmacokinetic Outcome Measures

The primary pharmacokinetic end-points for this study were defined as:

1. Levels of mAbs in Weck-Cel vaginal secretions 1, 8 and 24 hours post-1st dose, 12 and 36 hours post-12th dose

The secondary pharmacokinetic end-points for this study were defined as:

1. Levels of mAbs in cervico-vaginal lavage samples 24 hours post-1st dose, and 36 hours post-12th dose

2. Levels of mAbs in participant self-taken vaginal aspirate samples 24 hours post-1st dose, and 36 hours post-12th dose.
3. Levels of mAbs in serum samples 8 hours post-1st dose, and 12 hours post-12th dose.

2.7.2 Pharmacokinetic Sampling Procedures

This section describes the methodology for collecting samples for mAb pharmacokinetic analysis. Laboratory procedures for processing and analysis of samples are described in section 2.11.

2.7.2.1 Overview of sampling procedures

Women were examined in the lithotomy position using a disposable plastic vaginal speculum and samples were taken for measurements of mAbs at baseline (pre- 1st dose of the study gel), 1 hour, 8 hours and 24 hours post- 1st dose, 12 and 36 hours post-12th dose (see Table 2-2). 3 different sampling methods were used to collect genital secretions at times corresponding to the pharmacokinetic end-points outlined above. Weck-Cel and Cervico-vaginal lavage samples were performed by the SP and vaginal aspirates were taken by the study participants themselves. Standard Operating Procedures (SOPs) were devised and followed to try to minimise any variability.

Note: baseline pharmacokinetic samples were performed shortly before administration of the 1st dose of study gel, except for the 1st vaginal aspirate which was performed at the screening visit. This was done for logistical reasons to provide more time to instruct participants on the sampling method, and also to allow colposcopic evaluation to be carried out following the procedure to assess whether the aspirator caused any visible trauma. All sampling at other time-points was performed after initial colposcopic assessment (see section 2.8.3). This enabled appearances to be assessed before any samples were taken to avoid any trauma or bleeding that may have arisen as a result of the sampling procedure being erroneously attributed as having been caused by the study gel.

2.7.2.2 Order of sampling

To further standardise the sampling procedure, samples were taken in the following specified order, according to which were required at each visit.

1. Vaginal participant self- collected aspirate sample
2. Vaginal Weck-Cel
3. High vaginal swab and slide for the vaginal flora sub-study (see section 2.4.2)
4. High vaginal swab for candida culture and speciation (see section 2.7.2.6)
5. Cervico- vaginal lavage

2.7.2.3 Weck-Cels

Vaginal secretions were sampled primarily using Weck-Cel Ophalmic Sponges (Medtronic Xomed) (referred to as Weck-Cels), which were used at all pharmacokinetic sampling time-points. Weck-Cels are sterile, triangular- shaped cellulose sponge swabs on a polypropylene stem designed for wicking secretions during eye surgery. They have previously been used successfully by other research groups for measuring antibody levels in genital secretions following vaccination [525,526,527] and in HIV-1 positive individuals [562]. 2 Weck-Cel samples were taken per participant at each time point. Each Weck-Cel was grasped using a pair of disposable sponge forceps, placed inside the lateral fornix and held against the vaginal mucosa for 1 minute. Weck-Cels were placed in succession, one into each of the vaginal fornices. After removal, each Weck-Cel was placed sponge- end down into a Spin-X centrifuge tube (Corning) containing 300µl of extraction buffer. Each Spin-X tube was housed inside a 15ml centrifuge tube (Corning). Following sample collection, each Weck-Cel stem was removed to allow tube closure and avoid spillage but was retained to enable a post collection, pre-processing weight to be measured.

2.7.2.4 Cervico-vaginal lavages

CVLs were performed using 5ml of sterile normal saline at baseline, 24 hours post 1st dose and 36 hours post 12th dose. These were performed using a 10ml syringe with a plastic universal Kwill attached. Taking care to avoid contact between the vaginal mucosa and

the tip of the Kwill, the saline was gently expelled from the syringe to irrigate the lateral and posterior vaginal walls and fornices. The lavage fluid was then aspirated from the pool in the posterior fornix and transferred into a 15ml centrifuge tube.

2.7.2.5 Participant self-taken vaginal aspirates

Participant self-taken vaginal aspirates were performed using syringe-based volumetric devices (Rovumeter; Recipe Pharmaceuticals, Munich, Germany) which were designed for this purpose and were obtained from Professor Angela Kashuba, Centre for AIDS Research, University of North Carolina, Chapel Hill, North Carolina, USA. As mentioned in section 2.4.1, these aspiration devices have previously been used successfully to collect vaginal secretions for the analysis of levels of antiretroviral agents [538,539]. Participants were given verbal instructions and a demonstration by the SP on how to perform the procedure before being asked to self-sample their own vaginal secretions. These instructions were based on written instructions obtained from Professor Kashuba which had been provided to participants in one of her studies [539]. Before sampling, participants were required to lie supine on a couch for 15 minutes to allow time for pooling of secretions in the posterior fornix.

After removing the syringe from the packaging, the participants were advised to pull the plunger of the aspirator up and down to loosen the plunger and prevent it from sticking to the syringe. With the plunger pushed to the bottom of the syringe, the participants were instructed to gently insert the device into the vaginal canal until it touched the back of the posterior fornix - ‘push backwards but not upwards as far as the applicator with go until you feel it touch the posterior vaginal wall’. To perform the aspiration, the advice given was to ‘pull the entire syringe out slightly to avoid suction of tissue. With one hand holding the syringe in place, use the other hand to gently pull out the plunger to aspirate fluid. Stop pulling on the plunger if excess pressure causes discomfort. Continue to pull back on the plunger with one hand as you remove the syringe from the vagina with the other hand’.

After removing the syringe from the vagina, the participant handed the syringe to the SP or a research nurse who placed the tip into a 15ml centrifuge tube (Corning). Any

secretions collected were expelled by fully depressing the plunger, making sure the tip touched the side of the tube.

2.7.2.6 High Vaginal Swab for Candida Culture and Speciation

Species of the yeast Candida are common commensal organisms which colonise the vagina in 10 to 20 % of women of reproductive age without causing overt signs or symptoms (candidosis) [563,564]. The occurrence of clinical signs or symptoms related to candida e.g. vulvovaginitis (redness, soreness, itching, curdy adherent vaginal discharge), known as candidiasis, may result from a high organism load or from an interplay between the host immune system and Candida [565,566,567,568]. As many of the features of clinical candidiasis can mimic side-effects caused by topical microbicides it was important to screen for this infection at baseline and consider it as a differential cause of local adverse events (AEs) (see section 4.1.1.12).

In addition, Dunlop *et al.* have reported cross-reactive binding of C2G12 to the most common species of candida, *C. albicans*, and to the less common *C. tropicalis*, but not to *C. glabrata* or the related yeast *Saccharomyces cervisiae* [569]. It is therefore theoretically possible that the presence of certain Candida species in the vagina could have an impact on measurable levels of C2G12 due to a sequestration effect.

Thus, in our study, as is standard in microbicide trials, women with clinically or microscopically apparent Candida infections detected at screening were deemed screen failures and, unless treated and successfully re-screened subsequently, were not enrolled into the dosing phase. In addition, in view of the delay between screening and dosing (up to 6 weeks) and the possible pharmacokinetic interaction, it was decided to perform a high vaginal swab for quantitative Candida culture and speciation just before the 1st gel dose was given. It was envisaged that results of this test would not be available until the end of the dosing period, thus the presence of Candida would not exclude someone from dosing. However, the results would enable any potential impact on C2G12 analyses to be subsequently determined and could also be used as an adjunct in evaluating clinical AEs.

2.7.2.7 Serum

Serum samples for mAb analysis were obtained at the specified time-points by the SP or Research Nurse. 7.5ml of blood were collected into a brown top S-Monovette® (Sarstedt, NHS Supplies) serum blood collection tube.

At time-points where blood samples for safety analyses were also required, the samples were collected in the order recommended by the Clinical and Laboratory Standards Institute (formally the National Committee for Clinical Laboratory Standards (Standard H3-A6, 2007) to ensure minimum potential interference of tube additives on subsequent sample analyses [570].

2.7.3 Sampling windows

Sampling windows were determined in advance for each pharmacokinetic time-point:

- For the pre-1st dose samples, sampling had to be carried out on the same day as and prior to the delivery of the 1st dose.
- For the 1 hour post-1st dose time-point, sampling had to be carried out within 15 minutes less and 30 minutes more than the time indicated.
- For the 8 and 24 hour post-1st dose, and 12 and 36 hour post-12th dose time-points, sampling had to be carried out within 1 hour (either more or less) than the time indicated.

2.7.3.1 Timing of sample taking

The time to the nearest minute at which samples were taken was recorded from the start of each sampling procedure. That is from when the participant inserts the vaginal aspirator, the first Weck-Cel is inserted, the syringe and Kwill are inserted for CVL irrigation, or the needle is inserted into a vein for venepuncture.

Any samples taken outside of the sampling windows for each time-point were documented as protocol deviations.

2.8 Safety Evaluations

As discussed in section 2.3, the secondary objective of the study was to assess the safety of C2F5, C2G12 and C4E10 when applied vaginally.

2.8.1 Safety-Related Outcome Measures

The main safety-related secondary end-points for this study were defined as:

1. The number of grade 3 or above genital AEs during the dosing or follow up period.
2. The number of grade 3 or above other clinical or laboratory AEs confirmed at examination or on repeat testing respectively during the dosing or follow up period.
3. The number of events attributable to the study gel (i.e. adverse reactions (ARs)) leading to discontinuation of gel.

Additional safety end-points that would be analysed were:

- All grades of genital AEs.
- All grades of non-genital AEs (clinical or laboratory findings).
- Coagulation parameters (APTT and aCL)- if systemic absorption of the mAbs was detected in serum samples.
- Identification of *Lactobacillus* species, in vaginal secretions (to be analysed and reported as part of the vaginal flora sub-study). *Gardnerella vaginalis* and *Atopobium vaginae* (to be reported later).

2.8.2 Safety-Related Definitions and Classifications

Unless stated otherwise, all definitions followed for the purposes of safety-reporting in this study were obtained from the UK Medicines for Human Use (Clinical Trials) Regulations 2004 [571].

2.8.2.1 Definition of an Adverse Event

An Adverse Event is any 'untoward medical occurrence in a subject to whom a medicinal product (in this case, study gel) has been administered, including occurrences which are not necessarily caused by or related to that product'.

An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of an investigational medicinal product (IMP), whether or not considered related to the IMP.

In this study, however, in line with the North and East Yorkshire R & D Alliance Research Related Adverse Event Reporting Procedures, we were requested by the trial Sponsors to collect details of any untoward events arising in all trial subjects from the time of their providing informed consent at screening, whether the study gel had been administered to the subject or not. However, any AEs occurring prior to 1st gel administration were to be considered to be unrelated to use of the study gel and excluded from the main analyses.

2.8.2.2 Determination of Causality in Relation to Study Gel Administration

A judgment was made by the SP, in consultation with the CI, for each AE in relation to expectedness and likely relationship to the study gels. This was based on clinical judgement with reference to AEs observed in the previous human trials, where the mAbs were administered intravenously, and to data from animal studies (rats, rabbits and non-human primates) in which the Mabgel was applied vaginally, as reported in the MABGEL IB.

2.8.2.3 Definition of an Adverse Reaction

'Any untoward and unintended response in a subject to an IMP related to any dose administered to that subject' is defined as an adverse reaction (AR). Therefore, any AE for which there is at least some evidence or argument to support at least a possible causal relationship to the study gel qualifies as an AR.

2.8.2.4 Classification of AE Severity

All AEs were classified according to severity based on a pre-defined grading system. This system was based on the genital tract and clinical gradings devised and used by the UK Medical Research Council Clinical Trials Unit (MRC CTU) in the Microbicides Development Programme [572] and the laboratory gradings defined by US National Institute of Health Division of AIDS [573]. Common genital, other clinical and laboratory AEs were listed in tables with clearly defined cut-off boundaries for each grade.

2.8.3 Procedures for Assessing Safety

Clinical AEs were broadly categorised according to whether they were local to the genital tract, or affected an organ or body system outside.

Data on clinical AEs were collected through open questions at face-to face visits and during telephone calls. In addition, genital AEs were specifically solicited using questions related to unexpected vaginal bleeding, genital discomfort (itching, burning, soreness), discharge, dysuria and dyspareunia and from findings on colposcopy including erythema, oedema, and epithelial disruption. Findings were recorded on study proformas then transcribed onto CRFs.

Colposcopy was performed by the SP on all occasions using a Zeiss model 22/024282 colposcope. This was conducted in general accordance with the CONRAD/WHO Manual for the Standardization of Colposcopy for the Evaluation of Vaginal Products (2004), however no saline lavages or swabs were used apart from the study samples specified in the protocol to avoid impacting on pharmacokinetic evaluations [574]. Images were recorded using a USB200 Medicapture Unit to allow comparison across subsequent visits and verification by the CI.

Laboratory AEs were detected through blood or urine tests performed at scheduled time-points. Results were provided by York Hospital Pathology Laboratories as printed reports which were reviewed by the SP within 48 hours of receipt.

Details of any medications taken by participants during the trial were recorded, and where related, used in assessing the severity of AEs.

2.8.3.1 Timing of Safety Evaluations

Genital examination, including colposcopy, coagulation assays and serum aCL antibody titres were performed before the first dose of study gel, and repeated 8 hours after the first dose to check for any immediate genital AEs or aCL reactogenicity. Colposcopy was also performed at 1 hour post the first dose at the time of pharmacokinetic sampling visit.

In addition to the pharmacokinetic sampling visits, where safety data were also obtained, follow up face-to face safety evaluations were carried out on day 5 (+/-1) post 1st dose (colposcopy) and between days 7 and 21 of the subsequent menstrual cycle (10 days to 4 weeks after the 12th dose) (clinical examination, colposcopy, blood sampling, urinalysis and pregnancy test). Questions regarding AEs and concomitant medications were also asked during telephone calls at 2 days (+ /- 1) and 8 days (+ /- 1) post 1st dose.

2.8.3.2 Safety Data Derived from Sub-Study Analyses

As outlined in section 2.4.2 the impact of the study gels on vaginal flora was assessed by Dr Vicky Jespers and Dr Tania Crucitti, Institute of Tropical Medicine, Antwerp through analysis of vaginal swabs and Gram-stained slides taken at baseline, 36 hours post 12th gel dose and in the subsequent menstrual cycle. Results of these analyses were provided at the end of the trial and are summarised in Chapter 4.

At the end of the dosing period, as part of the qualitative interview sub-study, participants were asked whether they had experienced any problems with using the gel, had noticed any abnormal vaginal discharge or whether any features of the gel (such as consistency, smell) had bothered them. Data from the interviews was analysed separately from that from the main study and is presented in Chapter 5.

2.9 Dosing Interruptions, Withdrawals and Study Termination

As a condition of providing Informed Consent, any participant was free to withdraw from the study at any time, without giving a reason, and without their clinical care being affected. Interruption or discontinuation of gel use could also occur if the participant

desired to stop using the study gel. Interruptions to dosing or study withdrawal could also be initiated by the CI for safety reasons.

According to the trial protocol, a temporary cessation of study gel use should have taken place following a grade 2 or above solicited genital AE. Dosing could be recommenced after interruption following any AE at the discretion of the CI. A permanent cessation of study gel use would have taken place following a grade 3 or above solicited genital other clinical or laboratory adverse event. In the case of 3 women experiencing either a grade 3 or above genital AE or grade 3 or above clinical or laboratory AE, the study would have been temporarily halted pending unblinded review by the Data and Safety Monitoring Committee (DSMC) (see section 2.12.3). The decision to stop the study rested with the CI, with recommendations from the DSMC. The Sponsor also reserved the right to terminate the study at any time.

Wherever possible, participants who discontinued study gel use either by choice or following advice would be encouraged to continue to adhere to the follow up schedule up to the final visit. In the event of a discontinuation due to an SAE or AE, the participant would have been followed up by the study team until completion of the event.

If a participant was considered a screen failure, discontinued dosing or withdrew from the study at any time, data and samples collected up to that point were to remain available for analysis in accordance with the trial protocol.

2.10 Financial Re-imbursement of Study Participants

Participants were re-numerated to cover their time and expenses during the trial. A maximum of £565 was paid to each participant, having been pre-approved by the REC, based on the following breakdown:

- Screening visit, 1st dosing visit and final visit: £80 per visit.
- Other clinic visit requiring colposcopy and/or vaginal sample collection: £65 per visit.

Payment was made by cheque at the final study visit.

2.11 Laboratory Procedures

2.11.1 Screening and Safety Specimens

Screening microscopy was performed by the SP in the EMU according to SOPs based on those used in the York Sexual Health Clinic. Wet mounts were examined at x 400 magnification using a dark-field setting for *Trichomonas vaginalis,* clue cells and yeast. Gram-stained slides, examined with a bright-field under oil at x 1000, were considered indicative of *Candida* species infection in the presence of spores and /or hyphae. A Nugent score ≥7 was considered indicative of BV as was a vaginal pH > 4.5 in the presence of clue cells and compatible white homogeneous discharge (see section 2.11.4). For safety reasons, it was not possible to use potassium hydroxide to perform an 'amine whiff test' in the EMU.

Vaginal pH testing was performed at screening and subsequent visits using a 3cm strip of pH paper (range 4.0 to 7.0, Whatman) grasped in sponge-holder forceps and held against the lateral vaginal wall. pH was determined by comparing the resulting colour change with a chart supplied with the pH paper.

Cervical swabs for *Chlamydia trachomatis* (PCR, Roche), *Neisseria gonorrhoea* (culture, chocolate agar, Oxoid (PB0963A)) and vaginal swabs for Candida culture and speciation (cultured on Sabouraud dextrose agar plus chloramphenicol, Oxoid (PO0161A); speciation by bioMerieux API 20 C AUX (20210)) were processed by the Microbiology Section of the York Hospital Pathology Laboratories according to their SOPs.

Mid-stream urine samples were tested for protein, nitrites, leucocytes and blood (Multistix®, Siemens) and for evidence of pregnancy (QuPID®-One Step, Stanbio) by research nurses in the EMU.

Blood specimens were processed for U and E, LFT, FBC, Clotting profile (APTT, PT), serology (HIV (4th generation combined antibody (Ab) and antigen (Ag) enzyme-linked immunoassay (ELISA)); Syphilis (*Treponema pallidum* enzyme immunoassay (EIA)); HCV, HBV (surface Ag), and aCL IgG) by the York Hospital Pathology Laboratories according to their SOPs.

2.11.2 Initial Processing and Storage of Specimens for mAb Analysis

2.11.2.1 Overview of Procedures for Initial Processing and Storage

Initial preparation of reagents and sample collection equipment took place either in the HYMS EMU laboratory or in the Immunology and Infections Unit (IIU), University of York. Vaginal and serum samples obtained for mAb pharmacokinetic analysis were initially processed and stored in the HYMS EMU laboratory or transported and stored in the IIU in accordance with study-specific SOPs. Following collection, all vaginal samples were transported to the relevant laboratory on ice and stored at 2 to 8 °C to be processed within 2 hours. Serum samples were kept at room temperature for 1 hour then stored at 2 to 8 °C and processed within a 4 hour window of collection. After initial processing by a laboratory technician, samples were stored in fridges and freezers which were alarmed and data-logged to ensure they were kept within the designated temperature ranges. Subsequently, samples were transported to Polymun Scientific in two batches, the first containing samples from the first 11 participants, the second containing samples from the remaining participants. Samples were transported by cold-transport courier on dry ice.

2.11.2.2 Preparation of Extraction Buffer Required for Vaginal Sample Processing

Processing of Weck-Cel, CVL and vaginal aspirate samples required use of an extraction buffer which was prepared as follows. Firstly, 1 vial of protease inhibitor (PI) cocktail set 1 (100x solution) (Calbiochem, Cat. № 539131) was reconstituted in 1ml sterile distilled water and vortexed for 30 seconds so that it dissolved completely. When diluted to 1x concentration this contained 500μM AEBSF, 150nM aprotinin, 1μM E-64 PI, 0.5mM EDTA, 1μM leupeptin. 1ml of the reconstituted PI cocktail was mixed with 20μl of a 10 % solution of sodium azide (Sigma, Cat. № 2002-25G), 1.5g Sodium Chloride (0.25 M final concentration) (BDH, Cat. № 102415k) and 1X Dulbecco's phosphate buffered saline (PBS)(pH 7.4)(prepared from 10X stock solution)(Sigma, Cat. № D148)) to make a final volume of 100ml. The resulting extraction buffer was filter sterilised and stored at 2 to 8°C for up to 1 month.

2.11.2.3 Preparation of Weck-Cel Sampling Kits

For Weck-Cel sampling, 300μl of the extraction buffer was pipetted into each Spin-X centrifuge tube (Corning Cat .N º 8160), 2 per participant per sampling time-point, prior to sample collection. Each Spin-X tube contained a 22μm pore filter insert. Each complete sample collection kit containing the Weck-Cel sponge plus the Spin-X tube containing 300μl extraction buffer was placed in a 15ml centrifuge tube (Corning Cat.Nº 430766) and weighed on a fine balance (Mettler-Toledo) so a pre sampling weight could be recorded. Weck-Cel sampling kits were stored at 2 to 8°C until sample collection.

2.11.2.4 Weck- Cel Sample Processing

Before processing, the sample collection kits were re-weighed on the same fine balance as before to determine the post-sampling weight. As mentioned in section 2.7.2.3 Weck-Cel stems which were removed to allow lid closure were retained and weighed with the rest of the sampling kit. After re-weighing, the Spin-X tubes containing the Weck-Cel sponge-heads were removed from the 15ml Corning tubes, placed in a bench-top refrigerated centrifuge (Fisher Scientific) and spun at 13,000xg for 15mins at 4°C. After centrifugation, another 300μl of extraction buffer was added to each of the Spin-X tubes containing the Weck-Cel sponge-heads and they were spun again. If the buffer had not been completely drawn through the filter, using forceps, the sponge head was transferred to a new filter, placed in the original Spin-X tube and centrifuged again for 5 minutes at the same speed and temperature as before. Filtered buffer and secretions eluted from both samples taken per time-point per participant were then combined into a single 2ml sterile-capped microtube (Sarstedt, Cat N º72-694-005). 5μl was removed and applied to Hemastix® (Bayer) testing strip for 1 min to test for blood contamination. 8μl of heat-inactivated fetal calf serum (HI-FCS) (Invitrogen, Cat. Nº 10108-165) was then added and the tube inverted gently 15 times to mix. The processed sample was then divided into 200μl aliquots in 2ml Sarstedt tubes, labelled with the participant's subject ID, visit number/sampling time point and sample type and stored at -80°C.

2.11.2.5 CVL sample processing

Sample volumes were recorded prior to processing. Samples were gently vortexed for 10 seconds. 10µl of each sample was then placed on a KOVA® slide (Hycor, Biomedical) and assessed for presence of red blood cells (RBCs) or spermatozoa by microscopy. Following the addition of 50 µl of the extraction buffer, each sample was transferred to a 15ml Corning tube and centrifuged for 6 minutes at 1000 x g at 4°C to pellet the cells. A 10µl aliquot of supernatant was assessed for presence of RBCs and sperm as before. 5µl of supernatant was applied to a Hemastix testing strip for 1min to test for the presence of blood. The supernatant was divided into 200µl aliquots in 2ml Sarstedt tubes, labelled with the participant's subject ID, visit number/sampling time point and sample type and stored at -80°C. The cell pellet was kept in the original 15ml Corning tube, labelled with the participant's subject ID, visit number/sampling time point and sample type and stored at -80°C.

2.11.2.6 Participant Self-Taken Vaginal Aspirate Processing

Aspirators were placed inside a 15ml Corning tube. The plunger was suppressed to eject any secretions, ensuring the tip of the aspirator touched the side of the tube to remove any residual sample. The tube was gently vortexed to ensure the sample collected at the bottom of the tube and the volume of sample obtained recorded. Samples taken at visits other than screening (i.e. post-baseline samples) were then diluted 1:1 with extraction buffer. Baseline samples obtained at the screening visit were stored neat. Samples were divided into 200µl aliquots in 2ml Sarstedt tubes, labelled with the participant's subject ID, visit number/sampling time point and sample type and stored at -80°C.

2.11.2.7 Serum Processing

S-Monovette® tubes (Sarstedt) containing blood samples were centrifuged at 1000 xg for 10 mins to ensure the serum was separated from the clotted blood. Serum was then removed and divided into 500µl aliquots in 2ml Sarstedt tubes labelled with the participant's subject ID, visit number/sampling time point and sample type and stored at -80°C.

2.11.3 ELISAs for Quantifying mAb Concentrations

Serum and vaginal secretion samples were analysed according to established SOPS at Polymun Scientific. The methodology has been used in the previous clinical studies involving the mAbs [501,502,504,505].

Briefly, C2F5 and C4E10 concentrations were determined by enzyme-linked immunosorbant assays (ELISA) systems using the peptides GGGLELDKWASL and KKWNWFDITNWGGG respectively for coating, and goat anti-human IgG gamma chain antibody (Zymed Laboratories Inc. 62-8420, USA) conjugated with horseradish peroxidise (HRP) for detection. Highly purified internal C2F5 and C4E10 antibodies were used as standards.

C2G12 concentrations were determined by an ELISA using the anti-idiotypic antibody M1G1 (in-house) for coating and goat anti-human IgG gamma chain antibody (Zymed Laboratories Inc. 62-8420, USA) conjugated with (HRP) for detection. A highly purified internal C2G12 antibody was used as a standard.

Quantification was performed in pre-coated 96-well microtiter plates. The standard and samples were run in a 2^8-fold dilution row. The concentration range of the standard was 1.563 - 200 ng/ml. After incubating the pre-coated plates with the added samples for one hour at room temperature and washing with PBS pH 7.2 including 0.1 % Tween 20, the conjugated antibody was added. After incubation for one hour at room temperature and washing with PBS pH 7.2 including 0.1 % Tween 20, the enzymatic colour reaction was started by addition of the chromogene substrate o-phenylenediamine dihydrochloride (OPD) and hydrogen peroxide and stopped by adding 2.5 M sulphuric acid. The absorbance was measured at 492 nm (reference wavelength 620 nm) and the results were evaluated with a fourth-degree polynomial (Software: Biolise for Windows).

The test was considered valid if the well with the highest standard protein concentration displayed an absorbance of at least 1.0 and if the absorbance of the blank vials was below 0.1. In addition, recovery of the standard must be within +/- 20% and the coefficient of variance between repeat sample wells on the same plate had to be below 15%.

2.11.4 Specimens for Vaginal Flora Sub-study

2.11.4.1 Initial processing and storage of swabs and slides

Glass slides containing vaginal secretion smears were heat-fixed then stored at room temperature. High vaginal swabs (flocked dacron swabs, Copan) were stored at -20°C. Swabs and slides were transported to Dr Vicky Jespers in a single shipment after all participants had completed the study.

2.11.4.2 Analysis of the slides

Slides were Gram stained, examined by bright-field microscopy and interpreted according to the method described by Nugent [561]. As shown in Table 2-4, this scoring system grades vaginal smears according to the visual quantification of characteristic bacterial morphotypes per oil immersion field (0 = no morphotypes; 1+ = less than 1 morphotype; 2+ = 1 to 4 morphotypes; 3+ = 5 to 30 morphotypes; 4+ = 30 or more morphotypes).
Scores assigned for each morphotype are added together to give an overall Nugent Score between 0 and 10 for each sample. Nugent Scores between 0 and 3 indicate the presence of normal flora; scores of 4 to 6 indicate an intermediate flora; scores ≥ 7 are graded as BV.

Table 2-4 Scoring System for Bacterial Morphotypes on Gram-stained Vaginal Smears

Score	*Lactobacillus* Morphotypes (large Gram positive rods)	*Gardnerella* and *Bacteroides* Morphotypes (small Gram variable rods)	*Mobiluncus* Morphotypes (curved Gram-variable rods)
0	4+	0	0
1	3+	1+	1+ or 2+
2	2+	2+	3+ or 4+
3	1+	3+	
4	0	4+	

2.11.4.3 Quantitative PCR for *Lactobacillus* Species

Real-time, quantitative PCR was performed for *Lactobacillus* species present in the vaginal secretions collected on the dacron swabs. Deoxyribonucleic acid (DNA) was isolated through capture by magnetic silica particles (BOOM-technology) (NucliSENS® easyMAG®, Biomérieux) according to the manufacturer's instructions [575]. PCR was performed using SYBR® Green PCR Mastermix (Applied Biosystems) on 5µl samples of extracted DNA. Specific primers were selected to detect Lactobacilli generally and also the individual species *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactobacillus vaginalis* and *Lactobacillus iners* (see Table 2-5). Primers were designed in-house for L. *vaginalis* but have been described elsewhere for generic *Lactobacilli* and for the other L. species [542,543,560,576].

Table 2-5 *Lactobacillus* Specific PCR Primers

PCR	Author	Regions	Used primers
L. species	M. Reza Zariffard 2002	16S r RNA	F-LBF: 5'-ATGGAAGAACACCAGTGGCG-3'
			R-LBR: 5'-CAGCACTGAGAGGCGGAAAC-3'
L. crispatus	Byun et al 2004	16S r RNA	LcrisF: AGCGAGCGGAACTAACAGATTTAC
			LcrisR: AGCTGATCATGCGATCTGCTT
L. gasseri	Renuka Tamrakar 2007	16S r RNA	LgassF: AGCGAGCTTGCCTAGATGAATTTG
			LgassR: TCTTTTAAACTCTAGACATGCGTC
L. jensenni	Renuka Tamrakar 2007	16S r RNA	LjensF: AAGTCGAGCGAGCTTGCCTATAGA
			LjensR: CTTCTTTCATGCGAAAGTAGC
L. iners	Ellen De Backer 2007	16S r RNA	InersFw: GTCTGCCTTGAAGATCGG
			InersRev: ACAGTTGATAGGCATCATC
L. vaginalis	In-House PCR	16S_23S r RNA	LvagF: CGGAAACCTACACATCGAAG
			LvagR: GTGCAAGCTTGAGAGTAAAC

2.12 Data Management and Quality Assurance

2.12.1 Data Management and Trial Monitoring

Responsibility for managing trial data and monitoring trial conduct was sub-contracted by the Sponsor to Covance Clinical Research Unit, Leeds. Monitoring was undertaken before any participants were enrolled in the study and at regular intervals by suitably qualified

and trained personnel in accordance with their own SOPs. The purpose of monitoring was to ensure: compliance with the protocol, adherence to regulatory and GCP obligations, proper maintenance of all study documentation, the completeness and exactness of the data entered on the CRFs, source data verification, accurate reporting of all adverse events,drug accountability and to maintain close liaison with the Investigators to clarify any problems which may arise during the study. Formal reports were sent to the Sponsor by the Monitor after every visit. Completed CRFs were forwarded to the Covance Data Manager by the Monitor after verification. Any data queries generated were forwarded to the Trial Management Group for clarification.

2.12.2 Trial Management Group

A Trial Management Group (TMG) was formed comprising the CI, the SP (i.e. the author) and the EMU Co-ordinator. The TMG was responsible for the day to day running of the trial and discussed the progress of the trial at least once a month. The TMG provided feedback to the Data and Safety Monitoring Committee

2.12.3 Data and Safety Monitoring Committee (DSMC)

A DSMC was established by the CI to review safety and pharmacokinetic data as the study progressed. This consisted of two Consultant GUM/HIV Physicians with appropriate research experience (Dr Steven Taylor, Heart of England NHS Trust and Professor George Kinghorn, Sheffield Teaching Hospitals NHS Trust) and Martin Bland, Professor of Health Statistics, Department of Health Sciences, University of York (Committee Chair). The DSMC met by teleconference on 2 occasions, 19th April and 14th June 2010, after 11 and 22 participants had been randomised and dosed respectively. On both occasions interim blinded AE data was made available by the TMG. For the initial meeting, interim blinded pharmacokinetic data was also provided by Polymun for the first 11 participants. The TMG attended part of the meeting to answer questions regarding the progress of the study, however the majority of the meeting was conducted in private. The Committee had access to the unblinding list for the trial in order that they be in a position to fully assess the data, however no unblinded results were communicated to the CI, Sponsor or any investigators involved in recruiting or reviewing participants during the study. Having reviewed the data, the Chair issued a report to the CI and Sponsor that the DSMC had no

concerns regarding the integrity of the pharmacokinetic data or safety of participants and were happy for the study to proceed. A final review of study data was conducted by the Committee Chair at the end of the trial.

2.13 Planned Statistical Analyses

An initial Statistical Analysis Plan (SAP) for the Mabgel Trial had been devised in June 2008 by the then Statistician for the trial, Dr Victoria Allgar (Statistician at HYMS and the Department of Health Sciences, University of York) when the study was first submitted for Sponsor approval. However, in the interim, the protocol had undergone a number of revisions without adjustment of this plan. On reviewing the SAP on his appointment as DSMC Chair, Professor Bland identified areas which employed statistical methods which were impractical given the expected nature of the data. In addition, the author and Professor Lacey had thought of several analyses that they felt ought to be included but which had been omitted from the original SAP. Thus, it was decided to produce a revised, final SAP for the trial, prior to performing any analyses. This final SAP and the rationale behind the chosen statistical methods, is outlined in section 2.13.1

As a result of the work put in by Professor Bland in his role on the DSMC and his consequent familiarity with the data, it was agreed by Dr Allgar, Professor Lacey and the Sponsor that he take over as Trial Statistician and complete the final analyses. In view of Professor Bland's dual roles, an independent expert medical statistician from another institution was asked, by the Sponsor, to review and approve the revised SAP, prior to its implementation. In addition, verification of all completed analyses was performed by both Dr Allgar and the independent statistician.

2.13.1 Final Statistical Analysis Plan

The revisions to the original SAP related to the analysis of pharmacokinetic data described in section 2.13.4. All other elements of the original SAP remained unchanged. Study objectives and end-points are described in sections 2.3,2.4,2.7.1 and 2.8.1 respectively. Key details from the SAP that aren't provided elsewhere in this thesis are given below.

2.13.2 Analysis Populations

Presentation of summary statistics for the mAb levels at each time point and comparison between arms was to be conducted on an intent-to-treat basis according to allocated treatment arm. Providing that all participants in the placebo arm were negative for all pharmacokinetic endpoints at all time-points, only data from those in the active arms (low dose and high dose mAbs) were to be included in subsequent analyses.

Analysis of safety data would also be carried out on an intent-to-treat basis; all participants who are randomised and thus entered into the dosing phase of the study were to contribute data to the safety analyses.

2.13.3 Data Derivations and Data Handling

For the purposes of the analyses, sampling time was to be presented according to the intended time of each visit but deviations from sampling windows were to be reported. Missing data was not to be imputed. Any missing data was to be explored, and unless there was any reason to believe otherwise, missing data was to be assumed to be missing at random and therefore cases were to be excluded from analyses where their data are required but unavailable. Should a participant withdraw from the study any data and samples collected up to the point of withdrawal were to be analysed.

2.13.3.1 Rules for Handling Safety Data

AE definitions and grading are provided in section 2.8.2. In accordance with the North and East Yorkshire R & D Alliance SOP on Adverse Event Reporting, all untoward occurrences that arose in a participant following informed consent had to be recorded as AEs. However, any events that were detected in a participant prior to them being exposed to study gel would be considered to be not related to study gel for the purposes of analysis unless they increased in severity and /or grade during the study.

For every AE, the duration of the event was defined as the number of days from the first occurrence to the first event free day. The grade reported would be the maximum grade reached during that period. Events were only to be considered to be AEs if detected and recorded during the required period of follow up for each participant described in the

study protocol, i.e. up to and including the final evaluation (visit 8), except for any participant experiencing a grade 3 or above event who was required to be followed up until resolution of the event.

2.13.4 Pharmacokinetic Analyses

2.13.4.1 Non-parametric vs parametric tests

Given the fact that no mAbs would be expected to be detected in the baseline samples in any of the 3 study arms, or any samples from recipients of placebo, the complete mAb ELISA data for all sample types was likely to be highly skewed with many observations recorded as being "<0.06", meaning not detectable (ND) within the limits of the assay. Thus, a non-parametric statistical approach, using the Kruskal-Wallis Rank and Mann Whitney-U tests [577], was felt to be the most appropriate way of comparing mAb levels between all 3 study arms and for direct comparisons between the 2 active Mabgel arms.

However, for more complex comparisons of mAb levels, involving other variables, it was thought justifiable to use parametric methods, such as analysis of variance (ANOVA), or regression, after transforming the data (using $\log_{10}$) so that it approximated a normal distribution [578]. Such analyses would only utilise mAb levels expected to be detectable, i.e. from post-dose visits in the 2 Mabgel arms, thus avoiding the difficulties of obtaining $\log_{10}$ of values which are ND (impossible) or < 1 (negative log). Should any observations < 1 be encountered, they would be assigned the value 1, becoming 0 after $\log_{10}$ transformation.

2.13.4.2 Primary and Secondary End-point Analyses (mAb concentrations detected in Weck-Cel, CVL, aspirate and serum samples)

For the mAb levels in all sample types, results would be summarised for each treatment group (high dose Mabgel, low dose Mabgel and placebo) as minimum, median, and maximum at each time-point. All 3 treatment groups would first be compared using the Kruskal-Wallis Rank test corrected for ties. Since this test uses only ordinal data, it copes well with zero or "ND" values.

Where the Kruskal-Wallis test is statistically significant, $P<0.05$, the two active treatment groups would be compared using a Mann Whitney- U test, to check for evidence as to whether the higher dose produces higher antibody concentrations. The test would be conditional on the Kruskal-Wallis test result to avoid multiple testing problems and the need to adjust P values for these [577].

2.13.4.3 mAb Half-lives

Drug levels in pharmacokinetic studies are often expressed in terms of t ½ (t ½) and the two previous studies of passive application of IgG to the FGT have both provided t ½ estimates [510,511]. It seemed sensible therefore to attempt to estimate the t ½ of each of the mAbs in this study, with the caveat that the accuracy would be restricted by the relatively limited number of sampling time-points.

Using data from Weck-Cel samples, t ½ would be estimated for each mAb using only the groups which received an active dose, and observations at 1, 8, and 24 hours after the first dose. The method would assume an exponential decay curve. If the data cannot be assumed to fit an exponential decay curve, a t ½ is not a meaningful estimate [579]. A log transformation of the observed mAb concentration was to be used and an analysis of covariance (ANCOVA) model fitted with time as a continuous predictor and participant as a categorical factor [577]. Two checks on the assumptions were to be made. Firstly, linearity would be checked by including and testing a time squared term. Uniformity of the t ½ would then be checked by testing an interaction between time and participant. If the assumptions are met, the t ½ could be estimated by log(2)/slope. The 95% confidence interval (CI) would be found from the usual CI for the slope. If the assumptions are not met, analysis would then be data-driven.

2.13.4.4 Adjustment for Dilution Factor

Analyses of mAb levels in all samples would be carried out using the 'raw' ELISA data, as reported by Polymun. In addition, antibody concentrations in Weck-Cel samples would also be analysed after application of a standard 'dilution factor correction' to adjust for variability in volumes of collected secretions and dilution occurring during processing.

This is discussed in section 3.6.3. All “not detectable” observations were to remain “not detectable” and would not be adjusted.

2.13.5 Safety Analyses

2.13.5.1 Grade 3 or Above AEs

The number of grade 3 or above genital, other clinical and laboratory AEs were to be reported for each participant and according to treatment arm. Attributed causality would also be provided. The proportion of participants in each arm experiencing a grade 3 or above AE was to be compared using Fisher’s exact test for appropriate combinations.

2.13.5.2 Events Leading to Discontinuation of the Gel

The reasons for any participants discontinuing or interrupting gel use were to be presented. Any reasons attributable to the study gel were to be reported according to treatment group however no statistical testing was to be performed.

2.13.5.3 All AEs

Data was to be reported according to treatment arm and attributed causality. For each type of event, the number of events experienced as well as the number and proportion of participants experiencing the event were to be presented. If AEs were recorded in a minority of participants overall, then the proportion of participants in each arm experiencing an AE of any grade was to be compared using Fisher’s exact test. However, should AEs be recorded in the majority of participants, the number of AEs per woman would be more important and relevant than their presence or absence for a woman. In this situation, counts of AEs per participant in each study arm were to be compared using negative binomial regression. Analyses would be performed for all events and for events classed as possibly or probably treatment related i.e. the latter would exclude all events that occurred before administration of study gel and any events that were deemed by the SP, in conjunction with the CI, to be unrelated to study gel.

2.13.6 Sub-Studies

2.13.6.1 Exploration of the variability of weights/volumes of vaginal secretion samples obtained using Weck-Cels and the relationship between sample weight/volume and mAb concentration

Given that the reliability of the Weck-Cel sampling method was key to the accurate determination of the primary end-point and the 'dilution factor correction' had never previously been applied in the context of secretions collected after application of a gel, it was decided to try to quantify the degree of variability in weights/volumes of secretion samples obtained and explore the relationship between sample weight/volume and detected mAb levels. Weights of Weck-Cel samples would be analysed to estimate the variation between paired samples for the same woman at the same visit, between women, and between visits, using the method of components of variance. Weights would be found by subtraction of the weight of a dry Weck-Cel sponge (in the sample collection kit) from the weight of the sponge plus sample (in the sample collection kit) (see sections 2.11.2.3 and 2.11.2.4). Assumptions of ANOVA would be checked and a data transformation used if appropriate. The relationship between weight of sample and Weck-Cel mAb level would be estimated for levels detected on samples taken after administration of active gels, using multiple regression. Data transformations were to be applied if required.

2.13.6.2 Comparison of participant self-sampled to clinician sampled vaginal secretions

It was planned that a comparison would be made between the levels of mAbs detected in secretions obtained using the participant self-sampling aspirator and those obtained using Weck-Cel and CVL sampling. Using data for the active treatment arms only, regression analysis would be performed to see how well the mAb levels detected by the Weck-Cel and CVL methods could be predicted from those obtained in the aspirates.

2.13.6.3 Investigation of the Impact of Mabgel on Vaginal Flora

A line listing of Nugent score, vaginal pH, and *Lactobacillus* species detected would be presented for each participant, for each sampling point. Treatment groups were to be compared by 2 methods. Firstly, the proportion of women in each study arm at each visit

who had were positive for each *Lactobacillus* species by PCR would be compared using Fisher's Exact test. In addition, quantitative counts for all *Lactobacillus* species would be compared across all 3 study arms using the Kruskal Wallis rank test, allowing for ties.

2.13.6.4 Investigation of the Experience of taking Part in the Clinical Trial

The analysis plan for this sub-study was devised separately from that for the remainder of the Trial and is described in Chapter 5. Analysis of the interview transcripts was to be performed qualitatively using the framework approach.

3. PHARMACOKINETIC RESULTS AND ANALYSES

3.1 Data Sets Analysed

After completion and verification by the study Monitor, carbon-copies of CRFs were forwarded to Covance Data Management for data extraction. A database was created by Covance using Oracle Clinical Version 4.5 software, with all ancillary data programmed, read and reconciled with the CRF data using SAS version 9.0. Final data sets were provided as Excel spreadsheets on 8th October 2010 following database lock on 5th October 2010.

3.2 Data Analysis and Reporting

Final data sets provided by Covance were analysed and interpreted by the author with reference to source data for clarification where required. Statistical analyses were performed in conjunction with Professor Martin Bland, Professor of Health Statistics, Department of Health Sciences, University of York, in accordance with the Final Trial SAP (see section 2.13).

Results are reported below in accordance with the CONSORT (Consolidated Standards of Reporting Trials) 2010 Statement [580]. An overview of the conduct of the trial from participant recruitment to analysis of results is depicted in Figure 3-1.

Figure 3-1 CONSORT Diagram Depicting the Passage of Participants Through the MABGEL 1 Clinical Trial

3.3 Study Participants

3.3.1 Accrual and Retention

49 women provided consent for the study and underwent screening to determine eligibility. Of these, 28 were enrolled into the dosing phase of the study. 4 women were initially ineligible and were enrolled after successful re-screening, thus there were a total of 53 screening visits (see section 3.3.3). All 28 women attended all study visits and completed the study (for protocol deviations see section 3.5). The study was terminated early on 29th July 2010 due to the expiry of the study gels at the end of July 2010.

3.3.2 Baseline Characteristics

A summary of the main baseline characteristics of enrolled participants is given in Table 3-1. Overall, 27 women described themselves as white and one as black African. The median age at enrolment was 25 years (range 19 to 43). Participants in the Low dose Mabgel arm had an older median age than the other two arms, and perhaps reflecting this, this arm had a larger proportion of women who had had children, with 2 women having had more than 2 children. However, the majority of participants in all 3 study arms were nulliparous. All current medical problems were mild in nature and considered insignificant by the SP and CI.

Although required to refrain from sexual intercourse during the dosing period, in view of the lack of safety data on exposure to the mAbs during pregnancy, all women had to be using a reliable form of contraception (as defined in the study protocol:- see eligibility section 2.5.3) at time of screening and agree to do so for the duration of the study. 13 women were using condoms consistently, 6 were using etonogestrel implants, 4 were taking a COCP, 2 had a levonorgestrel- releasing IUS in- situ, 1 had a copper IUCD, 1 was taking the desogestrel-containing POP and 1 was using DMPA injections. One of the participants using condoms (MAB014) was in addition taking a progestagen-only pill (Etynodiol, Femulen®, Pharmacia) that, if used alone, fell outside the contraceptive requirements of the protocol. Distribution of non-hormone (IUCD and/or condom) and hormone (IUS, DMPA, desogestrel POP, or COCP) users was fairly balanced overall

between study arms, however there were a preponderance of COCP users (3 of a total of 4) and fewer users of progestagen-based methods (2 vs 4 in each of the Mabgel arms) randomised to placebo compared to Mabgel. In view of this, fewer women in this arm had been amenorrhoeic > 3/12 before enrolment.

Table 3-1 Summary of Baseline Characteristics of Enrolled Participants

	Study Arm		
Demographic	**High Dose (n=10)**	**Low Dose (n=9)**	**Placebo (n=9)**
Ethnicity			
White British	9	8	9
White Other	0	1	0
Black African	1	0	0
Age (years)			
Number in Range			
18-24	4 (40 %)	3 (33%)	4 (44 %)
25-34	6 (60%)	3 (33%)	1 (11%)
35-44	0 (0%)	3 (33%)	4 (44 %)
Median Age	25	32	25
Height (metres)			
Mean	1.68	1.68	1.68
Weight (kg)			
Mean	73.15	75.67	62.28
Contraception			
Condoms	4 (40%)	5 (56%)	4 (44%)
IUCD	1 (10%)	0 (0%)	0 (0%)
COCP	1 (10%)	0 (0%)	3 (33%)
Cerazette (POP)	1 (10%)	0 (0%)	0 (0%)
IUS	1 (10%)	1 (11%)	0 (0%)
Implant	2 (20%)	3 (33%)	1 (11%)
DMPA	0 (0%)	0 (0%)	1 (11%)
Number of Pregnancies (Term)			
0	7 (70%)	5 (56%)	6 (67%)
1	2 (20%)	0 (0%)	1 (11%)
2	1 (10%)	2 (22%)	2 (22%)
>2	0 (0%)	2 (22%)	0 (0%)
Day of Cycle at 1st Gel Dose			
Number in Range			
7-10	2 (20 %)	4 (44%)	5 (56%)
11-13	4 (40%)	1(11%)	2 (20%)
Amenorrhoea > 3/12	4 (40%)	4 (44%)	2 (20%)
Median Day	11	9	12

3.3.3 Screen Failures

Twenty four women were found to be ineligible for the study following initial assessment. Of these, 4 were subsequently re-screened and enrolled into the dosing phase; 3 after successful treatment of infections detected on their initial screening (1x CT, 1x BV, 1x urinary tract infection) and 1 following renewal of her contraceptive implant. An additional 3 women were also found to have infections (2x BV, 1x CT) and were referred for treatment but did not wish to be re-screened.

Eleven women were judged by the SP or CI to have a significant current general medical illness and/or condition that had the potential to interfere with the evaluation of study objectives (i.e. current respiratory infections, recent diagnosis of genital HSV, severe depression with evidence of recent self-harm, severe recurrent headaches, recent transverse myelitis, significant vaginal prolapse, severe asthma requiring recent hospitalisation, diabetes, uncontrolled hypertension, latex allergy, severe allergic reaction of unknown aetiology). One woman was found to have a significantly elevated alanine transferase (ALT) level on LFTs at screening requiring investigation by her GP. Two women were deemed ineligible due to not using a reliable method of contraception. Another participant was judged to be unlikely to be able to comply with study procedures required in the protocol due to needle-phobia. Two women could not be enrolled into the dosing phase due to study investigators being unable to obtain a response from their GP within the required time-frame. One subject declined to be enrolled following successful screening having recently started a new job.

3.4 Blinding and Randomisation

Twenty eight women received the study gel; 9 received placebo, 9 low dose mAbs and 10 high dose mAbs. There was no indication to access the unblinding website during the study. All clinical investigators, including the SP, research nurses and the CI remained blinded until after database lock. Thus, double blinding was maintained for the duration of the study.

3.5 Compliance with Protocol

3.5.1 Gel usage and sexual abstinence

As far as could be ascertained from participant report, diary cards, and returned syringes, 16 women completed dosing in accordance with the study protocol. With the other 12 women there were either dosing-related protocol deviations or inconsistencies detected. In the case of 2 women there was an apparent discrepancy between the different measures of compliance. 1 woman (MAB004) returned a blank diary card at the end of dosing but verbally reported that she had applied all 11 doses in the evening and returned 11 empty syringes. Another woman (MAB013) stated both verbally and on her diary card that she had correctly used all doses of gel, however she only returned 10 empty syringes having discarded one by mistake.

Three women (participants MAB014, 018 and 023) returned 1 unused syringe with 10 empty ones. In all 3 cases the missed doses were reported to have occurred in the middle of the dosing period (doses 3, 4 and 9 respectively), thus minimising any impact on pharmacokinetic and safety analyses. In addition, by chance, each of these women had been randomised to a different arm of the study, further reducing any influence on study outcomes. In the case of MAB018 and 023 the doses were missed due to the participant simply forgetting to use the gel. With MAB014, the participant omitted the gel due to feeling unwell. This will be discussed further in section 4.1.1.4.

Participants were instructed to apply the gel at night at bedtime as this was thought by the Study Investigators to be the time that would be most convenient and impact least on their activities of daily living. However, it became clear as the study progressed that some participants were not following this instruction. This appears to be for two reasons. Firstly, we had inadvertently caused confusion as bed-time was not the same as night-time for some of our participants. We recruited a number of healthcare workers to the study who worked variable shifts, thus bed-time for them could be in the morning or early afternoon following or preceding a night-shift. In addition, a couple of participants reported that they preferred to use the gel in the morning as they actually found this to be more convenient. Following discussion with the DSMC and study Monitor, it was

concluded that since only the first and 12th doses were considered to be time critical with regards to pharmacokinetic sampling and analysis, provided that participants had applied each of the other successive doses within a calendar day of the previous one they would be unlikely to have reduced the integrity of the pharmacokinetic or safety data derived from the study.

No women reported having had oral or vaginal sexual intercourse during the specified abstinence period. This requirement was explored further in the qualitative interview study (see chapter 5).

3.5.2 Deviations Relating to Sample Collection or Analysis

3.5.2.1 Samples not obtained

MAB015 attended for visit 4 (24 hours post 1st dose) having mistakenly applied the 2nd dose of study gel that morning. Consequently, it was decided not to perform any pharmacokinetic sampling at that time since results would have been difficult to interpret.

3.5.2.2 Samples obtained outside of sampling windows

MAB019 attended for her 12 hours post 12th dose evaluation (Visit 6) at the scheduled time of 10.30. However, she had mistakenly administered the 12th dose of gel at 21.20 the night before rather than 22.30 as planned. Weck-Cel samples were obtained at 10.36 and venepuncture for serum performed at 11.15 (16 minutes and 55 minutes respectively outside the allowed sampling window of an hour either side of the 12 hour time-point). The scheduled time for this participant's Visit 7 the following day was brought forward by 1 hour to allow 36 hour post dose samples to be taken at the correct time in relation to the timing of administering the 12th dose.

2 other participants attended late for their 36 hours post 12th dose evaluations (Visit 7). As a result, the CVL sample for MAB007 was taken 4 minutes outside the allotted time window and the self-taken aspirate, Weck-Cel, and CVL samples for MAB023 were taken 22, 28 and 30 minutes outside respectively.

In accordance with the SAP (see section 2.13.3), results are presented according to the intended time-point of each visit.

3.5.2.3 Samples not Analysed

Weck-Cel samples from MAB006 visit 3 were collected, stored and transported, but were not analysed by Polymun as they arrived unlabelled and could not be confidently identified.

3.6 Pharmacokinetic Sampling and ELISA Data

3.6.1 Samples Obtained

Samples were obtained and processed in accordance with the study protocol and relevant SOPS except where outlined in section 3.5.2 above.

3.6.1.1 Weck-Cel Vaginal Secretion Samples

2 Weck-Cel samples were obtained per participant per sampling visit, except for MAB015 visit 4 where no sampling was performed. As described in sections 2.11.2.3 and 2.11.2.4, entire Weck-Cel sampling kits were weighed (in g) on the same fine balance before and after sampling; the difference in the weights (the difference between a 'dry' pre- and a 'wet' post-sampling Weck-Cel sponge) was calculated to be the weight of secretion obtained. Vaginal fluid has been shown to have a composition which is around 99% water [53], hence with the density of water being approximately 1g/ml, 1ml of collected secretion has been assumed to weigh 1g. Original values in g (corresponding to ml) were converted to µl by multiplying by 1000.

3.6.1.2 CVL Samples

As described in section 2.7.2.4, CVL samples were obtained through irrigating the vaginal walls with 5ml of saline, which was then re-aspirated, together with any cervico-vaginal secretions that had pooled in the posterior fornix. Recovered volumes were recorded to the nearest 0.5ml.

3.6.1.3 Participant-Self taken Vaginal Aspirate Samples

Participants were asked to collect samples of their own vaginal secretions using a volumetric aspirator as outlined in section 2.7.2.5. Unfortunately, due to difficulties encountered with the self-taken aspirator (discussed in section 3.8.6.5), measurable samples were only obtained at 36 (43%) of the 84 possible time-points.

3.6.1.4 Serum Samples

1 x 7.5ml of blood was obtained (as described in section 2.7.2.7) per participant at each time-point required in the protocol. Between 2 and 7, 500µl aliquots of serum were extracted from each sample (see section 2.11.2.7). 2 x 500 µl aliquots were sufficient for performance of the ELISAs, hence a more than adequate volume of serum was obtained at each time-point for each participant.

3.6.2 mAb Concentrations Detected on ELISAs

As outlined in section 2.11.3, concentrations of C2F5, C4E10 and C2G12 were analysed in Weck-Cel, CVL, vaginal aspirate and serum samples by Dr Brigitta Vcelar and technicians at Polymun Scientific, Vienna, Austria. All investigators involved in evaluating mAb ELISA results were blinded to participant treatment allocation. The lowest quantifiable standard concentration was 3.126ng/ml. To reduce non-specific background signals, all samples were diluted at least 1:20, resulting in a lower limit of quantification of 60ng/ml (0.06µg/ml). For reasons that Polymun were unable to determine, the ELISA for C2F5 resulted in background signals below or close to the quantification limit whereas the assays for C4E10 and C2G12 produced background signals above this limit.

As discussed in section 3.5.2, no samples were available for analysis for participant MAB006 visit 3 or MAB015 visit 4. For participant MAB010 no value was obtained for C4E10 on Weck-Cel sample analysis at visit 4 due to failure of the ELISA to produce a valid test result. ELISA data was only available for 27 (32%) of the 84 possible aspirate samples; for the others either no sample had been obtained or it was too small or thick to be pipetted out of the storage tube for analysis at Polymun.

3.6.3 Dilution Factor Correction For mAb Concentrations Detected in Weck-Cel Samples

The mAb concentrations reported by Polymun did not take account of the variable 'dilution factor' which exists as a result of variability in the volumes of vaginal secretions collected by Weck Cels and the addition of buffer during processing of these samples. An adjustment is commonly applied when using Weck-Cels to collect mucosal secretions for measurement of antibodies or cytokines occurring naturally or induced through vaccination [525,526,527,581]. It has also been applied, by some researchers, to levels of mAbs detected in vaginal and rectal secretions in macaques following i.v. administration, prior to SHIV challenge [K Klein and R Shattock, personal communication]. Thus, in order to enable more accurate comparison of our data with that from other studies, it was decided to apply a 'dilution factor correction' to the mAb concentrations detected by ELISA.

$$\text{The dilution factor correction} = \frac{(\text{Processing buffer volume} + \text{Secretion volume (in µl)})}{\text{Secretion volume (in µl)}}$$

As detailed in sections 2.11.2.3, 2.11.2.4 and 3.6.1.1, a total of 600 µl of buffer was used to process each sample and sample weights were calculated through weighing the entire sampling kits, containing the Weck-Cel sponge, processing tubes and filter and initial 300 µl of buffer, before and after sampling. 1 µl of secretion was assumed to weigh 1mg.

Each adjusted mAb concentration (per participant, per time-point) was derived by multiplying the reported ELISA value by an individually calculated dilution factor correction. As the eluants of the two Weck-Cel samples taken per participant per time-point were pooled after processing, before being divided into aliquots for analysis by ELISA, each calculated dilution factor correction used the mean of the two Weck-Cel sample volumes.

3.7 Statistical Analysis of Pharmacokinetic Data

All statistical analyses were performed in conjunction with Professor Martin Bland using STATA version SE 10.1, Stata Corp., College Station, Texas, released 1st October 2009. All analyses were conducted on an intention-to treat basis and in accordance with the final, revised SAP, outlined in section 2.13.1, unless specified otherwise.

3.7.1 Handling of Missing Data

As discussed in sections 3.5.2 and 3.6.2 above, Weck-Cel sample data was missing from MAB006 visit 3 (8 hours post 1st dose), Wecks, CVL and self-sampled aspirate data was missing from MAB015 visit 4 (24 hrs post 1st dose) and the C4E10 result from the Wecks samples was missing from MAB010 visit 4. There was no reason to consider that these data were not missing at random therefore analyses were performed without them using existing data.

3.7.2 Primary and Secondary Pharmacokinetic End-Point Analyses

The primary pharmacokinetic end-points for this study (as given in section 2.7.1) were the levels of mAbs detected in samples of vaginal secretions collected using Weck-Cels at all time-points post-gel use (1,8 and 24 hours post 1st dose of study gel and 12 and 36 hours post 12th dose of study gel), with a baseline (pre-1st dose) sample obtained for reference.

mAb levels detected in CVL, self-sampled aspirate and serum samples, at fewer time-points, were secondary end-points.

3.7.2.1 Summary Statistics

As discussed in section 2.13.4.1, for all sample types, the full data -sets of detected mAb concentrations display highly skewed distributions, with the lowest observations reported as < 0.06. It was thus considered more appropriate to use the median as the average measure and the range (minimum and maximum values) to describe the spread of the data than the mean and standard deviation respectively.

The ELISA-derived mAb concentrations were reported by Polymun to a varying number of decimal places and significant figures. For consistency, median, minimum and maximum mAb concentrations are provided, in Table 3-2 to Table 3-5, to the following degree of accuracy: values > 10 are given to zero decimal places, values > 1 but < 10 to one decimal place and numbers < 1 to one significant figure. Values recorded as < 0.06 are reported as ND = not detectable.

3.7.2.2 Statistical Tests Employed

Since the full-data set could not be assumed to be normally distributed, a non-parametric approach was followed. The Kruskal-Wallis rank test was performed initially, for each mAb at each sampling point, to determine whether there was evidence of a difference between the concentrations detected across the 3 study arms generally. If the result of the Kruskal-Wallis test was found to be statistically significant, the Mann Whitney U test was performed to determine whether the mAb levels in the high dose Mabgel arm were significantly higher than those in the low dose Mabgel arm. As the Mann Whitney U test was conditional on the result of the Kruskal Wallis test, no adjustments were needed to take account of multiple testing. Being based on ranked observations, neither test is affected by ND values. Allowances were made for ties [577].

P values (given to 1 significant figure), obtained using STATA, are displayed in Table 3-2 to Table 3-5. Any P value < 0.05 would be considered to be significant at the 95 % confidence level.

3.7.2.3 Primary End-Point Analyses of Data Obtained from Weck-Cel Samples

Analyses were performed both for the unadjusted concentrations as reported by Polymun and for concentrations that had been adjusted to correct for the variable dilutional effects of vaginal secretions and processing buffer. This 'dilution factor correction' is explained in section 3.6.3.

Table 3-2 Median, Minimum and Maximum Values for Concentrations of Each of C2F5, C4E10, C2G12 Detected in Weck-Cel Samples By Study Arm (Unadjusted Values

Table 3-2a Median, Minimum and Maximum C2F5 Levels Detected in Weck-Cel Samples

C2F5 (µg/ml)											
Visit	Placebo			Low Dose			High Dose			P values	
	Med	Min	Max	Med	Min	Max	Med	Min	Max	3 Groups	Low vs High
2 (pre)	ND	ND	ND	ND	ND	ND	ND	ND	ND	----	----
2 (post)	ND	ND	0.7	542	156	1207	1505	122	2709	0.0001	0.01
3	ND	ND	ND	126	30	424	220	1.5	614	0.0002	0.2
4	ND	ND	ND	9.5	3.8	33	15.4	3.2	61	0.0001	0.6
6	ND	ND	ND	34	8.8	337	154	2.2	1464	0.0001	0.4
7	ND	ND	ND	0.7	ND	23	0.3	ND	10	0.007	0.7
Number	9			9			10				

Table 3-2b Median, Minimum and Maximum C4E10 Levels Detected in Weck-Cel Samples

C4E10 (µg/ml)											
	Placebo			Low Dose			High Dose			P values	
	Med	Min	Max	Med	Min	Max	Med	Min	Max	3 Groups	Low vs High
2 (pre)	0.1	ND	0.4	ND	ND	0.2	0.06	ND	0.09	----	----
2 (post)	ND	ND	0.3	239	103	767	987	326	2116	0.0001	0.001
3	ND	ND	0.1	56	26	357	148	11.1	600	0.0002	0.3
4	ND	ND	0.2	6.3	ND	25	11	1.87	91	0.0009	0.4
6	ND	ND	0.09	33	4.0	400	96	1.5	281	0.0001	0.6
7	ND	ND	0.5	0.4	ND	4.7	0.5	ND	11	0.01	0.9
Number	9			9			10				

Table 3-2c Median, Minimum and Maximum C2G12 Levels Detected in Weck-Cel Samples

C2G12 (µg/ml)											
Visit	Placebo			Low Dose			High Dose			P values	
	Med	Min	Max	Med	Min	Max	Med	Min	Max	3 Groups	Low vs High
2 (pre)	ND	ND	0.6	ND	ND	0.14	ND	ND	0.3	----	----
2 (post)	ND	ND	0.4	619	123	2596	1620	138	4328	0.0001	0.07
3	ND	ND	0.2	5.2	1.7	135	23	0.1	690	0.0002	0.07
4	ND	ND	0.5	0.7	0.2	2.2	0.6	0.1	29	0.004	0.5
6	ND	ND	0.3	1.3	0.2	45	4.5	0.5	360	0.0002	0.6
7	ND	ND	0.3	0.1	ND	2.5	ND	ND	8.1	0.6	----
Number	9			9			10				

Median concentrations of each mAb over time have been plotted for both of the active gel arms in Figure 3-2 and Figure 3-3.

Note: The time quoted is the time since the previous dose of study gel was given. Graphs were created by combining data from sampling at 1, 8 and 24 hours post 1st dose and 12 and 36 hours post 12th dose. Obviously it is possible that there could be residual mAbs remaining from previous doses in addition to dose 12 detected at the latter 2 sampling time points, however, it is clear from the concentrations detected that the mAbs were virtually eliminated from the lumen by 36 hours post application (which would coincide with the 12 hr sampling point).

Figure 3-2 Median Concentrations (Unadjusted for Dilution Factors) of C2F5, C4E10, C2G12 Detected in Weck-Cel Samples Over Time in Participants Randomised to High Dose Mabgel Arm (20µg/ml of each mAb)

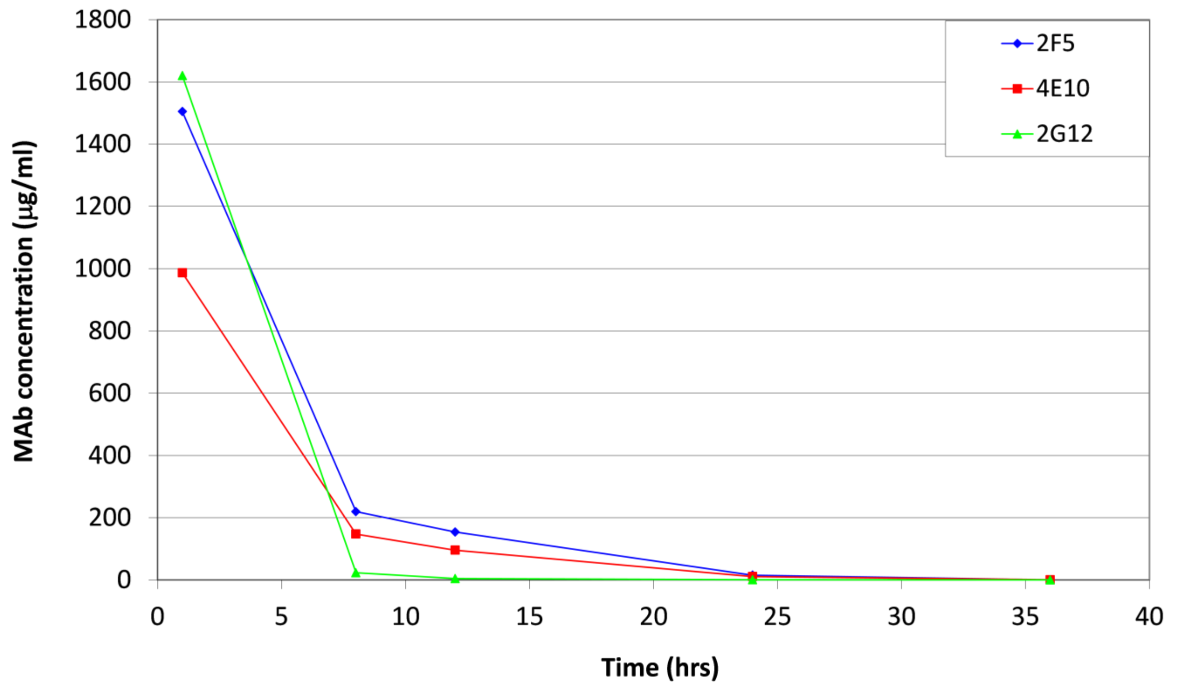


Figure 3-3 Median Concentrations of C2F5, C4E10, C2G12 (Unadjusted for Dilution Factors) Detected in Weck-Cel Samples Over Time in Participants Randomised to Low Dose Mabgel Arm (10µg/ml of each mAb)

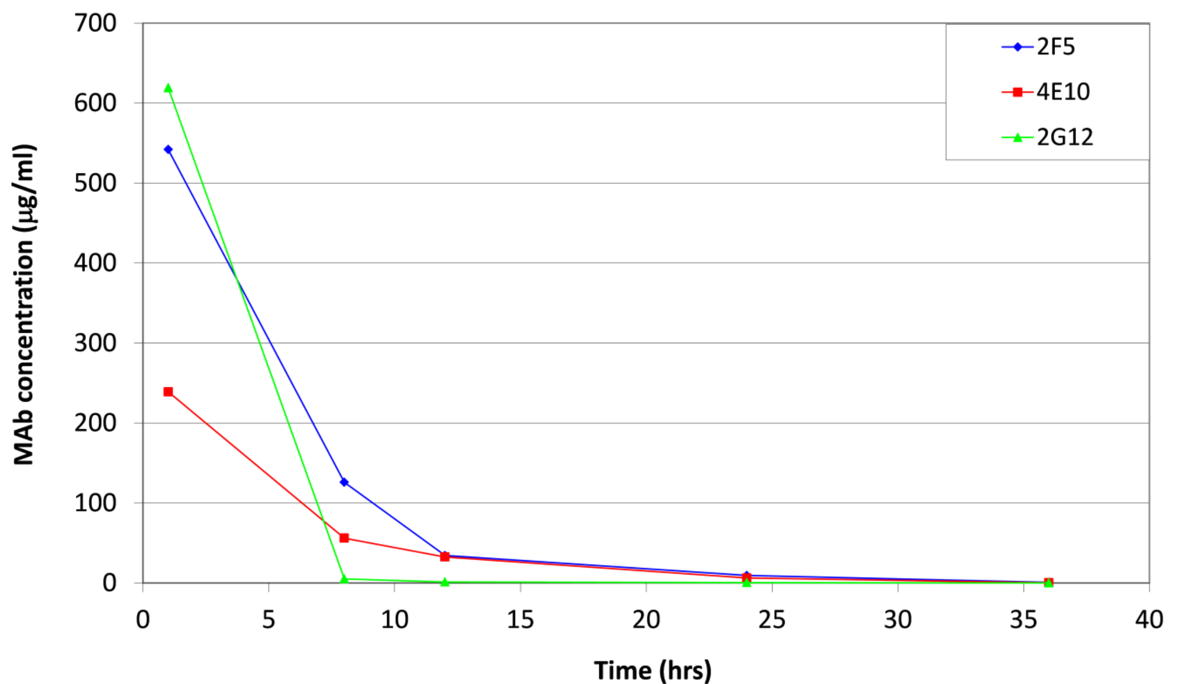


Table 3-3 Median, Minimum and Maximum Values for Concentrations of Each of C2F5, C4E10, C2G12 Detected in Weck-Cel Samples By Study Arm (After Adjustment for Dilution Factors)

Table 3-3a Median, Minimum and Maximum C2F5 Levels Detected in Weck-Cel Samples (post dilution factor adjustment)

C2F5 (µg/ml)											
Visit	Placebo			Low Dose			High Dose			P values	
	Med	Min	Max	Med	Min	Max	Med	Min	Max	3 groups	Low vs. High
2 (pre)	ND	ND	ND	ND	ND	ND	ND	ND	ND	----	----
2 (post)	ND	ND	3.8	2715	941	6073	7737	903	14377	0.0001	0.009
3	ND	ND	ND	910	342	3715	3161	30	6102	0.0001	0.06
4	ND	ND	ND	93	36	530	196	31	729	0.0001	0.4
6	ND	ND	ND	451	105	2146	1376	41	25334	0.0001	0.5
7	ND	ND	ND	8.1	ND	161	4.8	ND	118	0.006	0.6
number	9			9			10				

Table 3-3b Median, Minimum and Maximum C4E10 Levels Detected in Weck-Cel Samples (post dilution factor adjustment)

C4E10 (µg/ml)											
Visit	Placebo			Low Dose			High Dose			P values	
	Med	Min	Max	Med	Min	Max	Med	Min	Max	3 groups	Low vs. High
2 (pre)	1.1	ND	5.9	ND	ND	2.0	0.2	ND	1.6	----	----
2 (post)	ND	ND	2.7	1175	583	3849	5277	1442	13580	0.0001	0.002
3	ND	ND	1.6	547	302	3128	2505	176	6612	0.0001	0.1
4	ND	ND	1.6	64	ND	297	171	18.2	1088	0.0006	0.2
6	ND	ND	1.7	427	47	2548	870	28	2198	0.0001	0.7
7	ND	ND	4.8	5.8	ND	159	4.7	ND	119	0.008	0.8
number	9			9			10				

Table 3-3c Median, Minimum and Maximum C2G12 Levels Detected in Weck-Cel Samples (post dilution factor adjustment)

C2G12 (µg/ml)											
Visit	Placebo			Low Dose			High Dose			P values	
	Med	Min	Max	Med	Min	Max	Med	Min	Max	3 groups	Low vs. High
2 (pre)	ND	ND	8.6	ND	ND	6.8	ND	ND	6.7	----	----
2 (post)	ND	ND	3.3	3314	742	12622	7479	1021	27882	0.0001	0.06
3	ND	ND	3.0	63	19	1183	538	2.4	6953	0.0001	0.06
4	ND	ND	4.3	5.6	1.1	25	12	1.5	182	0.002	0.2
6	ND	ND	5.2	17	2.3	283	52	5.4	1982	0.0002	0.8
7	ND	ND	27.7	1.3	ND	17	ND	ND	160	0.7	----
number	9			9			10				

Figure 3-4 Median Concentrations (Adjusted for Dilution Factors) of C2F5, C4E10, C2G12 Detected in Weck-Cel Samples Over Time in Participants Randomised to High Dose Mabgel Arm (20µg/ml of each mAb)

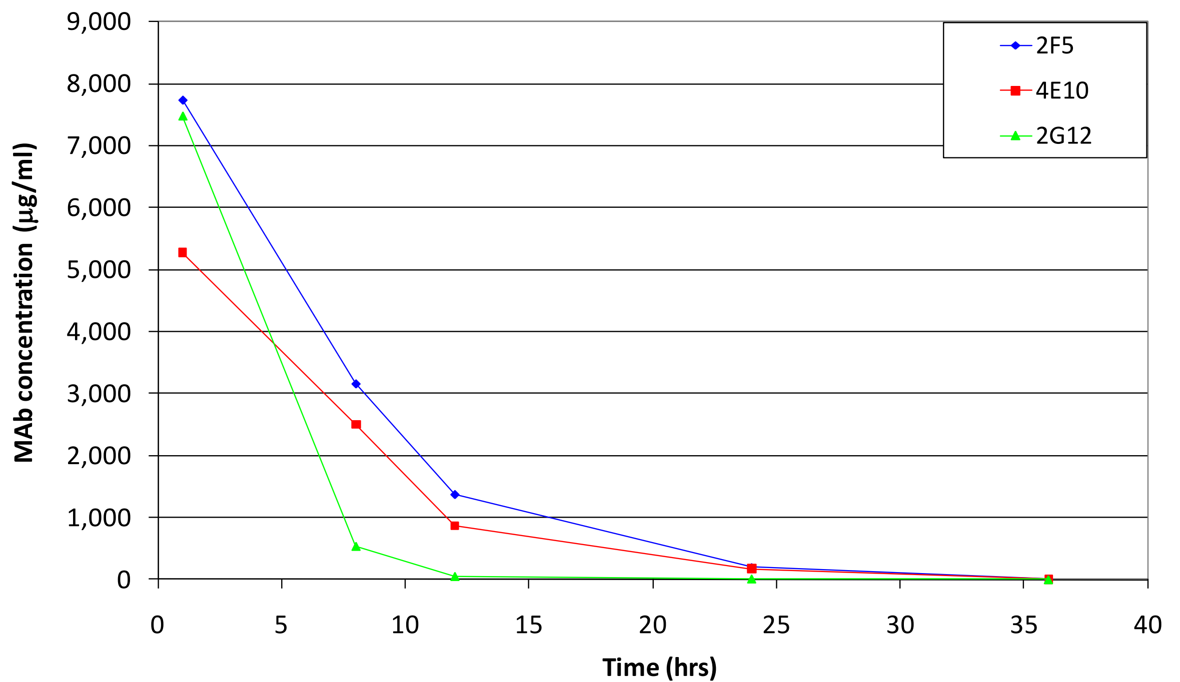
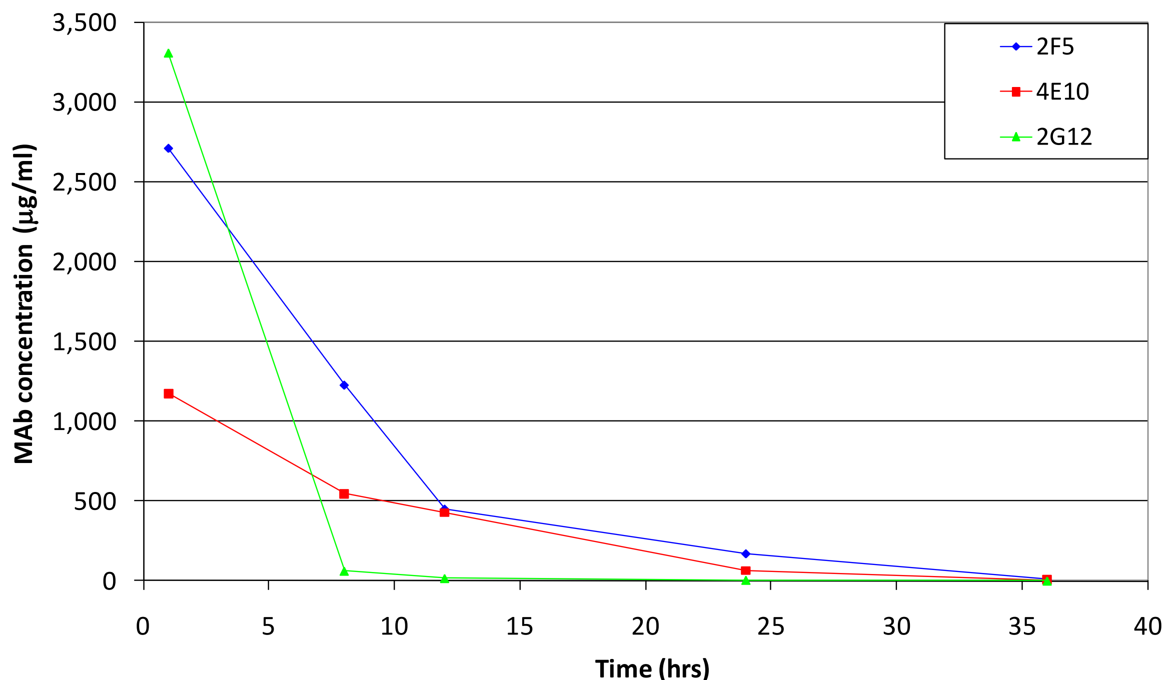


Figure 3-5 Median Concentrations (Adjusted for Dilution Factors) of C2F5, C4E10, C2G12 Detected in Weck-Cel Samples Over Time in Participants Randomised to Low Dose Mabgel Arm (20µg/ml of each mAb)



The overall pattern in MAb levels was the same for both the unadjusted concentrations and those adjusted for dilution factors. Concentrations of all 3 mAbs were maximal at 1 hour post dose and still detectable at low (nanogram) level at 36 hours post 12th dose in some participants. Although variable, on average, adjusting for dilution factors increased mAb levels by 5 times at visit 2 (1 hour post dose) and 12 times at other visits. For each antibody, there was strong evidence of a difference in levels between the three groups at visits 2 (1 hour post dose), 3, 4, and 6. For C2F5 and C4E10 there was strong evidence for a difference at visit 7, but not for C2G12. For C2F5 and C4E10 there was statistical evidence for a difference in the levels found between the high and low dose Mabgel arms at visit 2, 1 hour post dose, but not for C2G12. However, although the median antibody concentration was greater for the high dose arm than the low dose arm at most other visits, except for visit 7, these differences were not statistically significant.

3.7.2.4 Secondary End-Point Analyses of Data Obtained from CVL and Self-aspirate Samples

Analyses were performed for the mAb concentrations, as reported by Polymun, obtained from CVL samples. In view of the paucity of ELISA data available for samples obtained using the vaginal aspirator it was considered futile to perform any statistical analysis of the aspirate data or any formal comparison of the mAb levels obtained using the different sampling methods.

As detailed in section 3.7.2.2 above, P values (to 1 significant figure) were derived using the Kruskal Wallis rank test, allowing for ties, to compare all 3 treatment groups for each visit and, if this result was significant, using the Mann Whitney U test to compare low and high dose Mabgel arms for each visit.

Table 3-4 Median, Minimum and Maximum Values for Concentration of Each of C2F5, C4E10, C2G12 Detected in CVL Samples By Study Arm

Table 3-4a Median, Minimum and Maximum C2F5 Levels Detected in CVL Samples

C2F5 (µg/ml)											
Visit	Placebo			Low Dose			High Dose			P values	
	Med	Min	Max	Med	Min	Max	Med	Min	Max	3 Groups	Low vs. High
2 (pre)	ND	ND	ND	ND	ND	ND	ND	ND	ND	----	----
4	ND	ND	ND	2.3	0.1	3.9	1.03	0.07	3.3	0.0001	0.5
7	ND	ND	ND	0.1	ND	1.6	0.09	ND	0.7	0.02	0.7
Number	9			9			10				

Table 3-4b Median, Minimum and Maximum C4E10 Levels Detected in CVL Samples

C4E10 (µg/ml)											
Visit	Placebo			Low Dose			High Dose			P values	
	Med	Min	Max	Med	Min	Max	Med	Min	Max	3 Groups	Low vs. High
2 (pre)	ND	ND	0.2	ND	ND	0.3	ND	ND	0.1	----	----
4	ND	ND	0.08	4.5	0.2	14	2.9	0.3	13	0.0001	1.0
7	ND	ND	0.07	0.4	ND	4.1	0.3	ND	2.3	0.004	0.6
Number	9			9			10				

Table 3-4c Median, Minimum and Maximum C2G12 Levels Detected in CVL Samples

C2G12 (µg/ml)											
Visit	Placebo			Low Dose			High Dose			P values	
	Med	Min	Max	Med	Min	Max	Med	Min	Max	3 Groups	Low vs. High
2 (pre)	ND	ND	0.21	ND	ND	0.2	ND	ND	0.09	----	----
4	ND	ND	0.09	0.3	ND	1.5	0.4	ND	3.6	0.009	0.6
7	ND	ND	0.1	0.1	ND	1.2	ND	ND	0.7	0.2	----
Number	9			9			10				

There was statistical evidence of a difference between the three study arms for all 3 mAbs, at visit 4, but only for C2F5 and C4E10 at visit 7. There was no evidence for a difference between high and low Mabgel doses at visits 4 or 7.

3.7.2.5 Secondary End-Point Analyses of Data Obtained from Serum Samples

Overall, looking at the minimum, median and maximum values (Table 3-5), there were no apparent changes from baseline levels for any of the mAbs, although this was not tested statistically. There were no statistically significant differences in the levels of any of the mAbs between the 3 study arms at any time-point.

Table 3-5 Median, Minimum and Maximum Values for Concentration of Each of C2F5, C4E10, C2G12 Detected in CVL Samples By Study Arm

Table 3-5a Median, Minimum and Maximum C2F5 Levels Detected in Serum Samples

C2F5 (µg/ml)											
Visit	Placebo			Low Dose			High Dose			P values	
	Med	Min	Max	Med	Min	Max	Med	Min	Max	3 Groups	Low vs. High
2 (pre)	ND	ND	0.09	ND	ND	0.2	ND	ND	0.1	----	----
3	ND	ND	0.09	ND	ND	0.1	ND	ND	0.09	0.8	----
6	ND	ND	0.08	ND	ND	0.2	ND	ND	0.08	0.2	----
Number	9			9			10				

Table 3-5b Median, Minimum and Maximum C4E10 Levels Detected in Serum Samples

C4E10 (μg/ml)											
Visit	Placebo			Low Dose			High Dose			P values	
	Med	Min	Max	Med	Min	Max	Med	Min	Max	3 Groups	Low vs. High
2 (pre)	0.4	0.3	2.5	0.5	0.2	1.6	0.4	0.2	1.3	----	---
3	0.4	0.2	2.0	0.5	0.2	1.3	0.5	0.1	0.9	0.9	---
6	0.3	0.3	1.9	0.5	0.2	1.2	0.5	0.3	1.3	0.8	---
Number	9			9			10				

Table 3-5c Median, Minimum and Maximum C2G12 Levels Detected in Serum Samples

C2G12 (μg/ml)											
Visit	Placebo			Low Dose			High Dose			P values	
	Med	Min	Max	Med	Min	Max	Med	Min	Max	3 Groups	Low vs. High
2 (pre)	0.5	0.3	1.1	0.8	0.4	1.3	0.8	0.5	1.9	----	---
3	0.5	0.4	0.9	0.9	0.5	1.1	0.8	0.4	1.7	0.2	---
6	0.6	0.3	1.2	0.8	0.4	1.7	0.8	0.4	2.0	0.3	---
Number	9			9			10				

3.7.3 Analysis of mAb half-lives

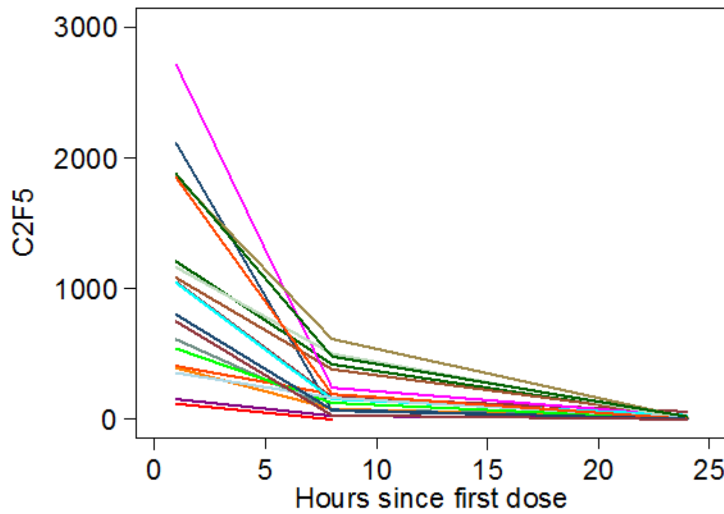
In addition to the primary and secondary end-point analyses, several additional analyses were identified in the final, revised SAP (see section 2.13.1). The first of these was to provide estimates of the residence half-life (t ½) of each mAb i.e. the time needed for half of the administered antibody present to be eliminated from the vagina.

This analysis was conducted in conjunction with Professor Bland, using data from Weck-Cel samples obtained at 1, 8 and 24 hours post 1st dose of study gel in the 19 participants who received either dose of the Mabgel. Data from all participants receiving either of the two doses were combined for this analysis. Estimates were derived both from the unadjusted concentrations as reported by Polymun and from concentrations that were adjusted to account for the 'dilution factor'.

3.7.3.1 C2F5 (unadjusted for dilution factor) and Methodology

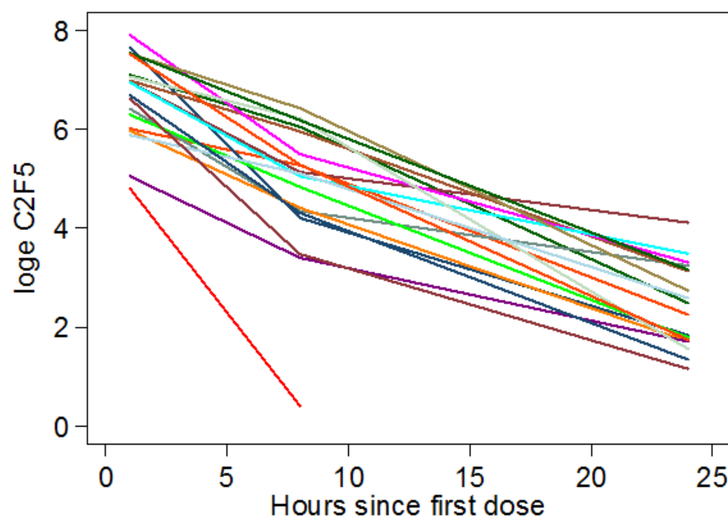
A meaningful estimate of the t ½ of each mAb can only be obtained if its decay curve matches an exponential decay, implying that its elimination follows 1st order kinetics [579]. The decay curve for C2F5 is shown in Figure 3-6.

Figure 3-6 C2F5 Concentration Since Application of 1st Dose of Mabgel



Each line represents a different participant. If decay is exponential, i.e. if the rate of decay is proportional to the amount present, the log of concentration should have a straight line relationship with time. This looks very plausible:

Figure 3-7 Log$_e$ of C2F5 Concentration Since Application of 1st Dose of Mabgel



An analysis of covariance model can be fitted to the log transformed data with time as a continuous predictor and participant as a categorical factor. Evidence of deviation from a simple straight line for each participant can be assessed by adding an hours squared term to the model, to fit a curve [577]. In this case, the added quadratic term has P = 0.02, so there is evidence that the linear fit is inadequate. Inspection of the graph suggests that that there are two outliers, Participants MAB015 and MAB028. MAB015 has the lowest C2F5 level at 8 hours, MAB028 has the lowest at 24 hours. If data from these 2 participants is excluded, the quadratic term has P = 0.1, so there is evidence that the linear fit is adequate in the reduced data set.

Evidence that the same slope does not fit all participants can be sought by including an interaction between participant and slope. This gives P = 0.1 for the full data set and P = 0.2 without MAB015 and MAB028. We have little evidence therefore that the slope is not uniform.

The half-life ($t_{1/2}$) is calculated as follows:

The equation of the decay curve is

$$\log(y) = a - bt$$

where *y* is the antibody concentration and *t* is the time, or

$$y = e^{a} e^{-bt}$$

If y_2 is half *y*, so that the time from *y* to y_2 is *t*, then

$$y_2 = e^{a} e^{-b(t+t_{1/2})}$$

Dividing one equation by the other gives

$$2 = \frac{y}{y_2} = e^{-bt-(-b(t+t_{1/2}))} = e^{bt_{1/2}}$$

Hence $\log(2) = bt_{1/2}$, $t_{1/2} = \log(2)/b$ [577].

The 95% CI is found by putting 95% confidence limits for *b* into this formula.

For C2F5, using the full data, the estimated slope of the line on the log scale is $-b = -0.1882865$, 95% CI for $b = 0.1669421$ to 0.209631. Hence the t ½ is estimated by log(2)/ 0.1882865 = 3.681343 = 3.7 hours, with 95% CI log(2)/0.209631 = 3.3065109 = 3.3 hours to log(2)/0.1669421 = 4.1520215 = 4.2 hours. These estimates appear quite plausible from the graph. If MAB015 and MAB028 are excluded, the estimated slope of the line on the log scale is $-b = -0.1840464$, 95% CI for $b = 0.1651004$ to 0.2029923. Hence the t ½ is estimated by log(2)/0.1840464 = 3.7661545 = 3.8 hours, with 95% CI log(2)/0.2029923 = 3.4146477 = 3.4 hours to log(2)/ 0.1651004 = 4.1983374 = 4.2 hours. There is little effect on the estimate and the full data estimate of 3.7 hours (95% CI 3.3 to 4.2 hours) can be taken as a reasonably robust estimate.

3.7.3.2 C4E10 (unadjusted for dilution factor)

Repeating the above procedure for C4E10 yields the following results:

The decay curve for C4E10 is shown in Figure 3-8 and the graph of loge concentration of C4E10 over time in Figure 3-9.

Figure 3-8 Concentration of C4E10 Since Application of 1st Dose of Mabgel

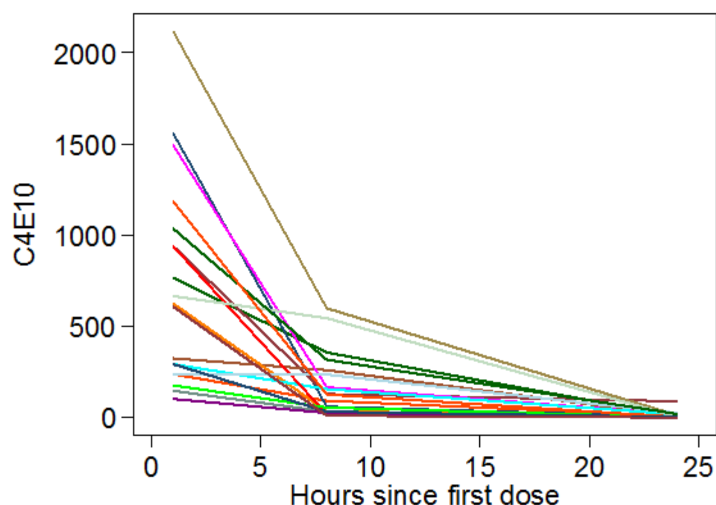
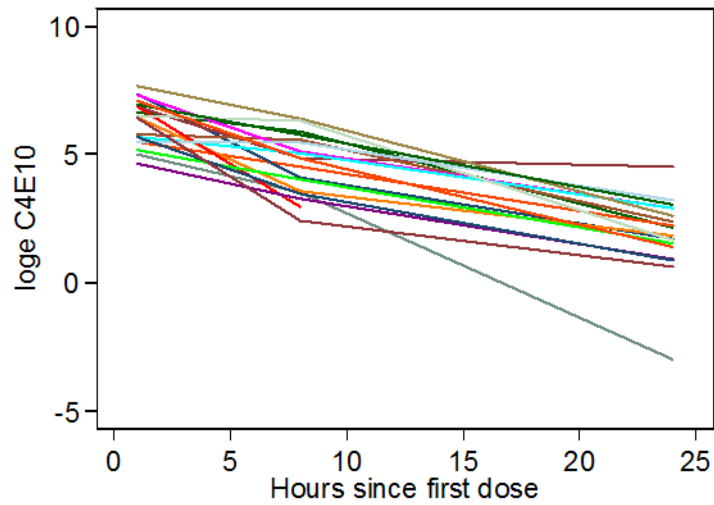


Figure 3-9 Log_e of C4E10 Concentration Since Application of 1st Dose of Mabgel



Evidence of deviation from a simple straight line for each participant: P = 0.2, so there is no evidence that the linear fit is inadequate.

Evidence that the same slope does not fit all participants: P = 0.08. There is a little evidence that the slope is not uniform, but it is not significant and it was felt reasonable for it to be ignored.

Estimated slope of the line on the log scale is $-b = -0.1891694$, 95% CI for b = 0.1622713 to 0.2160676. Hence the t ½ is estimated by log(2)/0.1891694 = 3.6641612 =3.7 hours, with 95% CI log(2)/ 0.2160676 = 3.2080107 = 3.2 hours to log(2)/ 0.1622713 = 4.2715328 = 4.3 hours. These estimates appear quite plausible from the graph. Thus the estimated t ½ of C4E10 = 3.7 hours (95% CI 3.2 to 4.3) which is essentially the same as that for the other MPER mAb C2F5.

3.7.3.3 C2G12 (unadjusted for dilution factor)

The same procedure was also repeated for C2G12.

Figure 3-10 Concentration of C2G12 Since Application of 1st Dose of Mabgel

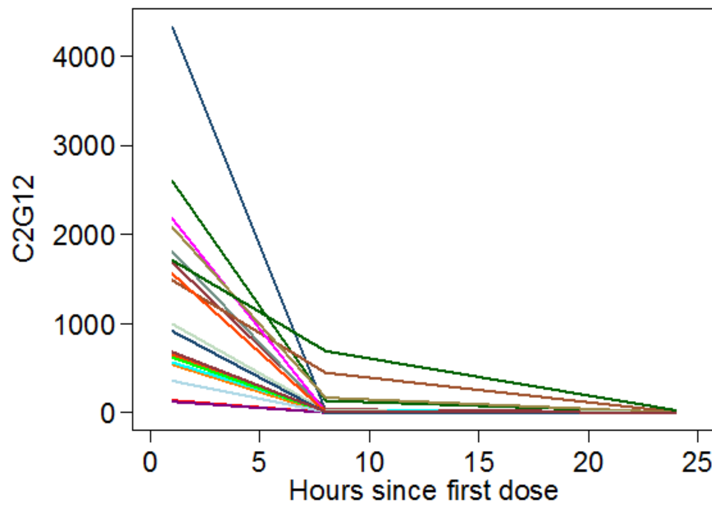
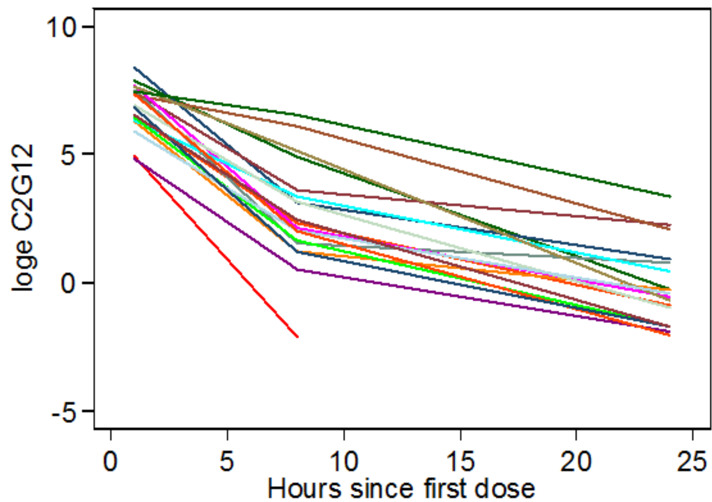


Figure 3-11 Log$_e$ of C2G12 Concentration Since Application of 1st Dose of Mabgel



Unlike with the other 2 mAbs, there is good evidence that the decay curve of CG212 is not a simple exponential decay. The linear fit of the relationship between log$_e$ of concentration and time is inadequate as addition of an hours squared term gives P <0.001. This is clearly shown in the graph. For most participants the log lines clearly get less steep from left to right. The t ½ has therefore been fitted to the 1 and 8 hour data only. Hence it is the early t ½.

Evidence that the same slope does not fit all participants: P = 0.9. There is little evidence that the slope is not uniform. However, using the 1 and 8 hour values only there are too few data points for this to be reliable.

For the early t ½ , estimated slope of the line on the log scale is $-b = -0.5949343$, 95% CI for b = 0.486288 to 0.7035806. Hence the t ½ is estimated by log(2)/0.5949343 = 1.1650819 =1.2 hours, with 95% CI log(2)/0.7035806 = 0.98517097 = 1.0 hours to log(2)/0.486288 = 1.4253841 = 1.4 hours. The estimated early t ½ of C2G12 = 1.2 hours (95% CI 1.0 to 1.4).

3.7.3.4 C2F5 adjusted for dilution factor

Analyses were repeated on the mAb values adjusted for the dilution factor

Figure 3-12 Concentration of C2F5 (Adjusted for Dilution Factor) Since Application of 1st Dose of Mabgel

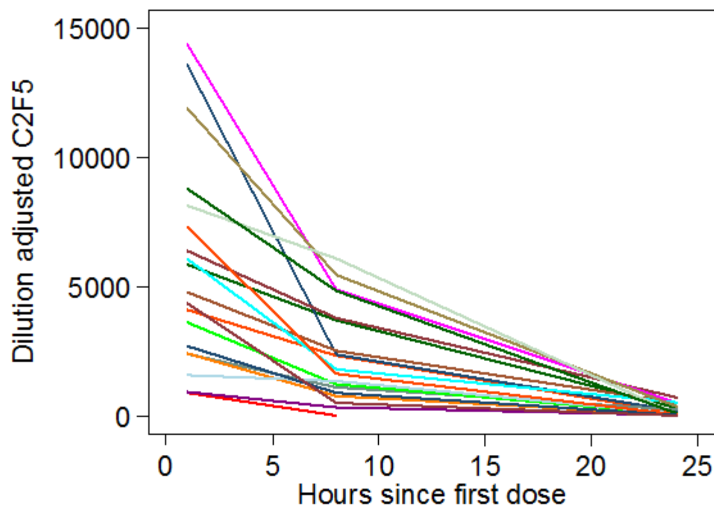
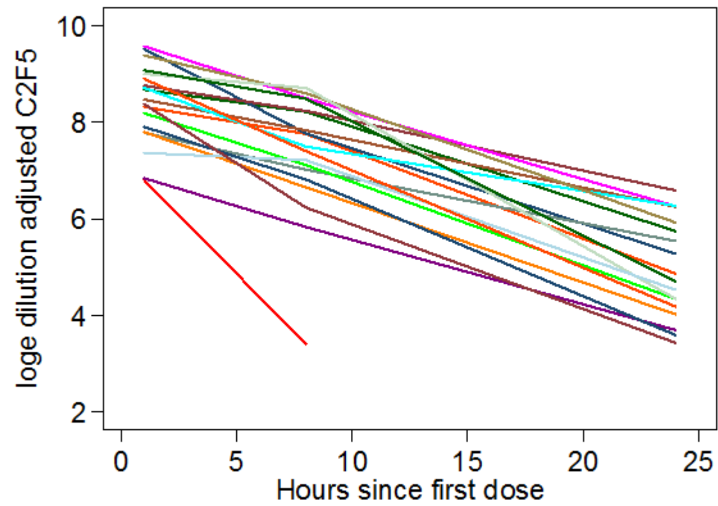


Figure 3-13 Log$_e$ of C2F5 Concentration (Adjusted for Dilution Factor) Since Application of 1st Dose of Mabgel



The linear model appears quite plausible and checking with a time squared term we get P = 0.9. However, there is evidence that there is an interaction between participant and time, P = 0.002, i.e. the same slope does not fit all participants. Unlike with the unadjusted levels, taking out MAB015 and MAB028 does not remove this, P = 0.008. There is no easy way of dealing with this interaction, so the final model was fitted without it and for all participants, leaving the interaction to be included in the error. Hence the CI for the mean t ½ will be wider because of it, and it should be borne in mind that the t ½ will vary between women.

The estimated slope of the line on the log scale is $-b$ = −0.1554724, 95% CI for b = 0.1384918 to 0.172453. Hence the t ½ is estimated by log(2)/ 0.1554724 = 4.4583295 = 4.6 hours, with 95% CI log(2)/0.172453 = 4.0193396 = 4.0 hours to log(2)/0.1384918 = 5.0049691 = 5.0 hours. We have estimated t ½ = 4.6 hours (95% CI 4.0 to 5.0). This is around an hour longer than the 3.7 hours (95% CI 3.3 to 4.2 hours) estimated for the unadjusted C2F5 concentrations.

3.7.3.5 C4E10 adjusted for dilution factor

Figure 3-14 Concentration of C4E10 (Adjusted for Dilution Factor) Since Application of 1st Dose of Mabgel

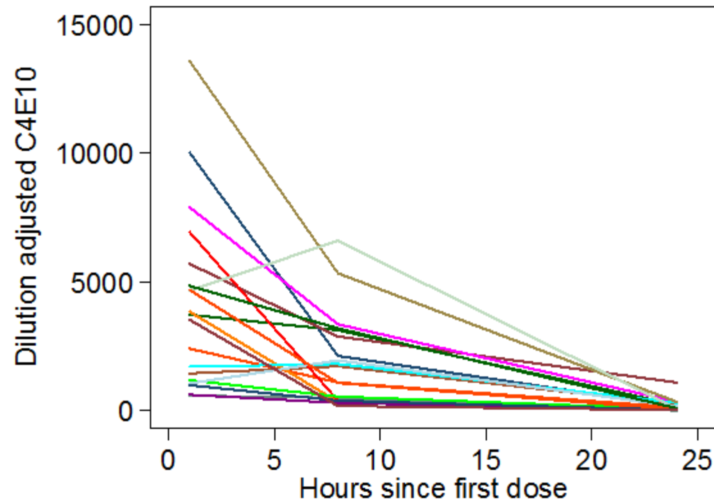
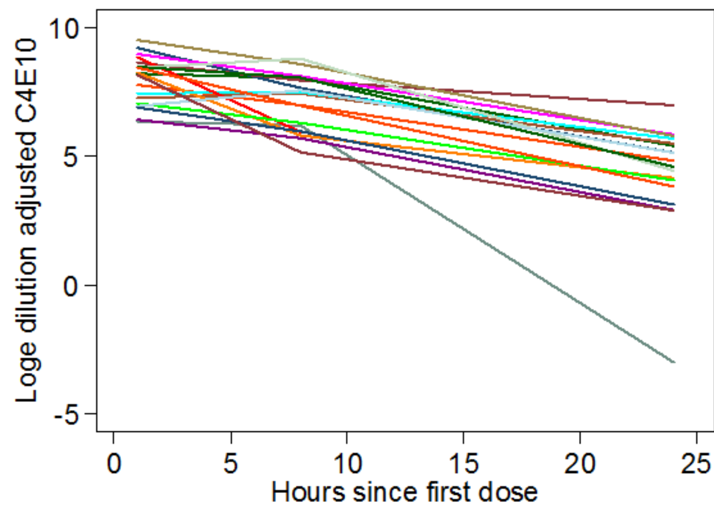


Figure 3-15 Log$_e$ of C4E10 Concentration (Adjusted for Dilution Factor) Since Application of 1st Dose of Mabgel



The linear model appears quite plausible, with one possible outlier, and checking with a time squared term we get P = 0.4. However, there is evidence that there is an interaction between participant and time, P = 0.01. If we omit the outlier, MAB007, the interaction

has P = 0.1, so we can try the estimate with and without MAB007, whose 24 hour post dose level was anomalously low.

Without MAB007, the estimated slope of the line on the log scale is $-b = -0.1462686$, 95% CI for b = 0.1260151 to 0.1665222. Hence the t ½ is estimated by log(2)/ 0.1462686 = 4.7388652 = 4.7 hours, with 95% CI log(2)/0.1665222 = 4.1624911 = 4.2 hours to log(2)/0.1260151 = 5.5005089 = 5.5 hours.

If MAB007 is included, the estimated slope of the line on the log scale is $-b = -0.1622861$, 95% CI for b = 0.1311617 to 0.1934105. Hence the t ½ is estimated by log(2)/0.1622861 = 4.2711432 = 4.3 hours, with 95% CI log(2)/0.1934105 = 3.5838136 = 3.6 hours to log(2)/0.1311617 = 5.2846767 = 5.3 hours. As expected, this CI is considerably wider, thus the estimate without MAB007 is preferable. Hence we have estimated t ½ = 4.7 hours (95% CI 4.2 to 5.5) for the dilution factor adjusted C4E10 concentrations. This is around an hour longer than the estimate of 3.7 hours (95% CI 3.2 to 4.3 hours) for the unadjusted C4E10 concentrations and is remarkably similar to the findings for C2F5.

3.7.3.6 C2G12 adjusted for dilution factor

Figure 3-16 Concentration of C2G12 (Adjusted for Dilution Factor) Since Application of 1st Dose of Mabgel

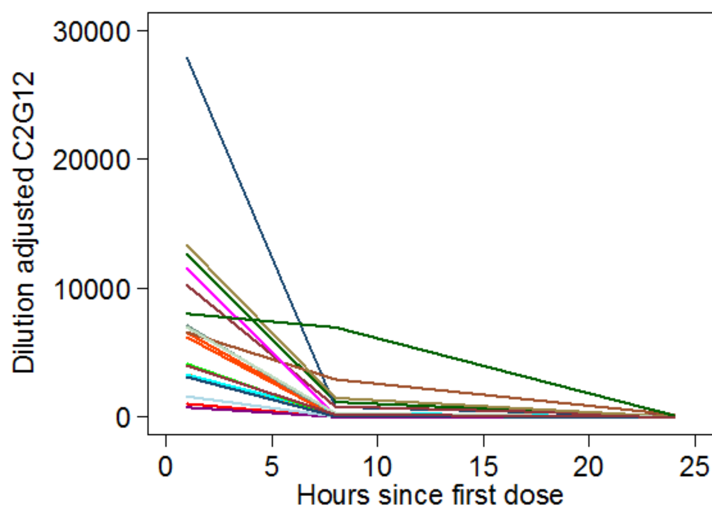
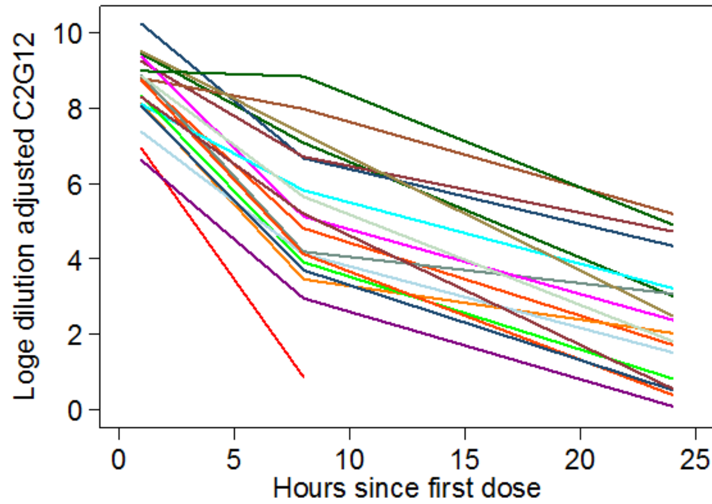


Figure 3-17 Log_e of C2G12 Concentration (Adjusted for Dilution Factor) Since Application of 1st Dose of Mabgel



As with the unadjusted 2G12 concentrations, there is evidence to suggest that these data do not fit with an exponential decay model. The linear fit does not appear plausible and checking with a time squared term gives $P < 0.001$. There is no evidence that there is an interaction between participant and time, $P = 0.8$ and most participants exhibit the non-linearity. As before, the 1 and 8 hour observations only will be used to estimate the early t ½.

The estimated slope of the line on the log scale is $-b = -0.4804366$, 95% CI for b = 0.3822727 to 0.5786004. Hence the t ½ is estimated by log(2)/0.4804366 = 1.4427443 = 1.4 hours, with 95% CI log(2)/0.5786004 = 1.1979722 = 1.2 hours to log(2)/0.3822727 = 1.813227 = 1.8 hours. Thus the estimated early t ½ for the dilution factor adjusted CG12 levels = 1.4 hours (95% CI 1.2 to 1.8). This is similar to the 1.2 hours (95% CI 1.0 to 1.4) estimated for the unadjusted C2G12 concentrations.

3.7.4 Analysis of Variability in Weck-Cel Sampling and relationship between sample weight and mAb concentration

The second of the additional pharmacokinetic analyses outlined in the final SAP (see section 2.13.1) was an estimation of the degree of variability in weights/volumes of samples obtained using Weck Cels from the same woman at the same visit, between women and between visits and an assessment of the relationship between sample weight/volume and detected mAb concentration.

Two Weck-Cel samples were performed on each of 28 women at each of five visits (Visits 2, 3, 4, 6, and 7) as described in section 2.7.2.3. At one visit, Visit 2, two pairs of samples were taken, one before and one after the first dose of study gel was applied. Sample weights were obtained as described in section 3.6.1.1. MAB015 had some missing data as no samples were taken at Visit 4 and for this part of the analysis she has been omitted.

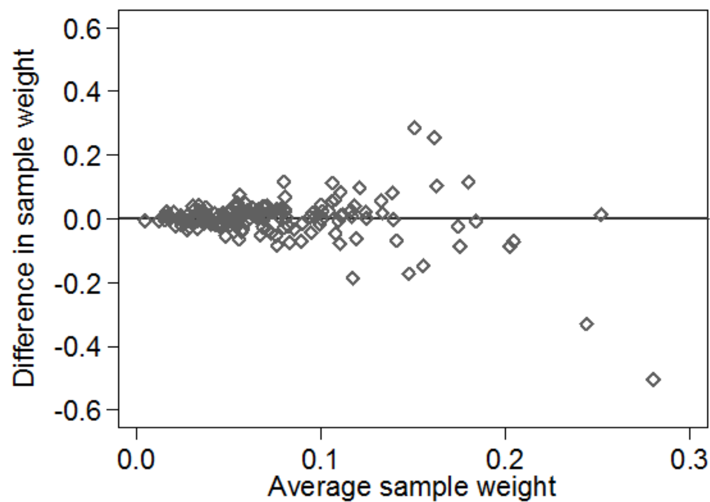
Note that the analyses have been performed using the actual sample weights recorded on the balance (in grams).

3.7.4.1 Variation in paired Weck-Cel sample weights obtained from the same woman at the same sampling time-point

Prior to performing any statistical analyses, variation in paired Weck-Cel sample weights obtained from the same participant at the same time-point was evaluated through presenting the data as a series of scatter diagrams.

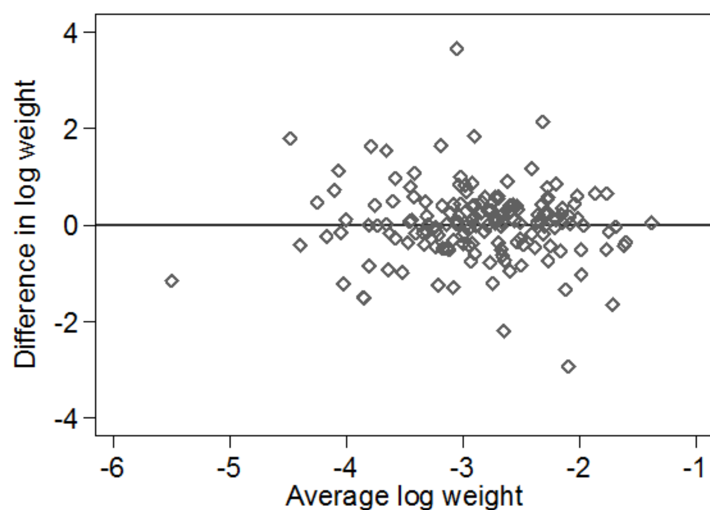
Firstly, to see how the variability of sample weights obtained was related to the magnitude of the weight, the difference between the weights of paired samples, taken at the same time-point in the same woman, was plotted against the mean of the two weights. As can be seen from Figure 3-18, there is a clear relationship with the heaviest samples (mostly those obtained at Visit 2, 1 hour post 1st dose (see section 3.7.4.4) having much greater variability than lighter ones.

Figure 3-18 Relationship between variability of weights of samples obtained at the same time-point in the same woman and magnitude of the mean sample weight (in grams)



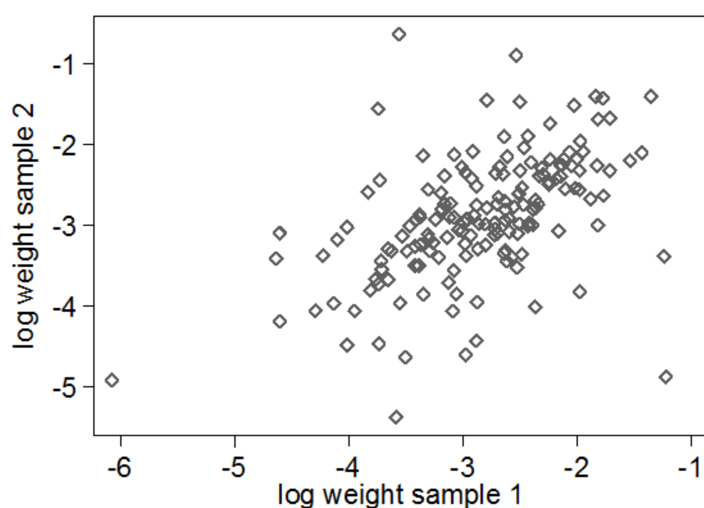
As can be seen in Figure 3-19, log transformation reduces the influence of the heaviest, most variable, samples on the overall distribution of the data and it is thus less skewed. This supports the decision to perform ANOVA of sample weights (see section 3.7.4.2) using log transformed data.

Figure 3-19 Relationship between variability of weights of samples obtained at the same time-point in the same woman and log $_{10}$ of the mean sample weight



As Figure 3-20 shows, the weights for paired, repeat samples are clearly related i.e. samples taken at the same visit from the same woman show a trend towards having similar weights, however there is a degree of variability observed.

Figure 3-20 Relationship Between the weights of paired samples obtained at the same time-point in the same woman



3.7.4.2 Analysis of Variance (ANOVA)

ANOVA is a statistical method which compares variation in repeated measurements obtained within a group of subjects to the variation between different groups [578]. It is often used to compare the means of samples from several different groups, e.g. participants in different study arms who have received different treatments or have different specific diagnoses i.e. where there are *fixed effects,* to see if there is a significant difference. In situations where 'groups' are actually individual members that can be considered to be a random sample from a larger population i.e. where there are *random effects*, e.g. individual participants on whom paired or serial measurements have been taken, it can also be used to estimate the variance between measurements in the same participant and between different participants, to look for sources of variability or measurement error, which is often of greater interest than comparing the means.

There are two assumptions for ANOVA: firstly, that that the observations within groups (called *residuals*) follow a Normal distribution and secondly, that the variances of these

distributions are the same. Of these, the assumption of uniform variance is the most important [578].

As shown in Figure 3-21 below, the raw sample weights (the residuals in this analysis) have a roughly symmetrical distribution, but with tails too long for the Normal, and very different variability within visits (with the samples from Visit 2, 1hr post 1st dose being the most variable).

The log transformed data (shown in Figure 3-22) have a more uniform variance, and although their distribution is slightly skewed, fit the ANOVA assumptions better. Thus, the analyses were conducted using the transformed data.

Figure 3-21a and b Distribution and within visit variability of raw sample weights (residuals)

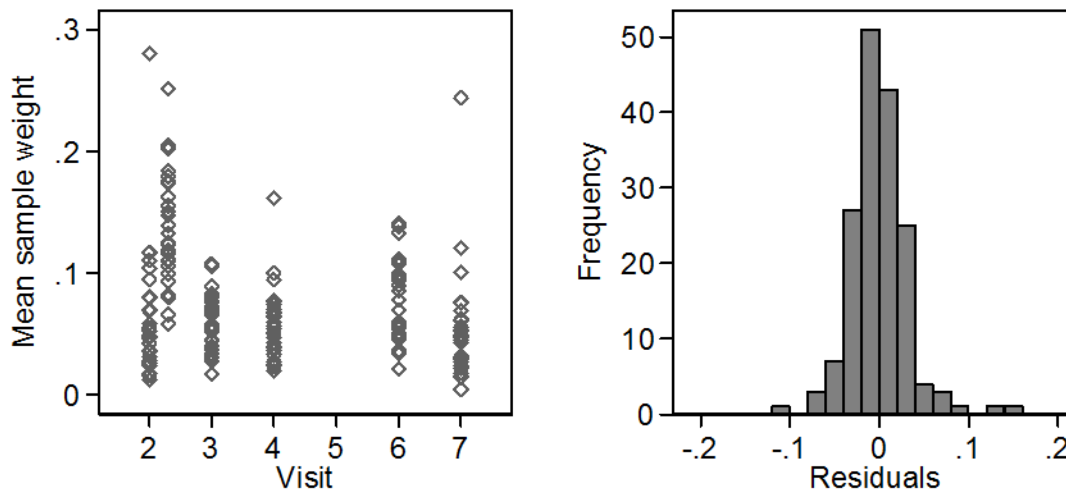
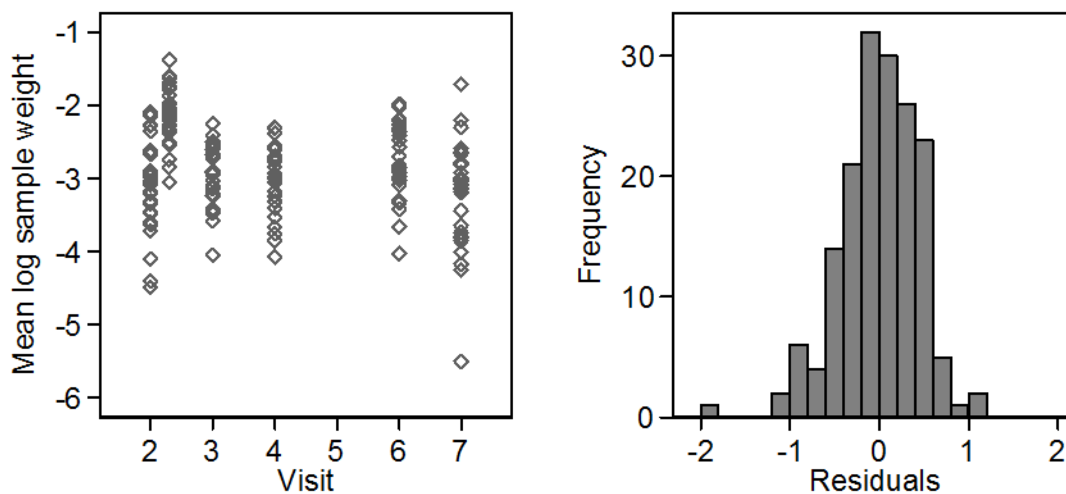


Figure 3-22 Distribution and within visit variability of $\log_{10}$ transformed sample weights (residuals)



A comparison of means of different groups of subjects using ANOVA is conducted through first obtaining an estimate of the common variance (S^2) of the residuals within the groups (assuming the variances are the same for all of the populations represented by the groups), known as the *within groups variance* or *residual variance*. To do this, the mean for each group of residuals is estimated from the data and a sum of squares about the mean is calculated for each group and then added together to give the *within groups*

(residual) sum of squares. The *degrees of freedom* are obtained from the total number of residuals in all groups, minus the number of estimated means. In general, for *m* groups of size *n* each, there are $nm - m = m(n-1)$ degrees of freedom.

residual variance = the within group sum of squares/the number of degrees of freedom

An estimate of variance is also obtained from the variance of all the group means. If there were no differences between any of the means in the populations from which the groups are derived, this *between groups variance* would be the variance of the sampling distribution of *n* observations, which is given by S^2/n (i.e. the square of the standard error) [578].

The null hypothesis (H_0) for the ANOVA is that the variance of residuals within the groups is the same as that between the groups. Thus, if this were true, *n* times the mean-derived (between groups) variance should be equal to the residual (within groups) variance. The ratio of these two variance estimates is called the *variance ratio (F ratio, or F value).* If H_0 were true and if the residuals were all from the same Normal distribution with uniform variance, then the variance ratio would follow a known distribution, F, with *m*-1 and *n*-1 degrees of freedom. If H_0 were true, the variance ratio (F value) would be expected to equal 1.0. The larger the variance ratio, the smaller the probability of it being compatible with H_0. If $P < 0.05$ then H_0 is rejected at the 95% confidence level.

As mentioned above, in situations where 'groups' are in fact individual subjects from whom repeated samples/measurements have been taken , ANOVA is most often used to provide estimates of both the *within subject variance* (σ^2_w) and the *between subject variance* (σ^2_b). This is known as the *method of moments of components of variance*. A single measurement observed from a single subject has variance $\sigma^2_w + \sigma^2_b$. If there are *n* repeated measurements (replicate residuals) in each of *m* subjects; σ^2_w can be estimated directly from the *within subjects mean square* (MS_w). The *between subjects mean square* (MS_b) provides an estimate of $m\sigma^2_b + \sigma^2_w$. The variance ratio, (F value) = MS_b/MS_w. In this instance, H_0 is that all subjects are the same, i.e. $\sigma^2_b = 0$. As above, if H_0 is true, the variance ratio would be expected to be 1.0. If $P < 0.05$ then H_0 is rejected at the 95% confidence level [578].

ANOVAs can be conducted for a single source of variability i.e. different groups or subjects alone; a one-way ANOVA, or for multiple sources of variability; a multi-way ANOVA.

3.7.4.3 Analysis of Variance (ANOVA) for log_{10} transformed Weck-Cel sample weights obtained at the same time-point (i.e. within the same woman and between different women within the same visit across all visits)

The following table shows an ANOVA table where individual women are the 'groups'; the row labelled 'women' represents the 'between subjects' terms and study visit is a second source of variation which was included in the analysis. The row labelled 'visits' represents the 'between visits' terms. The row labelled 'residuals' represents the 'within subject and within visit' terms. An assessment of the interaction between women and visits is also included to see whether there is any evidence to suggest that combining both potential sources of variation (i.e. comparing samples that have been taken both from different women and at different visits) produces greater variability than either factor on its own.

For the purposes of this analysis each woman is considered to be a random member taken from a larger population and it has been assumed that each sample weight is uninfluenced by study treatment arm. The latter assumption has been tested formally, as shown in section 3.7.4.4.

Table 3-6 ANOVA table 1 for log_{10} transformed weights of repeated Weck-Cel samples

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio (F)	Probability
Women	26	28.3966902	1.09218039	5.69	<0.0001
Visits	5	47.523177	9.5046354	49.56	<0.0001
Women x Visits	130	80.6929978	0.620715368	3.24	<0.0001
Residual	162	31.0714945	0.191799349		
Total	323	187.68436	0.581066129		

As can be seen from the table, there is a significant difference between the mean weights of Weck-Cel samples obtained from different women at the same visit and between those obtained from the same woman at different visits. The interaction term is also significant,

suggesting that weights of samples obtained from both different women and at different visits differ by a greater amount than samples obtained from either different women at the same visit or the same woman at different visits.

As the interaction term is significant, the main effect can also be tested against the interaction mean square rather than the residual (within groups) mean square. For visits we get $P<0.0001$, for women we get $P = 0.02$, so both main effects remain significant.

The variances relating to the different sources of variation can be estimated using the method of moments for components of variance. The residual (within groups) mean square gives us the within visit and woman variance, the variance of repeated measurements on the same woman at the same time-point. This is 0.191799349. The square root of this is the standard deviation, 0.43794903. This is the standard deviation of log weights sampled from the same woman at the same visit. The anti log of this is 1.55. This is approximately one plus the *coefficient of variation on the natural scale*, 0.55 or 55% [582]. Hence repeated samples on the same woman at the same visit will have a standard deviation approximately 55% of the actual weight. This great variability is reflected in Figure 3-18, where it can be seen that differences between pairs of weights can be as big as the average of the two.

The variability between samples taken from different women on the same visit can also be estimated. For this two estimates of variance are needed; for heterogeneity and between women [578]. For the heterogeneity variance, we have $(0.620715368 - 0.191799349)/2 = 0.21445801$. Between women we have $(1.09218039 - 0.620715368)/(2\times6) = 0.03928875$. The total variance between samples taken from different women on the same visit is the sum of these three variances: $0.03928875 + 0.21445801 + 0.191799349 = 0.44554611$. The square root of this is 0.6674924 and the antilog is 1.95. The coefficient of variation is 0.95 or 95%. Hence sample weights are hugely variable between women.

3.7.4.4 Variability between visits and by treatment arm

In the analysis above, no estimate has been made of the variability between samples taken at different visits. This is because variability between visits is not random but

systematic as there are experimental differences between the visits, e.g. differences in the timing post gel application.

As can be seen from Table 3-7, the mean weights of samples obtained at Visit 2, 1 hour post application of 1st gel dose are much higher than those obtained at other visits. There is little to suggest a difference in weights between study treatment arms, and this is demonstrated formally by the results of the ANOVA displayed in Table 3-8.

Table 3-7 Mean (and standard deviation) of paired Weck-Cel sample weights, in grams, by visit and treatment arm

Visit	**Treatment Arm**		
	Placebo	Low Dose	High Dose
2 (pre)	0.063 (0.033)	0.084 (0.079)	0.047 (0.031)
2 (post)	0.122 (0.047)	0.146 (0.056)	0.134 (0.036)
3	0.062 (0.026)	0.063 (0.013)	0.053 (0.029)
4	0.056 (0.018)	0.062 (0.025)	0.054 (0.043)
6	0.072 (0.041)	0.068 (0.024)	0.079 (0.038)
7	0.072 (0.072)	0.047 (0.029)	0.045 (0.020)
Number	9	9	10

Table 3-8 ANOVA table 2 for $\log_{10}$ transformed weights of repeated Weck-Cel samples

Source of Variation	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	**Variance Ratio (F)**	**Probability**
Treatments	2	1.1732163	0.58660813	1.07	0.3583
Women within Treatments	25	13.7103164	0.54841266		
Visits	5	21.3408999	4.26817999	16.62	<0.0001
Visits x Treatments	10	1.9832618	0.19832618	0.77	0.6552
Residual	124	31.8479446	0.25683826		
Total	166	70.0364077	0.42190607		

Table 3-8 shows a two-way ANOVA where study treatment arm is the grouping factor. It is a split plot analysis, with women being the main units/source of variation, and visits being sub-units within these main units. The row labelled ‘treatments’ represents the ‘between treatment arms’ terms. The row labelled ‘women within treatments’ represents the ‘between women within the same treatment arm’ terms and that labelled ‘visits’ the ‘between different study visits’ terms. The ‘residual’ row represents the ‘within treatment arm, within woman and within visit’ terms.

As can be seen from the ANOVA, there is no significant difference in the mean Weck-Cel sample weights between treatment arms ($P = 0.36$). This is good for the internal validity of the study as it suggests there was no systematic error or bias occurring in sampling between the different arms.

There is, however, a highly significant difference between sample weights obtained at different visits ($P < 0.0001$). The latter is largely driven by the much heavier samples from Visit 2 (1 hour post 1st dose). The samples obtained at this visit were visibly coated with gel, and it is this which has likely resulted in an increase in the recorded sample weight. By 8 hours post dose (Visit 3) there was very little gel visible in the vagina, hence less, if any would have likely been collected on the samples at other visits.

The composition of the study gels was adjusted so that each different type, placebo, low dose mAbs and high dose mAbs, had approximately the same density. Thus, any present in Weck-Cel samples, particularly those taken at visit 2, 1 hour post dose, should have had the same weight for the same amount present (g/ml). This seems to be supported by the lack of a significant difference in the sample weights between study arms.

3.7.4.5 Relationship between mAb concentrations detected in Weck-Cel samples and sample weights.

The final analysis performed with regards to sample weights was to examine the relationship between weight of samples and concentration of each mAb detected by ELISA. For this analysis data was only included from post-dose samples in participants receiving active treatment (low or high dose Mabgel). The few “not detectable” observations were set to 0.05 for this analysis, before transformation.

As shown in Figure 3-23 to Figure 3-28, in our study, there was an overall trend towards higher mAb levels being detected on heavier samples. However, as might have been predicted, the relationship was largely determined by the heavier, gel-laden, samples obtained at Visit 2 (1 hour post 1st dose), the time-point with peak mAb levels. A formal assessment of the relationship between sample weights, study visit, mAb concentration and treatment arm can be made through conducting a multiple regression analysis [577].

3.7.4.6 Multiple regression of log_{10} mAb concentration on log_{10} Weck-Cel sample weight, with visit, subject and visit X treatment interaction as categorical factors

In the following scatter diagrams, the symbol is the code number of the visit. 2.1 represents Visit 2 (1 hour post 1st dose). The mAb concentrations are highly skewed so have been log-transformed in addition to the sample weights. For comparison, the untransformed mAb levels and sample weights have also been plotted. It is clear that there is some over-transformation occurring but that that the log transformed values are more appropriate for regression analysis, which assumes that residuals from both variables being compared are from a Normal distribution with uniform variance.

Figure 3-23 Scatter plot of mean log_{10} Weck-Cel sample weight against log_{10} C2F5 concentration detected by ELISA (unadjusted for dilution factor)

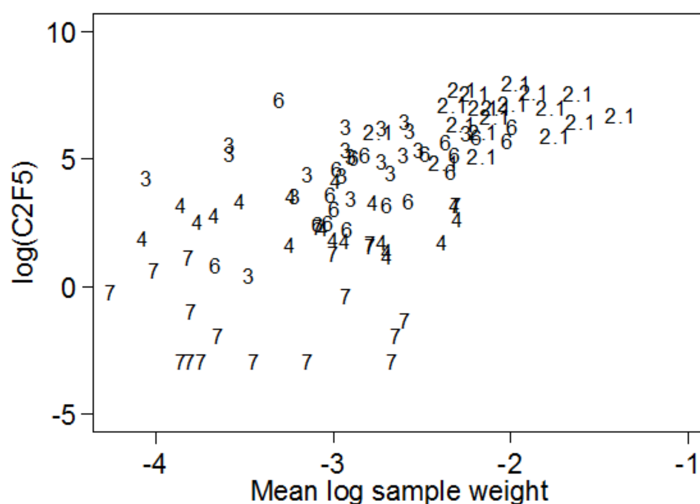


Figure 3-24 Scatter plot of mean Weck-Cel sample weight against C2F5 concentration detected by ELISA (unadjusted for dilution factor)

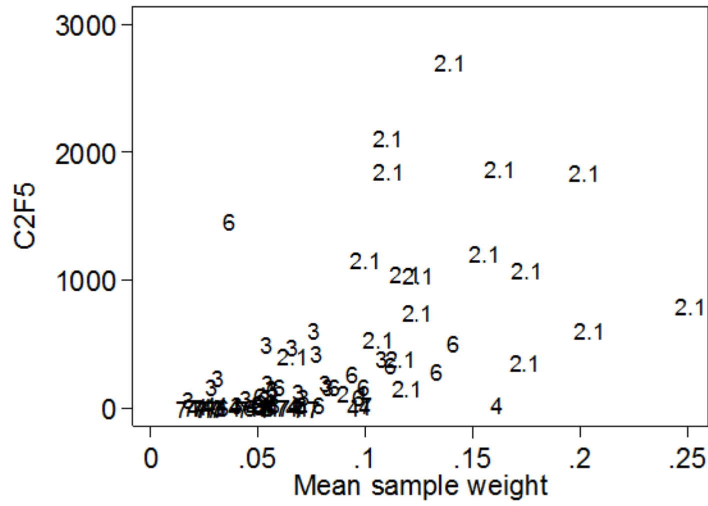


Figure 3-25 Scatter plot of mean $\log_{10}$ Weck-Cel sample weight against $\log_{10}$ C4E10 concentration detected by ELISA (unadjusted for dilution factor)

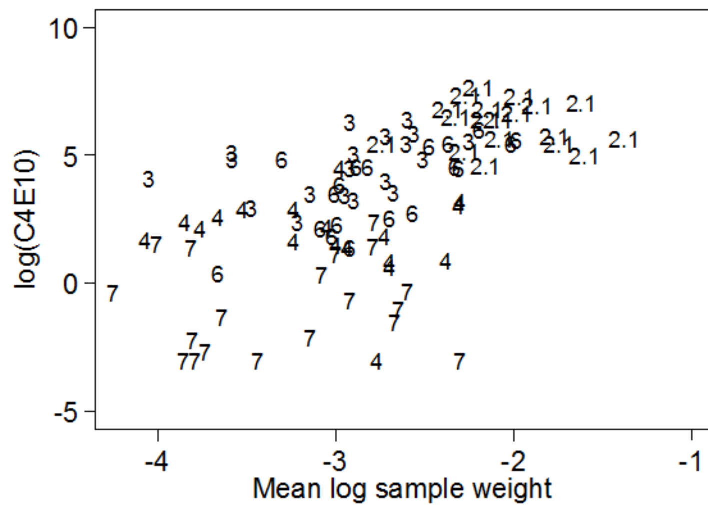


Figure 3-26 Scatter plot of mean Weck-Cel sample weight against C4E10 concentration detected by ELISA (unadjusted for dilution factor)

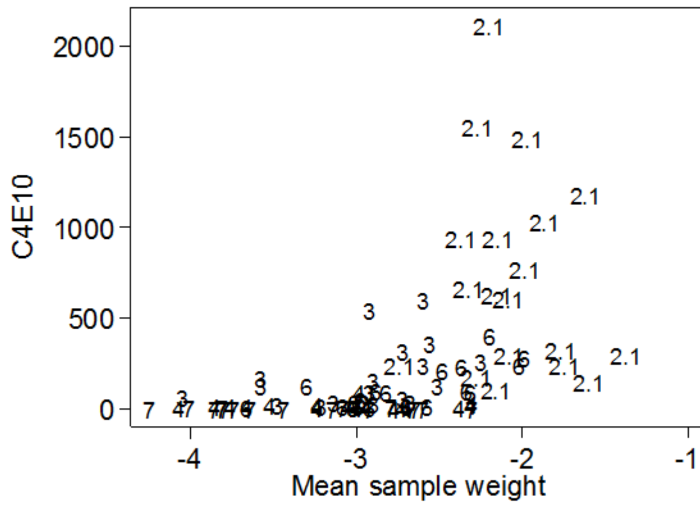


Figure 3-27 Scatter plot of mean log_{10} Weck-Cel sample weight against log_{10} C2G12 concentration detected by ELISA (unadjusted for dilution factor)

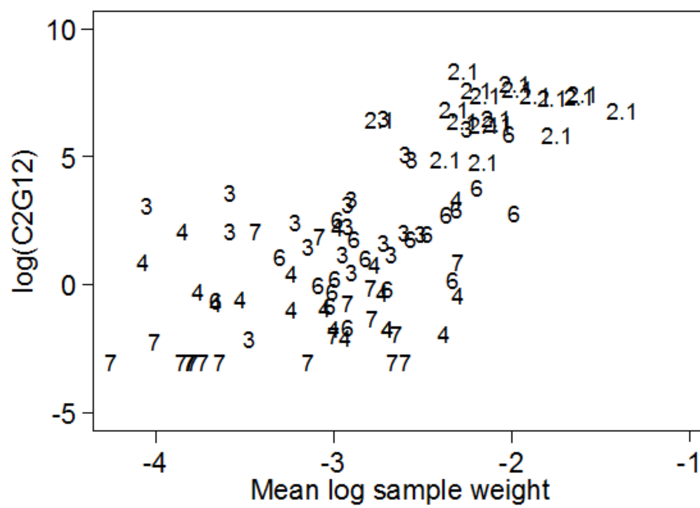
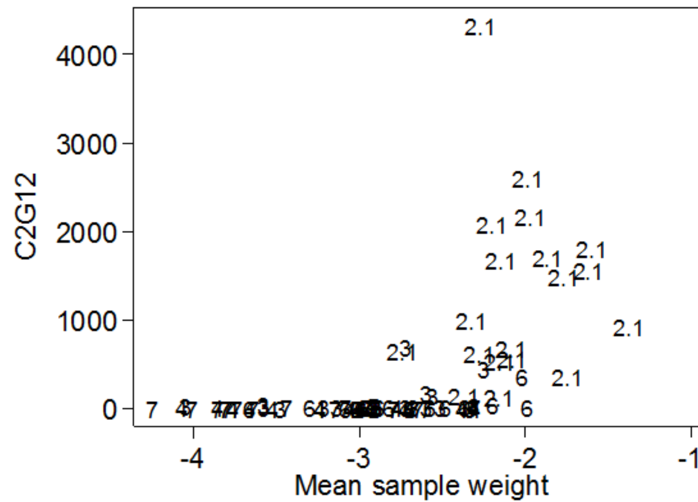


Figure 3-28 Scatter plot of mean Weck-Cel sample weight against C2G12 concentration detected by ELISA (unadjusted for dilution factor)



Multiple regression of log_{10} antibody on log_{10} sample weight was performed with visit, treatment, woman (participant), and visit×treatment interaction as categorical factors [577]. Visit was included in the analysis because of its strong influence on mAb levels and sample weight. Woman (participant) was included because it is a structural variable and it needed to be taken into account that the successive observations were on the same woman.

Table 3-9 shows the regression slope of log antibody on log sample weight, first adjusted only for woman, then adjusted for woman and visit. The table also shows the increase in R^2 when sample weight is added, first to the model with woman only as the predictor, then to the model with woman and visit. R^2 is the square of the multiple correlation coefficient (R) and represents the proportion of variability explained by the regression [577].

Table 3-9 Regression slope of log_{10} mAb concentration on log_{10} Weck-Cel Sample Weight

mAb	Adjusted for woman only		Adjusted for woman and visit	
	Slope for sample weight (95% CI, P)	R^2 increase when sample weight added	Slope for sample weight (95% CI, P)	R^2 increase when sample weight added
C2F5	3.44 (2.63 to 4.26, P<0.001)	0.439	1.10 (0.45 to 1.76, P=0.001)	0.018
C4E10	3.26 (2.44 to 4.07, P<0.001)	0.411	0.90 (0.23 to 1.58, P=0.009)	0.013
C2G12	4.53 (3.73 to 5.32, P<0.001)	0.560	1.67 (1.02 to 2.31, P<0.001)	0.031

For each mAb, there was a clear correlation between mAb concentration and sample weight overall, however, the slope was greatly reduced when visit was included in the model and the contribution of sample weight to the R^2 was much smaller. This showed that a large part of the relationship between mAb concentration and sample weight was explained by the influence of visit on both variables. This was largely driven by Visit 2 (1 hour post 1st dose), where both sample weights and mAb levels were at a maximum. However, it also showed that there was a small residual relationship which was not explained by visit i.e. that there was a slight general trend towards obtaining higher mAb concentrations from larger (heavier) samples independent of study visit.

3.8 Summary and Discussion of Pharmacokinetic Results

3.8.1 mAb Pharmacokinetics

Pharmacokinetics is 'the handling of a drug within the body, including its absorption, distribution, metabolism, and excretion' [583]. Current knowledge of these processes with regards to mAbs has largely been derived from the study of naturally occurring antibodies or mAbs administered parenterally [468,584,585]. To date, almost all mAbs licensed for therapeutic use are administered either IV (the majority), or subcutaneously (SC), for systemic activity [468].

A few mAbs have been developed for localised administration in humans. These include Catumaxomab (TRION Pharma and Fresenius Biotech, Germany), a dually targeted IgG mAb against epithelial cell adhesion molecule (EpCAM) and the T Cell Co–Receptor, CD3, which is infused intra-peritoneally for the treatment of malignant ascites [586] and the vascular endothelial growth factor A (VEGF-A) binding Fab fragment Ranibizumab (Lucentis®, Genentech, San Francisco, CA, USA (owned by Roche, Basel, Switzerland), used intravitreally to treat neovasular age-related macular degeneration [587]. Pulmonary delivery of mAbs for the treatment of lung cancer is also being explored [588]. Although humanized anti-HSV2 IgG mAbs have been developed, studies of vaginal application have thus far been confined to rodents, and have primarily provided data on protective efficacy [589, 590]. To our knowledge, MABGEL 1 was the first study to look at the pharmacokinetics (and safety) of any mAbs in the human FGT.

3.8.2 mAb Concentrations detected in vaginal secretions sampled using Weck-Cels (primary study end-points)

The primary end-points for the MABGEL 1 trial were the concentrations of the mAbs detected in vaginal secretions sampled using Weck-Cel sponges at 1, 8 and 24 hours post-1st dose, and 12 and 36 hours post-12th dose of study gel. Median mAb levels (µg/ml)(as reported by Polymun) were found to be maximal at 1 hour post dose in the high dose (1505, C2F5; 987, C4E10; 1620, C2G12) and low dose Mabgel (542, C2F5; 239, C4E10; 619, C2G12) groups and still detectable at very low (nanogram range) levels at 36 hours (post 12th dose) in some participants. On average, adjustment to account for dilution factors during processing increased mAb levels 5 fold at 1 hour post 1st dose, and 12 fold at other time-points. Although there were a couple of anomalous values in a small number of participants, the median levels are in keeping with application of a gel containing either 10mg/g or 20mg/g of each mAb.

3.8.3 mAb concentrations in other samples (secondary end points)

3.8.3.1 CVL and aspirate samples

CVL samples were taken at the last sampling time-point per gel dose, to allow direct comparison with the neat, self-aspirated secretions obtained by each participant as well as to those obtained by Weck -Cel. On visual inspection of the concentrations as reported by Polymun, in comparison with the values obtained from Weck-Cel samples (unadjusted for dilution factors) at the same time point and in the same woman, mAb levels obtained from CVL samples were generally lower (although by variable amounts). As is reflected in the median levels in Table 3-4, differences were greatest for visit 4 samples (24 hours post 1st dose) for levels of C2F5 and C4E10, with smaller differences seen at visit 7 and for C2G12 in general, where mAb levels were measured as being < 1mg/µl in both sample types.

Unfortunately, as discussed in section 3.8.6.5, there were difficulties encountered with using the self-aspirator, resulting in a paucity of analysable samples. A formal statistical comparison between the 3 methods was therefore not possible.

3.8.3.2 Serum samples (systemic absorption)

For C2F5, mAbs were undetectable or very close to the assay cut off (0.06 µg/ml) in most participants at most time-points. Levels between 0.1 and 0.2 µg/ml were detected in 2 participants ((MAB016) in the low dose and (MAB003) in the high dose Mabgel arms), however, in both participants, levels were greater at baseline (pre-1st gel dose) than at the 8 hour post 1st dose and 12 hour post 12th dose visits . There was no evidence of an overall difference in 2F5 levels between study arms.

For C4E10 and C2G12, mAbs were detected at low levels (0.4 to 2.0 µg/ml) in all participants at all time-points (including samples taken at baseline and in placebo-users). Looking at the data recorded for individual women, similar levels (within 0.2 µg/ml) were detected across all 3 samples, with baseline values being higher, in many cases, than those taken at the 2 post dose visits. There was no evidence of a difference between study arms. As discussed in section 3.8.6.7, it seems likely that the levels reported represent non-specific background signal in the assay rather than actual mAbs. Thus,

there appears to be no overall evidence from our data to suggest that any systemic absorption of the mAbs occurs.

3.8.4 mAb Half-lives

The half-life (t ½) is the time required for half of an administered agent present to be eliminated from the body or a specified compartment. For t ½ to be valid, the decay in the concentration of the agent must be exponential i.e. the rate at which it is eliminated must be proportional to the amount present. In turn, this implies that processes responsible for elimination of the agent, i.e. distribution and clearance, must follow 1st order kinetics and not be subject to saturation [579].

3.8.4.1 Estimates of the residence vaginal t ½ of C2F5, C4E10 and C2G12 obtained in our study

Using the concentrations adjusted for dilution factors, for all participants in both the high and low dose Mabgel arms, residence half-lives in vaginal secretions (Weck-Cel samples) were estimated to be in the range of 4 to 5.5 hours for both of the MPER mAbs (4.7 hours (95% CI 4.2 to 5.5) for C4E10; 4.6 hours (95% CI 4.0 to 5.0) for C2F5). t ½ estimates were approximately an hour shorter using the unadjusted values, with both adjusted and unadjusted data fitting a exponential model. In contrast, vaginal levels of C2G12 did not conform to a single overall exponential decay, displaying a more rapid initial rate of decline than the other two mAbs, which then slowed at lower concentrations. An estimate obtained for the early t ½ of C2G12, based on the 1 and 8 hour post 1st dose values, was 1.4 hours (95% CI 1.2 to 1.8).

3.8.5 Variability in Weck- Cel sample volumes

As demonstrated in section 3.7.4, there was a significant degree of variability in secretion weights/volumes between paired Weck-Cel samples obtained from the same woman at the same time-point (coefficient of variation 0.55 (i.e. standard deviation approximately 55% of actual weight), although, as might be expected, this was much less than the variation between samples from different women at the same time-point (coefficient of variation 0.95).

In our study, measures taken to try to make sampling as uniform as possible included following SOPs, using the same brand of Weck-Cel throughout the study, utilizing different sampling methods in an order/at time-points designed to minimise their impact on subsequent samples and having the same clinician (the author) conduct all visits. However, as detailed in sections 3.8.5.1 and 3.8.5.2 below, variables arise both within the same woman and between participants which are beyond investigators' control. In addition, as discussed in section 3.8.6, limitations with our sampling and laboratory processes, e.g. errors in recording weights, are also likely to have contributed to observed variability.

3.8.5.1 Factors which could produce variability in the same woman at the same time-point

Provided Weck-Cels are held against the vaginal mucosa for a fixed period of time, the main factors influencing the volume/weight of sample collected will be the absorbance properties of the sponge and the amount and consistency of secretions/gel present in that area of the vagina. Although some variation in absorbance may exist between Weck-Cels, especially if from different batches, this is likely to be small. In contrast, differences related to secretions/gel and the vaginal environment are likely to be much greater.

For anatomical reasons (see section 1.3) both gel and secretions may be more likely to pool in the upper vagina than the lower [51], therefore it is important to ensure sampling is consistently from the same region. In our study, all Weck-Cel samples were obtained from the lateral fornices (for paired Weck-Cels at the same visit, 1 was placed in each fornix on opposite sides). However, there is likely to be variation in the spread of gel and secretions even within this region.

As shown in Figure 3-18, variability was greatest for the heaviest samples, obtained 1 hour post 1st gel dose. This is unsurprising. At this time-point, in contrast to all others, Weck-Cels were often visibly coated with gel, with gel covering the surface of the sponge as well as being absorbed within it. Thus, the weight/volume of sample collected was likely to be less determined by the absorbance properties of the Weck-Cel sponge and more dependent on the amount of gel/secretion present. As demonstrated by Barnhart

et al., using Magnetic Resonance Imaging (MRI) post vaginal application of gels of various compositions/formulations and volumes, gel distribution is rarely uniform (throughout the lower FGT), and complete, contiguous mucosal coverage is not always achieved [591,592]. Coverage would be expected to increase with time, as the gel spreads linearly and laterally and mixes with vaginal secretions, before being diluted out and/or lost through the introitus [591,592,593,594]. By 8 hours post dose very little gel remained visible in the vagina, thus it is likely to have had less influence over the weights/volumes of samples obtained at other visits.

3.8.5.2 Additional factors which could produce variability in different women at the same time-point or the same woman at different time-points

Variation with regards to reproductive hormone status, timing and technique of (non-1st dose) gel application, degree of ambulation and hygiene practices could all in theory affect the quantity, consistency and/or distribution of secretions/gel within the vagina, hence the volumes/weights of samples obtained between different participants and from the same woman at different visits. Of the above variables, only the first has been analysed using available data.

As described in section 1.3, stage of menstrual cycle and use of hormonal contraceptives can influence the amount and composition of genital secretions. Endocervical mucus is at its most copious and stretchy at mid-cycle, due to the influence of rising levels of oestrogen, which peak and then fall prior to ovulation. In contrast, progestagens act to thicken the mucus and cause physiological sloughing of the vaginal epithelium, resulting in a heavier, more viscous vaginal discharge, containing exfoliated cells. The latter is seen in the luteal phase of the cycle and in those using progestagen dominant contraception [55,56]. In our trial, distribution of non-hormone (IUCD and/or condom) and hormone (IUS, DMPA, Cerazette or COCP) users was fairly balanced overall between study arms (see Table 3-1). In those women with a natural menstrual cycle, dosing was initiated in the follicular phase (between days 7 and 13) with visits 6 and 7 in the luteal phase. Median day for initial dose varied slightly between the arms (day 9, 11 and 12 for Mabgel high dose, Mabgel low dose and placebo respectively). However, as shown in Table 3-7, although some hormonal influence on individual sample variability is possible, there was little evidence of any difference in mean volumes/weights of samples obtained at visits 2

(pre-dose) and 3 compared with those collected later in the cycle (visits 6 and 7). There was also no evidence of any difference in mean Weck-Cel sample weights/volumes between treatment arms.

Differences between women could also arise due to variation in the volume and surface area of the vaginal cavity. As Barnhart *et al.* have shown through MRI imaging, vaginal dimensions vary markedly between individuals. In their study of 28 vounteers, the length of the vagina (from external cervical os to introitus) ranged from approximately 4.1 to 9.5 cm, transverse diameter (at the widest point, level with the vaginal fornices) varied from 2.6 to 8.3cm and surface contact area ranged from 106.8 to 185.0 mm ^{2}. Increasing age and parity were significantly associated with widening of the transverse diameter (due to laxity in the vaginal walls) and lengthening of the posterior and lateral fornices respectively. A non-significant positive association was found between weight and overall vaginal length and a weak negative association between height and transverse diameter, with no associations between any parameter and ethnicity [51]. In our study, women in the low dose Mabgel arm were on average older than in the other 2 arms (32 compared with 25 years) and had had a greater number of children (Table 3-7), thus may have had a larger average vaginal surface contact area. This does not seem to have influenced the volumes/weights of collected Weck-Cel samples overall (since there was no evidence of a difference between the study arms (Table 3-7)) but may have increased the distribution of gel *in-situ*, with a subsequent reduction in detected mAb concentrations (see section 3.8.5.3 below).

3.8.5.3 Factors which could produce variability in mAb levels measured in different women at the same time-point

As shown Table 3-2 the range of detected concentrations of C2F5, C4E10 and C2G12, across all time-points, was generally wide, reflecting the degree of variability in mAb levels in different participants. Many of the variables affecting the amount/ distribution of gel or secretions within the lower FGT will also influence detected mAb levels. In the multiple regression analysis (section 3.7.4.6), there was a clear positive correlation between detected concentrations of each mAb and Weck-Cel sample volume/weight. Although a large part of the relationship was explained by the influence of Visit 2 (1 hour post 1st dose), where both sample weights and mAb levels were at a maximum, there

was a small residual relationship, independent of study visit, suggesting a weak general trend for larger/heavier samples to give rise to higher mAb levels. However, the relationship between sample volume/weight and mAb concentration is not a simple one. At the same (non- 1 hour post dose) time-point, secretion samples may be heavy because they consist of remnants of gel or viscous/mucoid secretion, containing high concentrations of relatively undiluted mAbs, or the Wecks-Cel may have absorbed a large amount of relatively dilute watery discharge/secretion containing lower levels of mAbs.

Adjustment for 'dilution factors' corrects for the variation in mAb dilution by buffer during sample processing, due to the different weights/volumes collected, but it does not adjust for differences in *in-situ* dilution and mucosal spread. In theory, it provides a measure of 'real-life' mAb concentrations within the vaginal lumen, maintaining variability between individuals due to the presence of varying amounts of natural vaginal secretions and/or distribution e.g. differences in contact surface area, ambulation, hygiene practices etc. In our study, general trends in mAb concentrations were very similar both before and after 'dilution factor' adjustment and median levels obtained were in keeping with the quantity of mAbs applied in the gel (see section 3.7.2.3). Thus, its use appears to be valid. That said, the higher degree of variability in both sample weights/volumes and mAb levels observed at 1 hour post gel dose visit, and concerns regarding processing of these samples (see section 3.8.6.4) mean that we cannot be certain to what degree our findings at this time-point are accurate.

Another potential influence on vaginal mAb levels is infection, in particular certain *Candida* species. Pre-1st dose cultures showed a heavy (+++) growth of *Candida albicans* in 3 participants in the low dose Mabgel arm (MAB014, 019 and 027). Of these, MAB019 consistently had lower levels of all 3 mAbs compared to other participants in the same arm. This was not true of MAB014 and 027. Since cross-reactivity to *Candida* species' surface glycoproteins is a feature of 2G12 alone, the low mAb levels in MAB019 are unlikely to be explained by this mechanism. Although, it is possible that the infection contributed in another way, e.g. through recruiting neutrophils (which release proteolytic enzymes) and/or increasing dilution through production of vaginal discharge, this participant was only mildly symptomatic and only one of the women (MAB027) had any objective signs of inflammation on examination (see sections 4.1.1.6 and 4.1.1.12).

3.8.6 Limitations of study design and procedures

A number of limitations in the design and procedures employed in our study should be considered when assessing the accuracy and external validity of our findings.

3.8.6.1 Sample size and study power

Our study was the first time any of C2F5, C4E10 and C2G12 had been applied to the human FGT and although some data existed from the CEA macaque studies regarding the pharmacokinetics of the 20mg/g dose, there was insuffіcent data on which to base power calculations to determine the likely sample size required to demonstrate statistically significant differences in mAb levels across the 3 study arms and, in particular, between the 10mg/g and 20mg/g Mabgel arms. A sample size of 10 participants per arm was therefore seen as a reasonable target given the financial, time and personnel constraints that applied. Unfortunately, due to unforeseeable delays with obtaining study approval and the establishment of the EMU, there was only time to enrol 28 women prior to the expiry of the study gels. This sample size proved large enough to demonstrate statistically significant differences in the primary study end-point (levels of each mAb in the Weck Cel samples) across the 3 study arms at all time points, except 36 hours post 12th dose for C2G12 (Kruskal-Wallis rank). However, although observably higher median mAb concentrations were detected in the high dose Mabgel arm compared with the low dose arm at most time-points (except 36 hours (post 12th dose) when they were low (< 1mg/µl) in both arms) these only reached statistical significance at the 1 hour post 1st dose visit for 2F5 and 4E10 and not at all for 2G12 (Man-Whitney U). Thus, although it seems probable that real differences were seen between the levels (detected on Weck Cel samples) achieved with the two Mabgel doses at most time-points, the small sample size, non-parametric method of analysis, high degree of variability between individual participants and imprecision in the sampling and detection methods (see section 3.8.5) likely reduced the power of the study to show this. The ability of our study to demonstrate statistically significant differences in analyses of secondary end-points (CVL and serum samples) was even further reduced due to the generally lower mAb levels detected in these samples.

3.8.6.2 Number and timing of sampling time-points

As discussed in section 2.3.1, sampling time-points were chosen in our trial to try to determine post-dose mAb concentrations at times of greatest relevance to the clinical use of the product whilst minimising the number of invasive examinations and sampling procedures required per participant. Sampling was performed at 1, 8 and 24 hours post 1st dose and 12 and 36 hours post 12th dose; the split enabling the study to be conducted in a day-unit (such as the HYMS EMU) rather than an overnight research facility and aiding the recruitment of participants, who were able to fit dosing and sampling visits around existing family and work commitments.

In terms of primary end-points, sufficient information was obtained from our study to provide an estimate of the peak levels of mAbs within an hour of gel application (of most relevance if the product were to be used just prior to coitus), concentrations at later time-points (to provide information regarding possible duration of efficacy) and the likely time-scale for complete clearance of mAbs from the FGT lumen. However, our sampling schedule was not ideally suited to estimating the t ½ of each mAb in the vaginal lumen. To be certain of timings and avoid the possibility of detecting residual mAbs from previous doses, only those samples obtained following the 1st gel dose were included in the t ½ analyses. Thus, estimates of t ½ for each mAb were based on data from 3 time-points only which limited their accuracy. In addition, the spacing of measurements meant that it was not possible to determine if 2G12 was truly eliminated in 2, bi-exponential, phases or if it was removed from the lumen through processes governed by non-linear kinetics. To obtain more reliable t ½ estimates and to investigate the linearity of the log curve more fully would ideally require at least hourly measurements for 8 hours then as many as could be carried out up to 24 hours. Such an intensive sampling regimen was beyond the scope of our study and would likely require the use of a reliable, acceptable, self-sampling method to avoid the need for repeated invasive clinical examinations.

With regards to serum sampling, we are relatively confident that we have excluded any significant systemic absorption of the mAbs with the samples we obtained. 8 to 12 hours was the time-period post dose that we predicted that we might begin to detect mAbs in serum if they were absorbed through the mucosa, via the lymphatic drainage of the FGT, with the latter sample, taken following 12 gel doses, also able to detect any delayed

absorption. However, with very limited data from previous research regarding uptake of antibodies from the FGT mucosa into the circulation (see section 3.8.8.4), it is difficult to be certain of this and further samples, at 4 hours, 24 hours and 36 hours post dose would perhaps have provided us with greater reassurance.

3.8.6.3 Gel administration procedures

Apart from the 1st dose of gel, which was administered under supervision on the EMU, all other doses, including the final dose, on which the 12 and 36 hour pharmarmacokinetic evaluations were based, were applied by women at home. Thus, although efforts were made by the research team to ensure that women adhered to the dosing protocol, there is an element of uncertainty as to whether the 12th doses were applied at the stated, pre-arranged time. It is well-established that self-reported product adherence can be unreliable [595], but, the fact that women in the trial admitted to having applied earlier doses in the morning (rather than at night/bed-time as they had been instructed) (see section 3.5.1), and 3 women brought back unused syringes, could indicate that they were honest about when they actually used the gel. As described in section 3.5.2 , in a few cases, investigators were aware that samples had been taken outside of the allotted window either side of the pharmacokinetic time-point, due to participants arriving late for visits or mistakingly applying the final gel an hour before schedule. No sampling was performed, to our knowledge, beyond 1 hour of this window.

Another potential limitation with the dosing procedure was the requirement to transfer the gel from its syringe into an applicator before administration. Unfortunately, the fit between the luer-lock nozzle of the syringe and the Ortho applicator was not tight, thus, the potential existed for gel to be spilt during transfer. Although no gel-spillage was reported by participants, several commented on the awkwardness of this procedure in the final interview (see section5.4.5.1), and with hind-sight, it would have been better to have pre-packaged the gel in an applicator device.

3.8.6.4 Potential limitations of vaginal sampling by Weck-Cel and CVL

Weck-Cel sponges have been established as a reliable way of obtaining vaginal and cervical secretions for measurement of naturally generated biological mediators

[525,526,527]. However, ours was the first study to use them to collect specimens for detection of mAbs which had been topically applied in a gel. In contrast to CVL, where the entire ectocervical-vaginal surface is washed with saline and any pooled secretions collected, Weck-Cel samples are obtained through direct contact of the sponge with a small area of the mucosal surface, enabling the site of collection, e.g. vagina, ectocervix or endocervix, to be determined. In addition, the process of Weck-Cel sampling does not dilute vaginal secretions in situ, and there is no potential for error resulting from variability in absorption and recovery of lavage fluid. To normalize the latter requires measurement of total protein or the addition of a lithium tracer [528,529], neither of which was logistically possible in our study. However, although neat, the volume of secretions collected by Weck-Cels is variable and centrifugation, following the addition of a known volume of extraction buffer, is required to elute fluid, containing antibodies, or other proteins of interest, from the sponge [523,524]. Adjustments can be made to correct for the dilution factor introduced during processing (see section 2.13.4.4), but only if an accurate record is obtained of the weight of the Weck-Cel both before and after sampling, to determine the amount of secretion absorbed by the sponge.

In our study, rather than assume an average pre-sample weight (e.g. obtained from weighing 10 representative dry Weck-Cels as has been done by some previous researchers [526]) we measured both the pre and post-sample weights of each individual Weck-Cel using the same fine-balance. For ease of measurement (as described in section 2.11.2.3 and 2.11.2.4), weights were determined for the entire sample collection kit. However, although weighing each individual Weck-Cel pre- as well as post- sampling could in theory increase the accuracy of the calculated sample weights, increasing the number of occasions on which the balance was used and including the entire kit in measurements may have increased opportunities for measurement error. Unfortunately, on four occasions (MAB001 sample 1, Visit 2 (Pre-dose); MAB008 sample 1, Visit 4; MAB004 sample 1, Visit 7; MAB011 sample 2, Visit 7) the balance reading post-sampling was lower than the pre-sampling weight. This was possibly due to an error re-setting the fine balance or a failure to include the Weck sponge stem or other component of the kit in the post-sampling weight. In view of these values being impossible, on the advice of the study statistician, Professor Martin Bland, they were converted to the lowest recorded weight observed of all other samples in the study which was 0.01g

(corresponding to 10 µl). Although these errors will reduce the accuracy of the dilution factor correction applied to the mAb concentrations detected by ELISA at these time-points, the impact will be decreased by the fact that there were two Weck-Cel samples obtained per time-point (pooled prior to analysis) and the dilution factor is calculated using the mean of the 2 sample weights.

In theory, applying a dilution factor correction provides a better *in-vivo* estimate of mAb concentrations present than the unadjusted values [525,526,527]. If standardized, it should also allow more accurate comparison of results between research groups. However, although the weight of the sponge is likely to give an indication of the volume absorbed, the assumption that 1mg of extra sample weight equates with 1ul of fluid that will be diluted by buffer may not always be precisely accurate, particularly in the presence of a vaginal gel. In addition, differences in consistency or constitution of genital secretions could have an influence on the efficiency of mAb extraction from the Weck-Cel sponge. In our study it was noted that the Visit 2 (1 hour post 1st dose) samples were much thicker in consistency than those taken at other visits as they contained visible quantities of gel. This is also likely to be responsible for the larger mean sample weights seen at this visit (as shown in Table 3-7). These samples were much harder to 'spin down' and elute fluid from than those from other time-points; thus, it is possible that, in some participants, mAbs may have remained stuck to the spin-filter. A similar phenomenon has been noted to occur in the presence of cervical mucus, but whether or not it impedes the elution of Ig or other immune factors has not been elucidated [524]. Thus, there is a degree of uncertainty as to how closely the adjusted mAb levels reported in our study, particularly at 1hr post 1st dose, reflect those present in the vaginal lumen.

3.8.6.5 Self-taken vaginal aspirate sampling

The ideal sampling method would provide a large, undiluted specimen from which concentrations of antibodies, cytokines or other molecules could be determined directly, per unit volume of secretion, without the need for processing using extraction buffers and subsequent dilution factor adjustment. Unfortunately, use of the volumetric aspirator device, which had the potential to allow up to 1ml of neat secretion to be obtained through participant self-sampling, was not successful in our study. Eight women found them uncomfortable to use (see section 5.4.4.3) and when samples were obtained,

they were often of poor quality and/or low in volume with only 32% of possible samples suitable for ELISA. As mentioned in section 2.4.1, the aspirators were obtained from Professor Angela Kashuba, CFAR, Chapel Hill, USA, who has used them successfully on a number of ARV pharmacokinetic studies [538,539,596,597]. Although we are not experienced in using these devices, our participants were provided with face-to face verbal instruction in how to use them based on written information provided by Professor Kashuba and performed their first samples at the screening visit to allow greater time for explanation. With hindsight, it may have been useful to have obtained a 'dummy' pelvis for demonstration. The minimum time period of recumbency advised by Professor Kashuba prior to self-sampling is 15 minutes (A Kashuba, personal communication). In view of time constraints during study visits this is what was applied in the case of our participants. It is possible that extending this period may have resulted in improved comfort and sample collection by increasing the quantity of secretion pooled in the posterior fornix. However, this would have simultaneously reduced the convenience of using the device.

Although outside of the aims of our sub-study, which was to compare the utility of self-taken with clinician taken sampling for mAb measurement, the aspirator can also be used by clinicians to obtain samples. Although usually performed via direct vision at time of speculum examination [538,539,597], samples have recently been obtained without using a speculum [596,597]. Although there are no published direct comparisons, it may be that aspiration performed by a trained clinician may be less uncomfortable and more reliable than when self-performed. A comparison between clinician-obtained aspirates and Weck-Cel sampling of vaginal secretions may be worth conducting in a future study.

3.8.6.6 mAb degradation

The complex tertiary and quaternary conformation of immunoglobulins is susceptible to denaturation. As for other proteins, exposure to high temperature (> 60 °C), extreme pH, hydrophobic surfaces and shear forces can disrupt both covalent and non-covalent bonding, resulting in cleavage and/or unfolding of constituent light and heavy chains [598]. In our trial, study gels were kept at 2-8°C prior to dispensing and participants were advised to keep them refrigerated until use. This was in accordance with the MHRA-approved IMP dossier, based on data available at the time of initial submission. However,

by end-of-trial, the stability of each mAb in product buffer had been monitored for 60 months at 2-8°C and for 12 months at 37 °C at pH 7.4 and 5.5 No decay of purity, aggregation or fragmentation was detected by size exclusion chromatography (SEC), SDS-PAGE, and Western Blot, and neutralization activities were preserved (B Vcelar, personal communication). Thus, despite having little control of gel storage conditions (for doses 2 to 12) post dispensing, we can be reasonably certain that the mAbs were intact prior to administration.

To try to limit any degradation of mAbs post sampling, all vaginal samples were required to be kept at 2 to 8 °C prior to processing, a PI cocktail was added to the extraction buffer (into which Weck-Cel sponges were placed immediately post sampling, but which was added later in processing of other vaginal samples) to inhibit naturally occurring proteases, and processing had to be completed within 2 hours of collection. Blood samples had a longer processing window of 4 hours, but were refrigerated, at 1 hour post collection, if they couldn't be centrifuged at that time. Following processing, all samples were stored at -80 °C with temperature logging on freezers and during cold storage (dry-ice) transportation to ensure they remained frozen until time of analysis at Polymun.

However, although similar to those used in other studies [599,526], the above precautions do not guarantee the integrity of samples and we cannot exclude the possibility of mAb degradation between collection and analysis. In particular, it should be noted that the maximum collection-to-storage time-windows are somewhat arbitrary, being a compromise between optimal speed of sample processing, and feasibility in terms of available staff, transportation and equipment.

3.8.6.7 ELISA

An ideal ELISA should be specific for the antigen/antibody being detected (with low false positivity), sensitive to a low limit of detection (to limit false negativity) and give reliably reproducible results when repeated [600]. The ELISAs performed by Polymun were designed specifically for detecting C2F5, C4E10 and C2G12 and had been utilized successfully in a number of previous studies [501,502,503,504,505]. The assays were dependent on initial Fab binding, to specific epitopes (C2F5 and C4E10) or an anti-idiotype antibody (2G12) on the pre-coated plate, followed by subsequent recognition of

the Fc region (by a goat anti-human IgG gamma chain antibody), and would thus be predicted to only detect mAbs which were largely conformationally intact. The lower limit of quantification of the assay was determined through the use of known, standard concentrations of purified C2F5, C4E10 and C2G12. Concentrations in samples above this limit were then quantified through comparison of absorbance readings, using a computer programme, with a standard curve generated using the purified mAbs. Chromogenic substrates, such as OPD, tend to be less sensitive than chemifluorescent or chemiluminescent substrates but have the advantage of being directly visualisable and can be read by absorbance plate readers common to many laboratories. They are generally preferred in assays measuring proteins in the nanogram to microgram range (as opposed to the picogram or femtogram ranges) since they are easier to optimise and have a generally lower tendency to generate non-specific, background signal than newer, more expensive substrates [600].

In our trial, despite having the same pre-determined quantification limit and using similar methodologies, the assays for C4E10 and C2G12 had much more non-specific background detection than that for C2F5. This was seen for all sample types, but was most apparent for serum. The reason for this could not be ascertained by Polymun and, although efforts were made to remove any non-specifically bound IgG, e.g. through increasing washing steps or detergent (Tween) before addition of the anti –IgG antibody, the problem was not resolved. As a result, any values close to the assay cut-off for C4E10 and C2G12 (below about 1 µg) should be interpreted with caution as they may not reflect true levels. With respect to the Weck-Cel samples, applying a dilution factor correction to such values could artificially increase some low concentrations and potentially distort the data. In practice, this does not appear to have had much impact, with similar trends seen in both adjusted and unadjusted concentrations at all visits. With regards to the serum samples, it is possible that small amounts of absorption may have been masked by the imprecision of the lower cut-off of the ELISAs and we can be more confident of our findings with 2F5 than with the other 2 mAbs.

3.8.7 Comparison of mAb concentrations and t ½ with those from other studies

3.8.7.1 Comparison of mAb concentrations with those from the CEA macaque studies

The median MAb levels detected in Weck-Cel obtained samples in the high dose (20mg/g) Mabgel group in our study can be compared to those seen in the macaque 20mg/g Mabgel pharmacokinetic studies conducted at the CEA (CEA study THE0703) (discussed in section 2.3). Since no correction was made for dilution factors in the reported results from the CEA studies, comparison will be made with our unadjusted values. It should be noted, therefore, that some difference in levels may result from variation in volumes of natural secretions between humans and macaques. In the macaque studies sampling was performed using Weck-Cels and the same technique and volume of buffer was used for processing as in our trial. The constitution of the gel was also identical to that used in the high dose arm of our study. However, there were a few procedural differences between the human and macaque studies. Firstly, relating to the smaller size of macaque vaginas compared to humans, a 2g application of the gel was used in macaques whereas in women the syringes dispensed approximately 2.5g of gel. Secondly, in macaques, all sampling was performed at serial time-points following a single application of the gel. In women, for reasons of comfort and logistics, samples at the 12 and 36 hour time points were performed after the 12th dose. Thirdly, whereas all women were sampled at all time points in our trial, in the CEA studies, due to technical and staffing constraints, in addition to baseline, most of the 6 animals were only sampled 4 times over the duration of the study. The median at each post-dose time-point was thus derived from levels obtained from an average of only 3 animals. Detection of mAbs by ELISA was performed at Polymun Scientific, using the same SOPs as for our trial.

Table 3-10 Median mAb Concentrations (µg/ml) detected using Weck-Cels (before and after adjustment for dilution factors) in participants in the High Dose (20mg/g) Mabgel arm compared with those in macaques in the CEA THE0703 study

Sample Time-Point (hours Post dose)	Median mAb concentrations (µg/ml) detected following application of 20mg/g MABGEL								
	THE0703 Macaques			MABGEL 1 High Dose (unadjusted)			MABGEL 1 High Dose (dilution factor adjusted)		
	C2F5	C4E10	C2G12	C2F5	C4E10	C2G12	C2F5	C4E10	C2G12
Pre 1st dose	0.07	ND	0.25	ND	0.06	ND	ND	0.2	ND
1	1535	1505	2008	1505	987	1620	7737	5277	7479
4	691	793	1046	-	-	-	-	-	-
8	91	181	153	220	148	23	3161	2505	538
12	30	18	35	154	96	4.5	1376	870	52
24	1.7	1.40	3.6	15.4	11	0.6	196	171	12
36	-	-	-	0.3	0.5	ND	4.8	4.7	ND
48	0.15	0.21	0.35	-	-	-	-	-	-
72	0.06	0.30	0.06	-	-	-	-	-	-

ND= Not detected, - = not performed in protocol

As can be seen from Table 3-10, in the macaque studies, the median concentrations of all 3 mAbs were of a similar magnitude to each other at each time point. Thus, all 3 mAbs seemed to decline at a similar rate. Comparing the human and macaque data, C2F5 and C4E10 concentrations detected in our trial participants were similar to those seen in the macaques, with possibly even a slightly slower rate of decline. In contrast, C2G12 levels were lower in women than macaques at each time-point, suggesting a more rapid elimination from the human vaginal lumen.

The apparent differences between macaques and humans, particularly with regards to C2G12 elimination, are intriguing and warrant more thorough investigation. However, it should be noted that there was a high degree of variability in detected mAb levels between participants within each of the Mabgel arms in our study, as borne out by the large reported ranges at each visit (Table 3-2). Thus, although clear trends in mAb levels were seen overall, there are likely to be marked differences in mAb elimination on an individual level. This could potentially also be the case with macaques. With this in mind,

the small number of animals studied and the fact that only a proportion of them were sampled at each visit might limit the reliability of any comparison.

3.8.7.2 Comparison of our findings with vaginal IgG t ½ estimates from other studies

Very few other studies have investigated the t ½ of IgG after intravaginal application. Unfortunately, no t ½ estimates were derived in the CEA analysis of the concentrations detected in macaques following vaginal Mabgel administration. On review of the data, although six animals were sampled over a 72 hour period, due to technical and staffing constraints and faecal contamination, analysable samples were only obtained at three or more time-points in the first 24 hours, the period during which results were most reliably above the ELISA cut-off, in three individual animals, precluding robust t ½ estimates.

In the only previous study of residence vaginal IgG t ½ in humans, which remains unpublished, human polyclonal IgG solution (RhoGam™, Ortho Diagnostic Systems) was applied to the posterior vaginal fornices of 10 women with a catheter [511]. Serial vaginal fluid samples were obtained using filter strips and anti- Rho_o D activity measured in a functional assay using Rho_o D^+ human red-blood cells. Antibody levels were reported to have displayed a single phase, exponential decay, with t ½ = 8.85 hours.

Studies conducted in mice used three different techniques (radioactivity detection, functional neutralization and ELISA) following lavage at various time-points, to measure elimination of antibody (^{125}I- polyclonal mouse IgG, HSV2 specific monoclonal mouse IgG2a mAb and biotinylated polyclonal mouse IgG respectively) after intravaginal application [510]. Although the individual studies were small (n= 12, 3 and 6) and utilized a combination of cycling and DMPA-treated mice, they all provided generally similar estimates of t ½, increasing the robustness of the findings. In contrast to the findings in women, IgG was found to be eliminated bi-exponentially, in 2 separate phases; up to 90 % was lost from the vagina in the first few hours (α phase, with t ½ = 0.7±0.1 hours) with the remainder eliminated more slowly (β phase, with t ½ = 5±2 hours).

There are a number of factors which must be taken into consideration when comparing our data with findings from the above studies. Firstly, the antibodies used were all applied in liquid solutions as opposed to a semi-solid, HEC-based, gel. As discussed in

section 3.8.8.1, this could alter their retention and distribution in a number of ways which could impact on vaginal t ½. Secondly, different techniques were employed for measuring IgG, with different assay sensitivities, dilution and processing requirements potentially impacting on the accuracy of IgG detection. However, since the same technique was used throughout each individual study, impact on t ½ estimates would be expected to small. Thirdly, as with our trial there were logistical restrictions on the number of serial samples that could be performed on each study subject. In the mouse studies, each animal could only be sampled at a single time-point since lavages were performed of the entire vagina, thus for each experiment, t ½ estimates were derived from concentrations detected in different animals at different time-points over a 25 hour period. For the RhoGAM study, filter strips were used, enabling serial samples to be performed on the same individual. However, as this study was only presented as a conference poster, detailed information regarding methodology and t ½ calculation was not reported. Personal communication with one of the study investigators, R Cone, revealed that each woman was sampled 4 times in a 72 hour period (1, 24, 48 and 72 hours post application) with t ½ estimates derived from log transformed data using a similar technique to us (see section 2.13.4.3). Confidence intervals were wider than for our study, given the smaller number of subjects, with the lower limit similar to our estimate (t ½= 8.85 95 % CI (5.1 to 11.2). Stage of menstrual cycle, contraceptive use and parity of the participants is unknown.

Given the above, the t ½ estimates derived from our data are in-keeping with those from previous studies, although it is possible this is by chance. All of the studies have limitations and many potential variables such as differences between IgG molecules, surface area: volume ratios between women and mice and effects of reproductive hormones have not been accounted for.

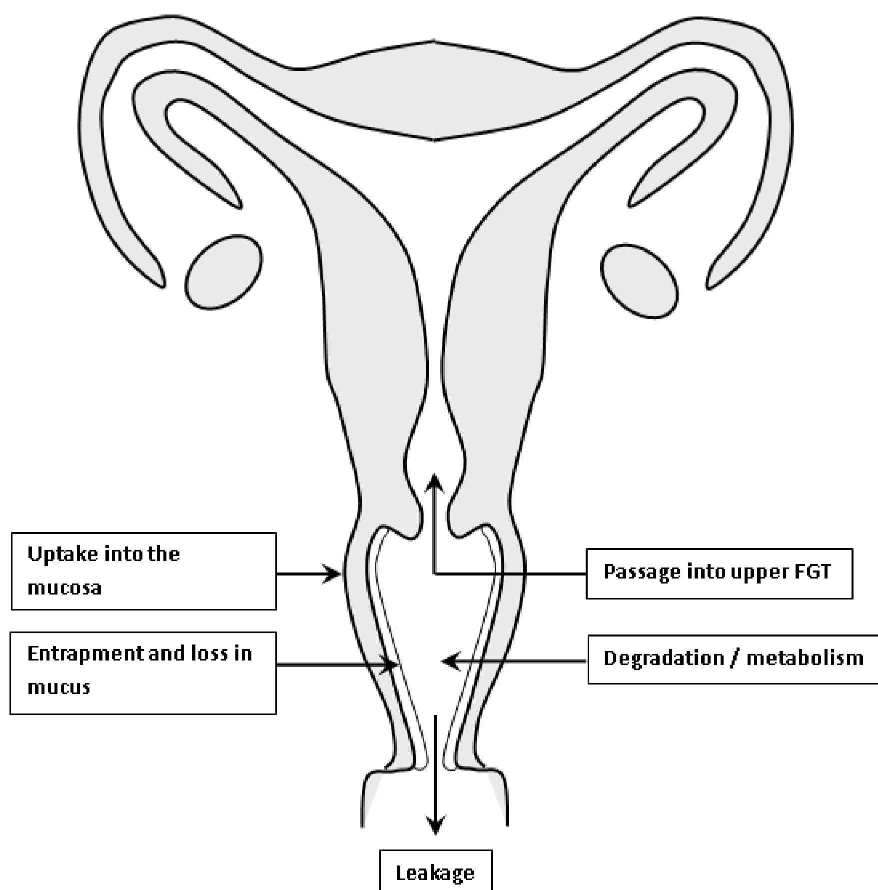
3.8.8 Putative mechanisms governing the elimination of the mAbs from the vaginal lumen

Given the importance of antibody in the protection of mucosal surfaces from pathogens, including STIs, a surprising paucity of research has been conducted in relation to humoral immunity within the FGT. As a result, the source, distribution and clearance of natural IgG within the vaginal mucosa is incompletely understood [84, 601]. Based on what is known, the anatomy and physiology of the vagina, research using other tissues and agents, and the study of parenterally administered mAbs, the following mechanisms could be envisaged as having a role in the elimination of the mAbs C2F5, C4E10 and C2G12 from the vaginal lumen:

1. Leakage
2. Entrapment and loss in mucus
3. Passage into the upper FGT
4. Absorption /uptake from the vaginal lumen
5. Metabolism/degradation

Understanding these processes and their influence on the distribution and t ½ of the mAbs in the FGT, may help to elucidate mechanisms which play a key role in protective humoral immunity in the FGT and provide insights into ways in which the activity of the mAbs may be enhanced or reduced.

Figure 3-29 Putative mechanisms governing elimination of mAbs from the vaginal lumen



3.8.8.1 Leakage

The simplest way in which topically applied antibodies may be lost from the vaginal lumen is via leakage. In the mouse studies described above, loss during the initial 2 hours was increased by administering the antibody dose in a larger volume of PBS, and was virtually eliminated through anaesthesia, where the animals were laid on their backs and unable to move or groom [510]. It was thus hypothesised by the authors that the initial α phase was due to leakage from the vagina.

If suitably formulated, applying mAbs within a semi-solid gel, rather than a liquid, should, in theory, reduce leakage whilst still allowing adequate distribution across the mucosal surface. As with mucus, the rheological properties of synthetic gels are largely determined by the nature of the matrix-forming polymer and its proportion relative to water and other constituents [602,603]. HEC is a non-ionic polysaccharide, produced through the expansion of cellulose, the major constituent of plant cell walls, by the

addition of sodium hydroxide and ethylene oxide [604]. A common gelling agent, it has a number of properties which make it highly suited for vaginal use and has been used in commercial lubricants, such as KY-Jelly (Johnson and Johnson, New Jersey, USA) and other microbicide formulations [512,513]. It displays pseudoplasticity and thermo reversible viscosity, becoming thinner and more stretchy at higher temperatures, enabling it to spread across the vaginal mucosa, whilst maintaining its integrity at low pH. In addition, it is mucoadhesive, forming cohesive interactions with the mucosal interface to increase retention (see section 3.8.8.2) [604]. However, producing a gel with ideal rheological properties is difficult, and even small changes in formulation can have a marked effect [602,603]. In comparison with the 'universal' HEC-based gel, developed and used by researchers from the International Partnership for Microbicides (IPM) and CONRAD [512,513], our gel vehicle contained a lower percentage of HEC (1.6 % vs 2.7%), different preservatives (methyl and propyl paraben vs sorbitol) and additional humectants (glycerin and maltose) to optimise the solubility and release of the mAbs. Thus, although the rheological suitability of our gel as a microbicide had been determined *in-vitro,* its behaviour within the complex physiological environment of the human vagina was uncertain prior to its use in the MABGEL 1 trial.

In our trial, overt gel leakage was reported by 5 women, and 7 women reported using pads or underwear as a precaution to protect clothes or sheets from gel (see section 5.4.6). Although difficult to quantify from history alone, most leakage was reported to occur on standing. This is unsurprising when considering the anatomy of the vagina (see section 1.3). Thus, although there is likely to have been some early leakage of gel from the lower vagina, particularly in those women who stood up soon after application, gel administered to the upper vagina has a greater chance of having remained in situ. However, it is not possible to determine the extent to which leakage contributed to mAb elimination in our study. In addition, given that the 1st gel dose was applied in the EMU and women remained (largely seated) in the examination room (as a safety precaution), until after the 1 hour post-dose examination, it is possible that the potential impact of leakage was reduced on mAb levels obtained at this time-point.

3.8.8.2 Entrapment and loss in mucus

As described in section 1.4.1, the vaginal mucosa is coated with secretions, including mucus. By adhering to mucus, polymers such as HEC not only increase the retention of gels but also the bioavailability of agents contained within them. HEC is water soluble and as it is spread and further hydrated at the mucosal interface, begins to swell and slowly dissociates. Interdiffusion and interpenetration can then occur between the chains of HEC polysaccharide and mucin polymers resulting in cross-linking, and the formation of hydrogen and/or electrostatic bonds [87, 604]. Such interactions open the gel matrix, allowing agents held within it to diffuse out. Thus, the mAbs should, in theory, be released into mucus and held close to the vaginal mucosa, whilst the HEC slowly dissolves away.

Naturally occurring antibodies are often found in mucus, having passed into it from the adjacent epithelium, and are thought to enhance protection against specific pathogens by trapping them and preventing their adherence to target cells [605,606]. This may be particularly important in the case of some nonenveloped capsid viruses (e.g, Human Papillomavirus (HPV), polio, adeno and rota viruses) which have evolved to pass efficiently through mucus and penetrate the underlying mucosa [87,607]. Although not fully understood, factors which influence the ability of a molecule or particle to diffuse through mucus include its size, hydrophobicity, its overall positive or negative charge and the thickness and viscosity of the mucus layer [87]. IgG mAbs, being approximately 20nm in diameter, should be relatively unimpeded by the mesh size of the mucin-matrix, which was originally estimated to be an average of 100nm in human ovulatory cervical mucus and may be even larger [607,608, 609]. However, antibodies contain multiple positive and negatively charged amino acids which, through both repelling and attracting the mostly negatively charged glycan moieties on mucin fibres, could enable them to form multiple transient, low affinity bonds. It has been proposed that these interactions may be sufficient to maintain antibody adhesion to mucus whilst allowing them enough freedom of movement to trap pathogens [607].

Since mucus is continuously produced, shed, and discarded from all mucosal epithelia, if the majority of the mAbs are retained in it post application then their residence time in the vagina would largely be determined by the clearance time for mucus. The typical

turnover time for mucus in the cervico-vaginal tract has not been elucidated, hence extrapolations must be made from other sites. In general, turnover appears to be fastest at surfaces with thin mucus layers, such as the nasal tract (20 mins) and slowest in the GI tract (4-6 hours), where there is a deep, 'unstirred' layer beneath the more rapidly cleared luminal mucus [610]. In addition, the mechanism of mucus transport will also have an influence. Mucus in the GI tract is dislodged by continuous peristaltic contractions, whereas in the respiratory tract it is wafted along by cilia. In the FGT, two processes act in opposing directions. Transport of sperm into and along the upper FGT is assisted through contractions of uterine smooth muscle (peristalsis) (triggered by endocrine and paracrine stmuli post coitus) and the ciliated secretory epithelium of the uterus and fallopian tubes. In contrast, mucus is mainly thought to be carried along the non-ciliated lower FGT mucosa to the introitus through the effects of gravity, the motion of the limbs and the activity of muscles in the pelvic floor and abdominal wall, which increase intra-abdominal pressure and/or squeeze the walls of the vagina together [611, 612]. Taken altogether, it is difficult to determine an exact transit time for mucus in the vagina. Although likely to be several hours, it may be highly variable and influenced by activity, coitus, infections and other factors.

3.8.8.3 Passage into the Upper FGT

A study using MRI visualisation, following vaginal administration of 5ml N9 in a single woman, demonstrated passage of gel through the external cervical os and into the endocervical canal within 10 minutes of application. The amount reaching the internal cervical os and beyond was small and could not be accurately quantified [613]. In contrast, in another study, comparing the distribution of 3ml and 5ml of Gynol II spermicidal lubricant before and after simulated coitus (with a model phallus), gel was not visible in the endocervical canal in any participant within the first hour of use or prior to simulated intercourse. At 6 hours, it was seen in the endocervical canal in 33% (2/6) of women after using either volume of gel. Endocervical gel was also present in 20% (1/5) vs 80% (4/5) of women, after using 5 and 3 mL, respectively, following simulated coitus 15-30 mins post gel insertion [594]. The above findings suggest that although gel passage into the endocervical canal occurs, its incidence and timing is variable. Entry through the external cervical os may be increased by coitus and may be influenced by the nature and

volume of the product. Factors such as BMI, parity and activity/ambulation, may also have an effect [614]. Little is known regarding gel passage beyond the internal os and higher into the upper FGT.

3.8.8.4 Absorption /uptake from the FGT lumen

With its thick, multi-layered epithelium, the lower FGT mucosa is not primarily designed for absorption. However, its large surface area and the rich vascular and lymphatic network in the submucosa assist in the uptake of certain agents, which, if they enter the blood-stream, avoid first pass metabolism by the liver [615]. Although, the columnar epithelium of the upper FGT presents a thinner barrier, (as discussed in chapter 1), the movement of molecules are restricted through the presence of inter-cellular tight-junctions [109]. Substances which are readily absorbed following vaginal application include small molecule drugs, such as atropine, morphine and vasopressin, and steroid hormones e.g. oestrogens and progestagens, for replacement or contraception [615]. In contrast to these molecules, which can diffuse transcellularly through most cell membranes, antibodies, being large and hydrophilic, must either pass between cells (paracellularly) or be 'engulfed' by cells via endocytosis [584,585,468]. Paracellular transport occurs through channels or 'pores' between adjacent cells, i.e. it is regulated by the 'tightness' of the inter-cellular junctions, whereas endocytosis can either be receptor-mediated or non-specific (pinocytosis) [584]. Despite IgG being the predominant antibody isotype found in human cervico-vaginal secretions, its source and the mechanisms of its distribution in the FGT have not been fully elucidated. However, as discussed below, there is some evidence to suggest that IgG may enter genital secretions via several routes and that some re-uptake by the epithelium may be possible.

Given the absence of a large local immune inductive area in the FGT mucosa, unlike the Peyer's patches in the GI tract, most IgG in the FGT lumen has historically been thought to be derived from the systemic circulation [601,616,617]. According to this model, IgG passes through the endothelium, into the submucosa, then through the cervico-vaginal epithelium via largely passive mechanisms, such as convection (paracellular carriage with fluid) and diffusion [584,585,617], with or without some additional active transport [616]. However, despite being debated for almost 50 years, no direct evidence has yet been presented to confirm or refute the theory that genital tract IgG originates primarily from

serum. Indirect support for a systemic origin includes the ability of IV administered anti-HIV-1 IgG1mAbs to protect macaques from vaginal SHIV challenge [491,492,493]. Also in keeping, is the finding that following IM vaccination, including against the sexually-acquired pathogens HBV and HPV, levels of specific IgG are detected in the same proportion (relative to total IgG) in both genital secretions and serum [618,619]. On the other hand, there are several lines of evidence which could suggest that at least some IgG is produced locally in the FGT. Higher specific:total IgG ratios have been detected in FGT secretions compared with serum following vaginal vaccination or genital infection [620, 621] and IgG sub-class distribution has been shown to differ between serum and cervico-vaginal secretions, with IgG1 predominating (>90% of IgG) in the latter [622]. In addition, IgG secreting plasma cells have been identified in the endocervical stroma, and to a lesser extent in that of the vagina and endometrium [616,623]. In support of an important role for the upper FGT in contributing to the antibody pool found in cervico-vaginal secretions, in post-menopausal women, levels of IgG decreased by half and those of IgA by 15 fold following hysterectomy [624].

What also remains unclear is whether IgG within the FGT sub-mucosa, either having been produced locally or extravasated, passes into secretions via an active or a passive process and whether the transport mechanisms in operation are the same in the upper and lower FGT given their different epithelial characteristics. In contrast to IgG, the mechanism through which most locally produced IgA and, to a lesser extent, IgM enters mucosal secretions is well understood and involves active transport by the polymeric immunoglobulin receptor (pIgR) [616,625]. Unlike IgG, which is monomeric, secreted IgM and IgA are found as polymers, joined at their Fc regions by a J-chain. IgA forms mainly dimers whereas IgM is pentameric [73]. After production by plasma cells in the lamina propria adjacent to the mucosal surface, IgA or IgM polymers bind to pIgR, which is expressed on the basolateral membrane of the epithelial cell, and are taken up into an endosome, within the cell, via receptor mediated endocytosis. Through being bound to pIgR, the Ig polymer passes through the cell and is targeted to the luminal plasma membrane. Once on the outside of the cell, proteolysis of the receptor occurs, and the Ig polymer along with a portion of the receptor known as the secretory component (SC), is released to diffuse into the lumen [625].

A possible candidate transporter of IgG in the FGT is the neonatal Fc receptor (FcRn). This is a heterodimer composed of a membrane-bound major histocompatibility complex (MHC)- class 1-like heavy chain joined to β_2 microglobulin (β_2m) [626,627]. It was first identified as the receptor which mediates the transport of IgG from mother to baby for passive immunity [628]. Expressed in the intestine of neonatal rodents, it transports IgG from ingested maternal milk across the intestinal epithelium [629]. In contrast, most passage occurs antenatally in humans, with FcRn transporting IgG from maternal blood to fetal capillaries through its expression on syncytiotrophoblast [630,631]. Although both systems involve FcRn-mediated transcytosis of IgG across a polarised epithelial cell layer, the mechanism by which this occurs is slightly different in each case. FcRn binds to the Fc portion of IgG in a pH dependant manner [632,633]. It can bind with high affinity at acidic pH (< 6.5) but not at physiological pH (7.4). Expressed on the brush border of enterocytes, it binds IgG in the acidic milieu of the lumen, internalises it, and releases it at neutral pH on the basolateral side [634,635]. In syncytiotrophoblasts, which are bathed in maternal blood (pH 7.4), IgG is instead internalised via pinocytosis, where it enters the endosomal pathway. FcRn is expressed on the endosomal membrane and binds IgG as the contents acidify. At the foetal side of the cell, the vesicle fuses with the membrane, and the physiological pH leads to the dissociation of IgG from FcRn [636,637].

FcRn has now been shown to be expressed and functionally active in a range of adult human epithelial cells and tissues, including in the intestine [638,639], respiratory tract [640], renal tubule [641,642], liver [643] and vascular endothelium [644,645]. It is also found in cells of myeloid origin [646,647]. In some mucosal tissues, such as the intestine and lung, potential for bi-directional transport has been demonstrated [639,640]. Using a model in which adult FcRn α-chain-deficient mice express intestinal FcRn under the control of the human FcRn promoter, Yoshida *et al*. found that FcRn transcytosed submucosal IgG across the epithelial layer to the luminal side of the cells. Antibody then bound to cognate antigen, and the resulting immune complexes were then transcytosed back in the reverse direction, being released at the basolateral membrane. Immune complexes were taken up by submucosal DCs which were shown to migrate to the draining LN where they elicited antigen-specific T Cell responses. The researchers then extended their findings to the respiratory tract of mice [648]. Thus, they demonstrated an additional role for FcRn in immunosurveillance of mucosal surfaces.

The above findings offered the potential that FcRn could be exploited for delivery of therapeutic proteins and vaccines across mucosal surfaces. Bitonti *et al.* demonstrated proof of concept by producing an Fc-fusion protein (a therapeutic protein fused to the Fc portion of IgG), depositing it in the lungs of NHPs by inhalation and detecting it in serum 20 hours later [649]. They subsequently demonstrated similar findings in humans [650]. Recently, Lu *et al.* have tested this strategy for vaccination. Mice were immunized intranasally with IgG Fc fused to HIV-1 Gag protein (Gag-Fc fusion protein) as an immunogen and CpG oligodeoxynucleotides as an adjuvant. Animals developed local and systemic immunity, including durable B and T cell memory. Gag-specific immunity was sufficient to protect against subsequent intravaginal challenge with recombinant vaccinia virus expressing the HIV Gag protein [651].

Recently, Li *et al.* have published data in support of a role for FcRn in the FGT [652]. They have demonstrated expression of FcRn mRNA (by RT-PCR) and protein (by Western blot) in 3 primary human female genital epithelial cell lines (End1, endocervical; Ect 1, ectocervical; and VK2, vaginal) and in HEC-1A, an endometrial adenocarcinoma cell line. In addition, they have shown, using immunohistochemistry, that it is present in human endometrial and vaginal tissue, with strong staining in the epithelium and weak scattered staining in the lamina propria, the latter, presumed to represent macrophages or dendritic cells, being more apparent in the endometrium. The authors were also able to demonstrate selective transcytosis of IgG *in-vitro*, using HEC-1A cells and a commercially available, primary human vaginal epithelial tissue (Human Epi-Vaginal Tissue Model (vaginal-ectocervical (VEC)-100 FT), and showed that it could occur in both basolateral-to-apical and apical-to-basolateral directions.

Experiments in mice appear to support these findings [652]. Using prepubertal mice, to minimise variation due to the estrus cycle, levels of biotin-labelled mouse IgG in vaginal washings were measured in both wild-type (WT) and FcRn knock-out (FcRn-KO) B6 mice 12 hours after intra-peritoneal administration. To compensate for the reduction in circulating IgG that is seen in the absence of FcRn (see section 3.8.9.2), FcRn-KO mice received double the dose (200 vs 100 µg) of IgG. Although serum concentrations were similar in both sets of mice, biotin-labelled IgG was only detected in vaginal washings in WT mice, suggesting that FcRn is required for the transport of IgG from the blood into the

genital tract lumen. In a parallel experiment, biotin-labelled IgG was administered intravaginally into both WT (50 µg) and FcRn-KO (100 µg) mice in 30 µl PBS, and only detectable, at 12 hours, in the serum of WT mice, supporting the idea that FcRn also mediates transport in the opposite direction. However, the results of this research must be interpreted with caution. Appropriate isotype and genetic controls were not shown for these experiments, and findings have not been corroborated by other researchers.

Using both adult and pre-pubertal WT and FcRn-KO B6 mice, Schäfer found no evidence, from measuring relative titres of endogenous IgG, and IV administered biotinylated mouse IgG and human IgG, in serum and genital tract secretions, that IgG accumulates differently in the FGT in the presence and absence of FcRn. In addition, although FcRn expression was detected in stromal immune cells, by immunofluoresence, it was not found in vaginal, ectocervical, endocervical or uterine epithelium in WT mice. Intravaginal administration of biotinylated mouse IgG and human IgG did not result in any detectable translocation to serum at time-points between 1 and 24 hours post dose. As has been found by other researchers, in post-pubertal mice, levels of genital tract but not serum IgG (both endogenous and exogenous) varied markedly with the menstrual cycle, being highest during metestrous (post-ovulation) and lowest during proestrous (pre-ovulation). Similar patterns were seen in both WT and FcRn-KO animals suggesting that a local hormonal effect on FGT epithelial and/or vascular permeability, or on another as yet undiscovered transporter, may underlie this [K Schäfer, unpublished, PhD thesis, 2012].

Schäfer's experiments have been confined to mice. However, due to differences in the structure of the promoters governing FcRn gene expression in humans compared with those of rodents and other mammals [653,654], significant inter-species variation is seen. For example, in humans FcRn is expressed by intestinal epithelial cells in both the fetus and adult whereas its presence diminishes in mice after the neonatal period [655,656]. Respiratory tract expression occurs mainly in the upper airways of humans and other primates and the bronchiolar and alveolar epithelium in cows and rats [640,657,658]. Thus, further investigation is needed to verify or refute the hypothesis that FcRn is present and mediates bi-directional transcytosis *in-vivo* in the human adult FGT. In addition, it is unclear if FcRn would have the same function at all sites in the FGT. It is easy to visualise how IgG could be transported in both directions across the single-cell

thick, columnar epithelia of the endocervix and endometrium. At these sites, under normal conditions, the basolateral and luminal extracellular environments are at neutral pH, and IgG transcytosis could be conducted in a similar manner to that seen in syncitiotrophoblast. Such a mechanism is supported by the supplementary finding of Li *et al*. that in HEC-1A cells, FcRn co-localised with EEA1, a protein found in early endosomes [652]. In contrast, hypothesised transport across the multilayered, stratified epithelium of the vagina is more problematic. Although FcRn appears to be expressed in VK2 cells and in the mature, human vaginal epithelium [652], its localisation within the latter has not been clearly elucidated. The vaginal epithelium consists of cells at various stages of maturity, making it possible to identify four different layers, namely the basal, parabasal, intermediate, and superficial layers [659]. Full thickness transport of IgG would conceivably require the expression of FcRn in all of these layers but whether this occurs has not been ascertained. Studies in skin have shown that FcRn is present intracellularly and is functional in cultured human keratinocytes, but its expression is confined mainly to the basal and suprabasal layers in normal human epidermis [660]. The fact that the human vaginal lumen is acidic (pH 4), under normal circumstances, would also present a barrier to the basolateral to luminal transport of IgG by FcRn. Although bidirectional transcytosis was possible in the Epi-Vaginal tissue model [652], the fact that this was grown as a monolayer and incubated in non-acidic culture medium renders it markedly different from the *in-vivo* environment.

It has been proposed that, in the human vagina, rather than primarily mediating IgG transport across all of its many layers, FcRn may play a role in the sequestration of IgG near the lumen and/or in the delivery of IgG-antigen complexes to antigen presenting cells in the epithelium [652]. The acidic milieu of the vaginal lumen may enable FcRn to bind IgG for relatively long periods on the epithelial cell surface and allow it to capture cognate antigen whilst still holding onto it [661]. Such IgG could potentially be held 'in-reserve' until a particular infection was encountered. IgG-Ag complexes could then be recycled into the cell for transcytosis and gradually transported from cell to cell until a dendritic cell or macrophage was encountered.

Our data, like that of Schäfer, shows no evidence of antibody uptake from the FGT lumen into the systemic circulation, and does not support the existence of bi-directional

transcytosis. However, the possibility of an IgG sequestration or surveillance role of FcRn in the vagina is not excluded. It is also possible, but unlikely, that luminal to basolateral mAb transport could have taken place and gone undetected i.e. if the mAbs were passed into the sub-mucosa but did not get taken up by the lymphatic system or if only a low level of transport occurred. Interestingly, a different mechanism through which antibodies may be retained by the ectocervical-vaginal epithelium has recently been demonstrated by Blaskewicz *et al.* In an experiment in which Cy-3 conjugated human IgG was administered intraluminally to cultured cervical explant tissue, IgG was shown to permeate into the most superficial 3 to 4 cell layers of ectocervical epithelium (which are devoid of tight-junctions) but did not penetrate into deeper layers or through the endocervix [241]. It could thus be envisaged that antibodies may be sequestered in the most superficial layers of the ectocervico-vaginal epithelium, providing a potential zone for binding to micro-organisms and preventing them breaching deeper into the mucosa.

Support for the retention of antibody by the mucosa is provided by findings from the mouse studies discussed in section 3.8.7.2. To determine the efficiency of lavage at removing IgG from the vagina, each individual mouse receiving topical ^{125}I-IgG, had at least 4 lavages taken at a single time-point which were analysed separately in a gamma counter. The first lavage removed approximately 70 +/- 10% of the ^{125}I-IgG from the lumen and the amount of ^{125}I-IgG recovered in each subsequent lavage decreased by a factor of ten. Lavaging four times was sufficient for obtaining all of the ^{125}I-IgG that could be removed from the vaginal lumen by lavage, which was 80 +/- 10% of the dose. Thus, this suggested that as much as 20% of the delivered antibody adhered to the vaginal mucosa in a way that resisted repeated lavaging. The authors verified this by removing the reproductive organs from some of the animals and showed that most of the residual radio-activity remained associated with the vaginal tissue [510]. To demonstrate uptake of antibody into the FGT mucosa in our trial participants would have required vaginal biopsies to have been taken for analysis by immunohistochemistry. However, biopsies were not included in our study protocol. Although not yet done, it would be feasible to conduct another study using mice, perhaps with immunofluorescent-labelled antibodies, to evaluate their distribution in tissue post vaginal administration.

3.8.8.5 Metabolism/degradation

IgG is susceptible to proteolysis by a number of enzymes including Plasmin, Pepsin, Trypsin, Papain, Cathepsins, elastases and metalloproteinases [598,662,663,664,665]. These mediate cleavage at or around the hinge region, which contains disulphide bonds between the Fab arms and the two carboxy-terminal domains (C_H2 and C_H3) of both heavy chains (see Figure 1-8). Cleavage at the hinge by Papain generates individual Fab and Fc fragments, whereas cleavage above (in C_H2), by Pepsin and below (in C_H3), by Plasmin, create (Fab)2 and Fabc (antigen and complement binding) molecules respectively. IgG subclasses vary in their susceptibility to these enzymes, with IgG3, which has a larger, more accessible hinge, being most susceptible, IgG2 most resistant and IgG1 and IgG4 intermediate [666]. In addition to proteases, immunoglobulins, like all proteins, are also substrates of peptidases. These are a family of enzymes which act to remove amino acids from the amino (NH_2) and carboxy (CHO) terminals of peptide chains, resulting in their modification or breakdown [667].

IgG breakdown can occur intra- and/or extracellularly in various body tissues and fluids. A number of genital tract pathogens have been shown to produce proteases, which cleave immunoglobulins as a means of evading detection. These include *Trichomonas vaginalis Neisseria gonorrhoea*, BV-associated bacteria and the urinary tract pathogen *Proteus mirabilis* [668,669,670,671]. In addition, enzymes are released by polymorphonucleocytes (PMNs) [672], which although increased in number in the presence of STIs and candidiasis, are also found in the healthy vagina [72].

Intracellular IgG degradation in the FGT has not been formally studied. The extent to which it contributes to mAb elimination will depend on how much enters the epithelium and whether it is taken up by cells. One mechanism through which endogenous IgG may be catabolised is following FcγR–mediated endocytosis by macrophages and monocytes. However, only FcγRI is bound and activated by monomeric IgG; FcγRII and FcγRIII subtypes requiring interaction with multiple IgG molecules bound to antigen within an inmmune complex [143,147]. Given the relatively high affinity of IgG for FcγR and the high endogenous concentrations of IgG in plasma (around 10g/L) and genital secretions (around 1g/L), it has been argued that FcγR-mediated elimination is unlikely to occur to a significant degree for monomeric IgG, but becomes a more dominant mechanism for IgG

immune complexes [584,585]. Thus, although macrophages are commonly found within the cervico-vaginal mucosa, it seems probable that FcγR-mediated clearance contributed little to mAb elimination in our study. However, FcγRIII-triggered endocytosis of mAb-HIV virion complexes may have played a role in the elimination of the mAbs following i.v. administration to HIV infected individuals (see section 3.8.9), and could also potentially contribute to the efficacy of a mAb-based microbicide (see section 6.2.3.2). The latter possibility is strengthened through the finding that FcγRIII is expressed by a range of genital tract ECs [652].

Intracellular IgG catabolism in the FGT may also be influenced by the exact location and function of any FcRn present. Following endocytosis, proteins enter the endosomal pathway and are targeted for destruction in lysosomes. However, as described earlier, in some tissues FcRn is located on the endosomal membrane where it acts to capture and return IgG to the surface. In certain cell types, such as endothelium, hepatocytes, and those of myeloid origin, instead of transcytosing IgG, FcRn may simply return it to the same portion of the membrane, hence in effect, recycling it [643,647,673]. The same occurs with albumin. The long plasma t ½ of most endogenous IgG, and albumin, in comparison to other antibody classes and plasma proteins (21 vs < 7 days) is thought to largely result from this process [142, 674, 675]. Thus, if present in the vagina, FcRn could potentially both recycle and sequester IgG, resulting in a prolongation of its t ½.

3.8.9 Possible explanations for the difference in pharmacokinetics between 2G12 and the MPER mAbs

The apparent difference in rates of vaginal luminal elimination seen in our study between C2G12 and the MPER mAbs is intriguing. Whereas the concentrations of C4E10 and C2F5 appeared to follow an exponential decay, with remarkably similar t ½s, levels of C2G12 declined non-exponentially, with a more rapid decrease seen at higher concentrations, in the 1st 8 hours post dose, becoming slower thereafter. Differences in the pharmaco kinetics of the mAbs have also been observed previously when they were administered intravenously to HIV positive individuals [501,503,505,504,676]. In a long-term pharmacokinetic study, high doses (1g, 1g and 1.3g respectively) of C2G12, C4E10 and C2F5 were given in combination to 14 HIV-1-infected individuals at weekly intervals over

a 3 month period [676]. A slightly higher dose of C2F5 was administered because it had been shown to have the shortest t ½ of the 3 mAbs in previous studies [501,503]. In general, the distribution and elimination pharmacokinetics of all 3 mAbs appeared typical of systemically administered antibodies [584]. The plasma concentrations declined bi-exponentially over time, compatible with a two-compartment pharmacokinetic model (plasma and tissues). Each antibody had a rapid distribution phase, followed by a log-linear terminal elimination phase. Volumes of distribution were similar for all 3 mAbs, with a small central volume (V_c), equivalent to the assumed plasma volume, and a 3 times larger steady state volume (V_{ss}), suggesting that significant amount of antibody entered the tissues. Despite these apparent similarities, C2G12 was found to accumulate far more on multiple dosing than the other 2 mAbs, achieving significantly higher trough and peak plasma concentrations. In contrast to the situation in the vagina, it was shown to display a much longer elimination t ½ (t ½ β) in plasma (21.8 +/- 7.2 days) compared to C4E10 (5.5+/- 2.2 days) and C2F5 (4.3+/-1.1 days) [676].

Analysis of the findings following both systemic and vaginal administration may help to elucidate the reason(s) behind the observed pharmacokinetic differences between the mAbs. However, it should be borne in mind that the studies differed in the nature of their participants (HIV positive vs negative) and time-scales (3 months vs 12 days). Although this discussion will concentrate on factors that may influence mAb pharmacokinetics in general, such as variation in the intrinsic properties of the antibody molecules, differences in mAb-HIV interactions and clearance via the reticuloendothelial system (RES) may also have played a role in the IV dosing studies.

3.8.9.1 Origin of constant (Fc) and variable (Fv) regions of the IgG1 mAbs

It seems probable that most of the t ½ variation seen between the mAbs may be explained by differences in their physico-chemical properties. It is thus important to fully understand what features are shared between the mAbs and which are not. In addition, any characteristics that make them atypical from usual IgG1 antibodies may also be of relevance. All 3 mAbs have fully human IgG1 sequences but were expressed in Chinese hamster ovary (CHO) cells. Their variable (Fab) regions are unique, each being derived from an individual hybridoma created through the fusion of peripheral blood lymphocytes from HIV-1 positive donors with the CB-F7 myeloma cell line [508]. In contrast, they are

thought to share identical, typically conserved IgG1 constant (Fc) regions [509]. However, whereas 2G12 was initially produced as an IgG1 antibody, hybridoma-derived 2F5 and 4E10 mAbs were originally generated as IgG3 subtypes. IgG3/G1 class-switched variants were subsequently generated in CHO cells through recombinant expression of the kappa light chain (κLC) and variable heavy chain (V_H) mRNA sequences from the original hybridomas with those encoding IgG1 C_H1-C_H3 domains [509]. The IgG1 sequences were obtained from an IgG1-producing human hybridoma cell line (known as 1B1) that had been created at the same time as those producing the other mAbs.

3.8.9.2 Influence of both Fc and Fv regions on FcRn-IgG binding

IgG3/G1 Isotype switching was primarily performed to increase plasma t ½. Typical IgG antibodies from sub-classes 1,2 and 4 have a t ½ of around 21 days whereas that for IgG3 and other antibody classes is < 7 days [677]. As described in section 3.8.8.5, the relatively long serum t ½ of most IgG isotypes has been primarily attributed to recycling by FcRn in endothelium and other tissues [645]. The structural basis for the FcRn-IgG interaction has been well characterised. The binding site for FcRn is in the C_H2-C_H3 hinge region of the IgG Fc and is distinct from that of other Fcγ receptors [627,678]. Binding requires a hydrophobic interaction between an isoleucine residue at position 253 of IgG and a tryptophan at 133 on FcRn. Salt bridges between anionic residues on the α2 helix of FcRn and histidine residues on the C_H2-C_H3 of Fc are crucial for stabilizing the interaction [645,678]. These bonds depend on protonation of the histidines, thus explaining the pH dependence of binding. The exact number of histidines present varies between different antibody isotypes and between IgG from humans and other species, helping to explain different binding affinities to human FcRn [679]. Mutational analyses have shown that single or combinations of amino acid substitutions at or adjacent to these key sites can significantly enhance or reduce systemic IgG clearance by up to 3-fold [680,681,682,683]. Since pH dependence is important for efficient recycling, mutations which improve binding at acidic pH alone result in much greater increases in plasma t ½ of the variant IgG than those which also increase affinity at neutral pH [684]. Enhanced binding or decreased dissociation at pH 7.4 may actually increase elimination, of the mutant and/or other IgG, through being degraded, after failing to be released at the cell surface, or competitively inhibiting the recycling pathway [685,686,687].

It is interesting to note that whereas the plasma t ½ of C2G12 is similar to that of natural IgG1, despite being class-switched, those of C4E10 and C2F5 remain more in keeping with their original isotype, IgG3, which is not bound by FcRn. In addition, in the vagina, the initial rapid loss of C2G12 at high concentration (in the 1st 8 hours) with slower loss thereafter, might be in keeping with a saturable mechanism, like receptor binding, unlike the exponential reduction of the other 2 mAbs. The elimination of IgG from plasma is known to be concentration dependant, with the recycling capacity of FcRn exceeded, and IgG t½ reduced, following administration of high doses of IVIg [677]. However, owing to the large quantity of endogenous IgG that is present in the body (around 10g/L in plasma; 50 to 100g total body), total doses of 3.3g of the mAbs given iv would not be expected to increase the overall concentration of IgG sufficiently to lead to a significant reduction in plasma t½ [584]. In contrast, since IgG levels in vaginal secretions are lower (approximately 0.5 to 1mg/ml) it is possible that administering just 25 to 50mg of C2G12 to the vaginal lumen may be sufficient to saturate any FcRn present in the mucosal epithelium. However, since all 3 mAbs possess the same Fc sequence, it might be expected that they would bind to FcRn with identical affinity. Although it is possible that mutations in the FcRn binding region may have arisen spontaneously during CHO cell expression, this is felt unlikely, and is not supported by amino acid sequencing performed by Polymun as part of their quality assurance analyses (B. Vcelar, personal communication). Interestingly, recent observations suggest that the IgG Fv domain may also impact on the FcRn-IgG interaction. mAbs with the same wild-type human Fc sequences but different Fab domains have been shown to exhibit marked variation in their binding affinities for FcRn at acidic pH and in their dissociation at neutral pH [688]. This supports the earlier finding that Fc-fusion proteins bind with lower affinity to FcRn than mAbs which possess the same IgG1 Fc region [689]. Although the mechanism through which the Fv may influence FcRn binding has not yet been determined, it is hypothesised that the overall tertiary or quaternary structure of the IgG molecule may impact on the stability of the interaction or the ability of FcRn to form functional dimers [688]. To date, no direct assessment has been made of the relative affinities of C2F5, C4E10 or C2G12 for FcRn but it would be an important future investigation.

3.8.9.3 Unique structural characteristics of the mAbs

The nature of the Fab regions or overall structure of the mAbs may be key to explaining their pharmacokinetic differences. 2G12 is a very unusual antibody. It has been shown to bind with nanomolar affinity to Man α1-2Man-linked moieties on oligomannose-type sugars that are covalently-attached, as a cluster, to gp120 [473,474]. This is interesting for three reasons: 1) oligosaccharides attached to the viral coat proteins are processed by the host and are, therefore, considered unlikely to be recognised as 'foreign' and immunogenic; 2) glycosylated proteins are normally synthesized as a collection of glycoforms, in which a range of different sugars can be present at a single site, which dilutes any potential antigenic response, and 3) because carbohydrate-protein interactions are usually much weaker (in the micro-molar range) than protein-protein interactions [690]. However, for reasons that are yet to be fully understood, intrinsic properties of the gp160 structure, together with steric constraints related to trimerisation, mean that the protein does not undergo the usual processing of sugar residues that occurs within the medial Golgi [691]. This gives rise to an unusual predominance of oligomannose-type glycans on gp120 including a particularly dense 'patch' of residues, not normally found on mammalian proteins, in which the 2G12 epitope is located [474].

Studies have shown that 2G12 achieves its strong antigen affinity through possession of an atypical 3D conformation, which enables it to bind multiple adjacent carbohydrate epitopes in an array [692]. As shown in Figure 1-8, in a typical antibody molecule, the 2 Fab domains are formed from the pairing of the light chain variable (V_L) and constant (C_L) domains with the variable and 1st constant domains of the heavy chain (V_H and C_H1). However, in 2G12 the two V_H domains have swapped their V_L partners ('domain-exchange'), resulting in a more tightly-packed, twisted orientation of the V_H domains with respect to the C_H1 domains compared to a standard Fab (see Figure 3-30). As a result, rather than being free to rotate independently around the hinge region, the two 2G12 Fabs are arranged side-by-side with their respective binding sites facing in the same direction. Thus, instead of containing 2 separate antigen binding sites separated by a variable distance (usually between 10 to 15nm), the two carbohydrate binding sites created by the V_H and V_L domains of 2G12 are separated by a fixed distance of only

3.5nm, with two additional binding sites created at the interface of the two V_H domains [692]. Such an arrangement results in 2G12 possessing an extended linear conformation instead of the usual Y- or T-shape.

Figure 3-30 Atypical structure of 2G12

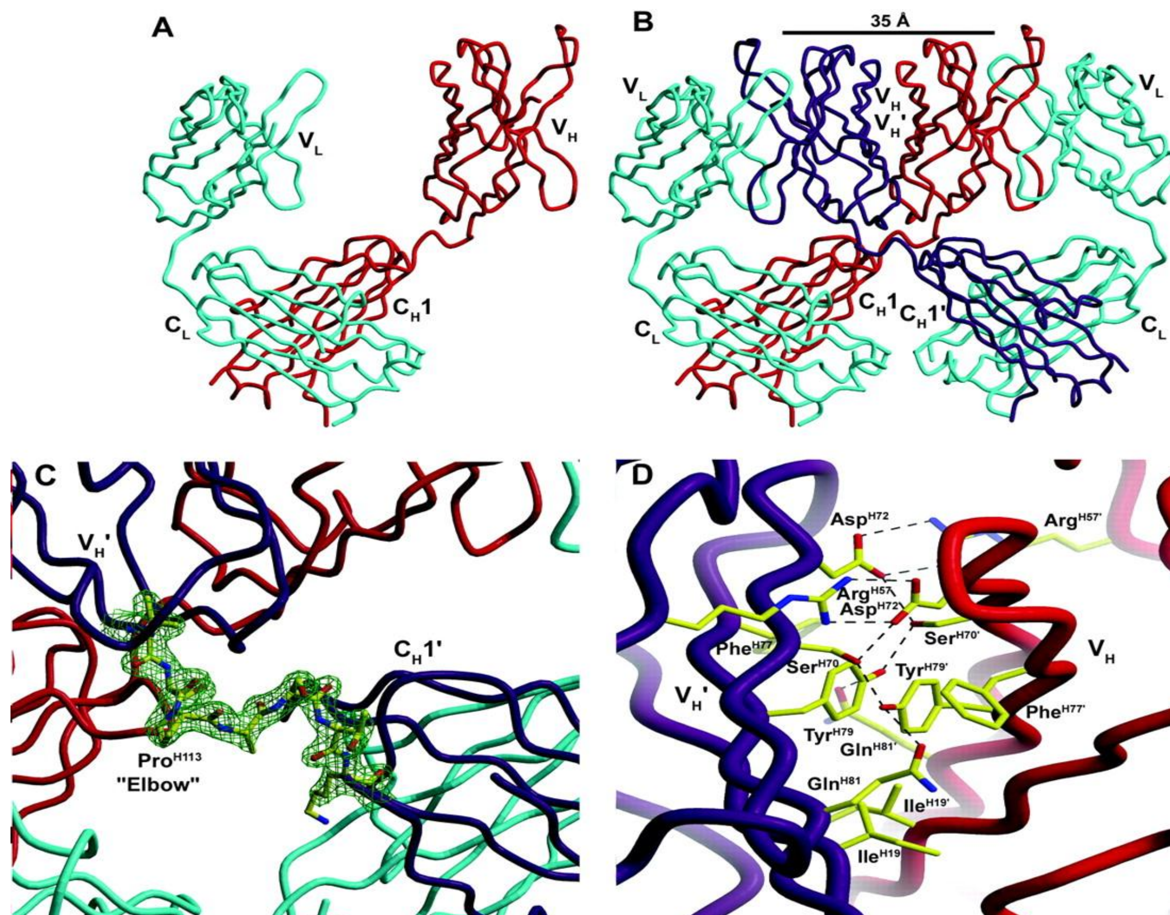


Figure reproduced from Calarese *et al.* Science, 2003 [692]. (A) Crystal structure of monomer of Fab 2G12 showing that the V_H domain clearly separates from its typical interaction with the V_L. The light and heavy chains are shown in cyan and red, respectively. (B) Crystal structure of the two domain-swapped Fab molecules assembled as a dimer. Both light chains are shown in cyan, with the heavy chains from Fab 1 and Fab 2 shown in red and purple. The fixed distance between the two combining sites is shown. (C) Elbow region between the C_H and V_H domains illustrating the domain exchange. The location of an unusual amino acid residue, ProH113, that may favor the domain exchange, is shown. (D) Close-up view of the V_H/V_H' interface between the variable heavy domains. Potential hydrogen bonds are shown with dashed black lines.

In comparison with 2G12, 2F5 and 4E10 exhibit much more typical antibody structures. However, they do show some adaptations which enable them to bind their epitopes, which lie on the portion of gp41 directly adjacent to the HIV viral membrane (the MPER). Both 2F5 and 4E10 possess longer and more hydrophobic (neutrally charged) third

complementarity determining regions (CDR H3) on the variable part of their heavy chain than found in other IgG molecules, and have been shown to interact with a range of membrane-associated lipids [479,481,693]. This propensity is greatest for 4E10, which has been shown to bind with high affinity to cholesterol in addition to several glycolipids and phospholipids, including cardiolipin [694]. In contrast, studies have demonstrated mainly low affinity interactions between 2F5 and several anionic and neutral lipids, and no evidence of any lipid binding by 2G12 [506,518]. It has been shown, using phosholipid membrane model systems, that 4E10, and to a lesser extent 2F5, interact with zwitterionic and anionic lipid membranes before and/or during epitope binding and that whilst this is not essential for epitope binding, it is required for efficient neutralization [695,696]. Recent studies have been conducted to explain the apparent greater membrane affinity of 4E10 compared to 2F5, as well as its more potent neutralizing effect. Although the linear peptide epitopes recognised by each mAb lie close together on the MPER, the NWF(D/N)IT core (bound by 4E10) is more deeply inserted into the lipid bilayer than the ELDKW core (bound by 2F5). In addition, 4E10 appears to interact more deeply within the membrane to promote the extraction of the MPER whereas 2F5 binds on top of its epitope at the surface of the membrane, with only the tip of its CDR H3 loop entering the bilayer [697,698].

Figure 3-31 Structures of 2F5 and 4E10

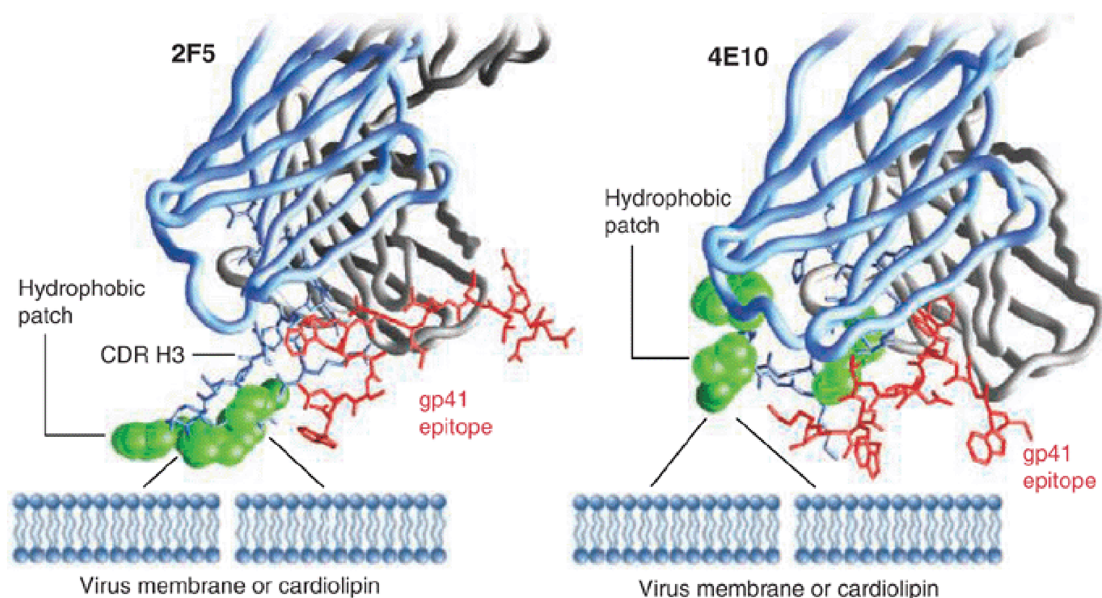


Figure reproduced from Nabel GJ, Science 2005 [699].

3.8.9.4 Effect of molecular charge on mAb pharmacokinetics

As well as impacting on epitope recognition and FcRn binding, the overall structure and polarity of an antibody can also influence its non-specific interaction with cells and tissues. A fundamental property of all proteins is their isoelectric point (pI) which is defined as the pH at which the molecule carries no net electrical charge [700]. Being mostly determined by the proportion and position of charged amino-acid residues within a molecule, the pI can largely be predicted from a protein's mRNA sequence and overall structure [700]. However, certain chemical modifications, such as glycosylation, deamidation, isomerisation or oxidation, which can arise during production or storage of proteins, including antibodies, can give rise to charge heterogeneity even within a single mAb preparation [598]. Thus, when pIs are confirmed experimentally, using immunoelectrophoresis (IEF), it is not uncommon to see multiple bands, representing different isoforms of the same mAb with a range of slightly different pIs. Indeed this is what is seen with the anti-HIV mAbs: 2F5 produces bands in pI range 9-10; 4E10 close to 9.5 and 2G12 in pI range 7.6- 8.6 (B. Vcelar, personal communication). Thus, although variable, 2G12 molecules generally have a lower, more anionic pI than the MPER mAbs.

In general, the more basic (higher) isoelectric point of most antibodies compared with other serum proteins (pI 6.5 to 9 vs < 5.5) tends to favour their retention in tissues, as it renders them more likely to bind to negatively charged glycans on cell surfaces or within the extracellular matrix [700,701,702]. In addition, electrostatic interactions between positively charged antibodies and negatively charged cell membranes have been shown to enhance cellular uptake by pinocytosis [703,704]. Thus, in theory, mAbs with higher pIs would be predicted to be taken up more readily into tissues than those with lower ones. The effect of charge on systemic pharmacokinetics has been specifically studied using chemically modified IgG. Overall, there is indeed a trend for antibodies to show greater uptake and binding by tissues, and a resulting increase in their plasma clearance, with increasing cationization. In contrast, although anonized antibodies generally show less tissue uptake, they can display either increased or decreased plasma and total body clearance [700]. The latter may depend on the site and nature of the modifications and whether or not they impair binding to FcRn. To date, only one group have looked specifically at the effect of varying charge in the Fab region in antibodies which share

identical constant regions. Igawa *et al.* produced several charge-modified humanized IgG4 antibodies through selective mutagenesis of amino acids in the Fv region and evaluated their pharmacokinetics in both normal and β_2m KO mice [705]. In their studies, they saw a 2.4 fold increase in the plasma elimination t ½ and a 4.4 fold reduction in total body clearance between the antibody with the highest pI (pI 9.2; t ½ 10.9 days) and the one with the lowest (pI 7.2; t ½ 26.1 days). Similar results were seen in WT and β_2m KO mice confirming they were independent of FcRn. This suggests that variation in charged residues in the IgG Fv region can produce pharmacokinetic changes of a similar magnitude as altering FcRn binding affinity.

One might predict from their higher pIs, and possibly their greater affinity for lipids, that the MPER mAbs could be taken up more readily by tissues than 2G12. This could explain their more rapid plasma clearance. In addition, it could lead to them being retained in greater concentrations in the superficial layers of the vaginal epithelium from which they could later diffuse to the mucosal surface. However, in the long-term systemic dosing study, the Vss/Vc ratios were alike for all 3 mAbs, suggesting their tissue distribution kinetics were similar. However, as Joos *et al*. point out, there were a number of limitations to their two-compartment model [676]. Firstly, predicted values of Vss and Vc were derived from their data assuming that a rapid equilibrium in concentration was reached between the central (plasma) and peripheral (tissue) compartments for each mAb. Any binding/retention of the mAbs in tissues would potentially invalidate this assumption and result in an underestimation of Vss. Secondly, when significant elimination occurs from peripheral compartments in addition to the central compartment, it is not possible to obtain accurate estimates of Vss unless tissue concentrations are measured in addition to those in plasma. This was not done in this particular study. Since the study was conducted in HIV positive individuals, at least some binding of mAbs to HIV virions and infected cells would be expected to take place in tissues in addition to in the systemic circulation, with resulting clearance by the RES. Thus, it is possible that differences occurred in the uptake and clearance of mAbs in tissues which could not be detected from the available study data.

3.8.9.5 Potential influence of structure and polarity on retention in mucus

As mentioned in section 3.8.8.2, entrapment of antibodies in mucus is believed to result from their propensity to form low affinity bonds with mucin fibres. Mucin fibres consist of long flexible strings of protein monomers, densely coated with short glycans, which are commonly tipped with a negative charge (carboxyl or sulfate groups). These glycosylated and highly hydrophilic regions are separated by "naked," relatively hydrophobic regions of the protein that are believed to fold into globules. Thus, by presenting alternating hydrophilic and hydrophobic regions, an array of low-affinity bonds can develop between the flexible fibers of the gel and other molecules [87]. Whether antibodies bind mucin directly is a matter of contention. Several researchers have tried and failed to demonstrate binding between immunoglobulins and purified mucins *in-vitro* [706,707,708] and intermediaries, such as SC (for s-IgA) or an IgG Fc binding protein, have been suggested to facilitate the interaction [709]. However, regardless of the mechanism, it is possible that the charge distribution and/or unusual linear configuration of 2G12 are less favourable for binding mucus than those of the MPER mAbs resulting in more rapid initial loss from the vaginal surface.

3.8.9.6 Effect of structure on stability to degradation/metabolism

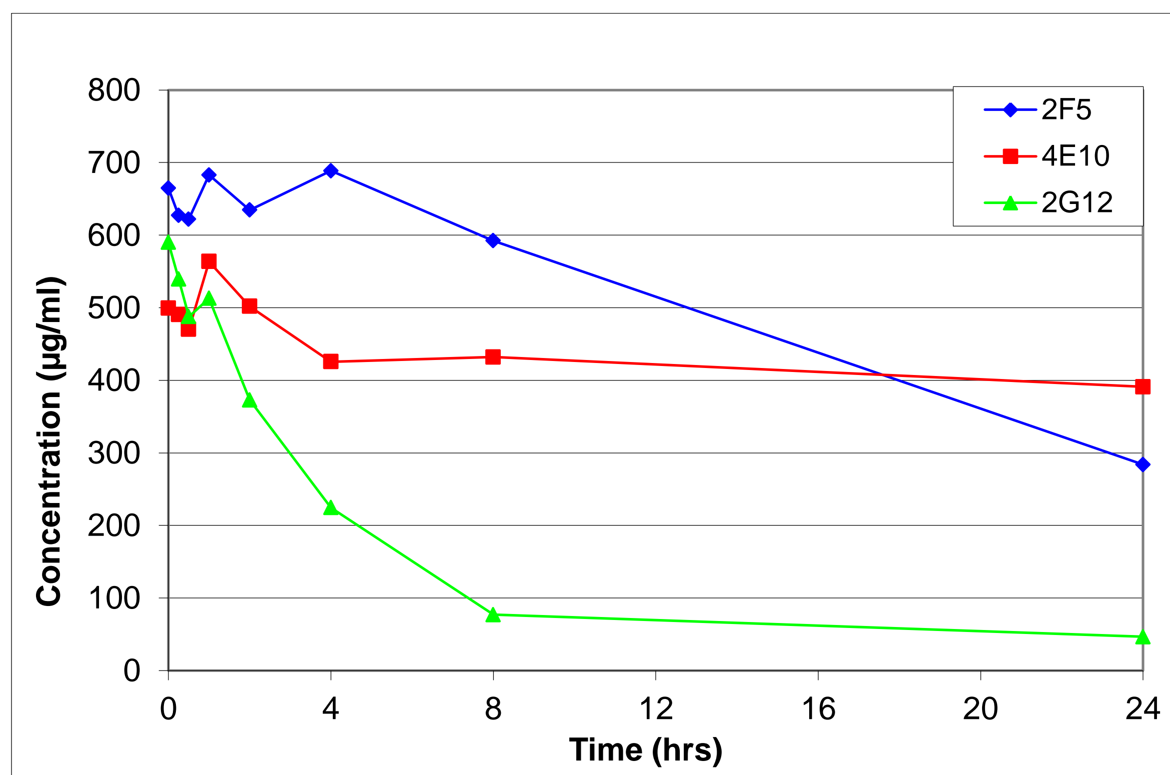
Given its unconventional structure, it is possible that 2G12 has a greater susceptibility to degradation or enzymic breakdown within the vaginal environment than 2F5 and 4E10. In an immunochemical analysis, C2G12 was found to be more sensitive to low pH than typical IgG. In contrast to b12 and IVIg preparations, which were unaffected even after 4 hour incubations, substantial aggregation of C2G12 molecules occurred after only 5 minutes exposure to 0.2M glycine sulphate (pH 2.3); a reagent commonly used to elute proteins from affinity chromatography columns. Acid-induced aggregates displayed large numbers of tightly-associated rings, with similar appearances on electron microscopy to C2G12 Fab arms generated after digestion by Papain [710]. The study authors suggested that acid treatment disrupts the normal V_H-V_L non-covalent interactions, but in contrast to typical IgG molecules, which re-associate rapidly upon a rise in pH, the twisted H-chain elbow region in C2G12 makes re-association less easy/favourable. Instead, unpaired V_H and V_L domains in one C2G12 molecule bind complementary partner domains in 2 other 2G12 molecules leading to aggregation. Although a sample of the Mabgel product (pH

5.5) was tested and remained intact under 'stressed' temperature conditions (25 °C and 37 °C for at least 3 months) prior to the start of the MABGEL 1 trial, the mAbs had not been evaluated at physiological vaginal pH (i.e. around pH 4.0). Nor had their stability been assessed in the presence of vaginal secretions. The plan had been to carry out such stability testing using the baseline samples of neat vaginal secretion obtained in the MABGEL trial using the aspirator (to which no buffer/PIs had been added). However, in view of the paucity of secretions in the aspirates, these analyses were only completed recently. Using vaginal secretions collected from 3 healthy women (as part of a vaginal flora study conducted by Dr Vicky Jespers, ITM), 1 ml of neat secretion from each participant (pool of 3 samples) was mixed with 1 ml of mAb preparation (mix of the 3 mAbs containing 10 mg C2F5, 10 mg C2G12 and 10 mg C4E10 / ml plus 2 mM acetic acid, 10 % maltose, pH 4). Samples were vortexed briefly and incubated at 37 °C. 100 µl samples were taken from this mixture at the following times: before incubation (mAb mix only), post incubation (mins): 15, 30, 60, 120, 240, 480, and 24 hours. 100 µl samples were diluted with 900 µl buffer (PBS 7.2-7.4 plus 1% bovine serum albumin) and frozen at – 70° C. Samples were shipped on dry ice to Polymun and analysed by specific mAb ELISAs (as per section 2.11.3) (B. Vcelar, personal communication). Median mAb levels per time-point are displayed in Figure 3-32 . As can clearly be seen, whereas concentrations of C2F5 and C4E10 were relatively stable in vaginal secretions, declining slowly over a 24 hour incubation period, levels of C2G12 fell more rapidly, suggesting it is more susceptible to degradation by the low pH, proteolytic enzymes or both. Further investigation is needed to elucidate the exact mechanism.

It would be interesting to determine whether a similar phenomenon occurs in vaginal secretions from cynomolgus macaques (*Macaca fascicularis*). Comparisons of the vaginal microflora between humans and macaques, both cynomolgus and the larger pig-tailed macaque (*Macaca nemestrina*), have shown similarities in the range of bacterial species identified, through microscopy and culture, but differences in the proportion in which each is present. For example, Lactobacilli are generally found in lower density and Viridans streptococci in higher density in macaques than is usual for the healthy human vagina, with most hydrogen-peroxide production attributable to the latter rather than the former [711,712]. In general, vaginal pH is higher in macaques, being 5.5 to 8 in *M. fascicularis*, and 4 to 8.5 in *M. nemestrina*, with 6 to 7 being most common [712, 713].

This compares with 3.8 to 4.5 in healthy women (see section 4.3.3) [714]. Interspecies differences in vaginal pH, and perhaps in other, as yet undetermined, variables e.g. in the enzymic activity of mucosal secretions, could underly the observed differences in 2G12 pharmacokinetics in the CEA studies compared with the Mabgel Trial.

Figure 3-32 Concentrations of C2F5, C4E10 and C2G12 after incubation with human vaginal secretions at pH 4.0



4. SAFETY RESULTS AND ANALYSES

4.1 Main Safety Data

4.1.1 Adverse Events

In total, 88 AEs were recorded for the 28 participants enrolled into the dosing phase of the Mabgel trial, of whom, 27 experienced at least one event. Only MAB026 was event-free. 77 of the 88 events were assigned by the SP, in conjunction with the CI, as being possibly or probably related to use of the study gel (see section 2.8.2.2).

Table 4-1 displays the distribution of AEs by study arm sub-divided according to Medical Dictionary for Regulatory Activities (MedDRA) category and nature of AE.

4.1.1.1 Grade 3 AEs and SAEs

There were no AEs occurring in the study that were graded as severe (grade 3) or higher and no serious AEs (SAEs) as defined in the UK Medicines for Human Use (Clinical Trials) Regulations 2004 [571].

4.1.1.2 Grading of other AEs

Eighty four AEs (95.5 % of the total) were graded as mild (grade 1) in accordance with the protocol (see section 2.8.2.4). The remaining 4 AEs were graded as moderate (grade 2). Two of the moderate AEs occurred in a single participant in the placebo arm of the study (MAB006), the other 2 occurred in 2 separate participants, both in the low dose Mabgel arm (MAB001 and MAB014).

MAB006 recorded 2 moderate AEs. These were intense vulval itching lasting 2 hours on a single occasion in the middle of the dosing period and PV bleeding like menses, at the end of the dosing period. These were both assigned as being possibly related to study gel use, although the participant reported that the latter started following a missed dose of her low dose COCP (Loestrin 20). Of note, this particular participant had 7 AEs recorded during the study, the most of any of the study participants.

MAB001 noticed light PV bleeding (reported initially as a brownish discharge and confirmed on examination (OE)) following the first dose of study gel. This settled after a couple of days but then returned later in the dosing period and was reported as intermittent between Visit 7 and the final visit (Visit 8). On the advice of the study Monitor, any AEs consisting of symptoms/signs that were intermittent or recurrent on > 2 occasions during the study in the same participant with the same attributed causation were collected together and reported as a single AE of longer duration rather than multiple separate AEs. Thus, in view of its total duration, the bleeding was classified as a single moderate AE rather than multiple mild AEs. As this participant was using an etonogestrel implant as contraception, it is possible that her bleeding was related to this rather than the use of the study gel (and she had become more aware of it whilst taking part in the study). However, since the symptom was reported as being new in onset after the start of gel dosing, and was not observed OE at the screening or pre-dose Visit 2 evaluations, it was attributed as being possibly related to the study gel.

MAB014 reported feeling generally unwell and lethargic for <48 hours starting on the 3rd day of dosing. This was reported to the study team at Visit 5, by which point she reported feeling back to her usual self. As this episode had resulted in her going to bed earlier than usual but had not prevented her from going to work or required any intervention apart from paracetamol, it was recorded as a moderate AE and assigned as being possibly related to the study gel.

Table 4-1 AE Distribution by Study Arm

Organ system/Nature of Event	Study Arm		
	Placebo (n=9)	10mg/g Mabgel (n=9)	20mg/g Mabgel (n=10)
At least 1 adverse reaction	9 (100%)	9 (100%)	9 (90%)
Reproductive System			
Non-menstrual bleeding	6 (67%)	5 (56%)	2 (20%)
Discomfort (soreness, burning, pruritis)	6 (67%)	2 (22%)	1 (10%)
Vaginal discharge	3 (33%)	2 (22%)	2 (20%)
Vulval or vaginal erythema	1 (11%)	3 (33%)	0
Epithelial disruption	1 (11%)	0	0
Constitutional Symptoms: Lethargy	1 (11%)	1 (11%)	0
Headache	4 (44%)	3 (33%)	1 (10%)
Gastrointestinal Disorder			
Abdominal pain (lower)	2 (22%)	1 (11%)	2 (20%)
Abdominal pain (upper)	0	1 (11%)	0
Nausea, bloating, vomiting or diarrhoea	0	1 (11%)	2 (20%)
Musculoskeletal: Back Pain	1 (11%)	1 (11%)	0
Rash	0	0	1 (10%)
Haematological Disorders			
Neutropenia	1 (11%)	1 (11%)	1 (10%)
Low total WCC	1 (11%)	0	1 (10%)
Increased PT	3 (33%)	0	3 (30%)
Biochemical Investigations			
Increased blood bilirubin	0	0	1 (10%)
Increased blood creatinine	0	1 (11%)	0
Infections			
Vaginal candida	0	3 (33%)	0
Bacterial vaginosis	0	1 (11%)	0

4.1.1.3 AEs Where There were Uncertainties as to Grade or Causation

MAB004 experienced PV bleeding for 4 days beginning on the morning of visit 6 that she described as being 'like a period'. According to the dates she gave us on the 1st dosing visit, the bleeding started on day 26 of her menstrual cycle and she reported having a regular cycle of around 28 to 30 days (she had a natural cycle as she was not using hormonal contraception). A decision was made by the study team, in conjunction with the CI, that this probably represented her normal menstrual period, as, when questioned, the participant had not been 100% certain regarding the start date of her previous period before study enrolment. However, on discussion with the Monitor, it was decided that it should be recorded as an AE as there was a chance that the bleeding could have been triggered by repeated examinations or use of the study gel. It was designated a mild AE but would have been assigned a moderate grading based on the duration and nature of the bleeding had it been felt to be non-menstrual in nature. This AE thus demonstrates two important points; firstly, the importance of the reliability of participant's histories regarding menstrual cycle and symptomatology when assessing microbicide safety and secondly, the subjective nature of assigning severity grading and causality to AEs. Measures such as reviewing stored images after colposcopy, having clearly defined AE gradings in the study protocol, ensuring hospital emergency departments are informed about clinical trials (and alert study teams if any trial participant presents) and good study monitoring, all help to increase objectivity and prevent AEs from being missed. However there still remains a degree of reliance on participants reporting AEs accurately and them being documented and interpreted appropriately by investigators.

4.1.1.4 AEs Resulting in Discontinuation of Study Gel

As noted in section 2.13.5.2, according to the Trial Protocol, any AE graded as moderate (grade 2) should have resulted in a temporary discontinuation of dosing. However, in practice this only occurred for MAB014.

The bleeding reported by MAB001 was only assigned as (cumulatively) being a moderate AE after the end of the dosing period and that experienced by MAB006 began on day 12 of dosing, being reported to the study team at Visit 6. The vulval itching episode occurred on day 3 of dosing and had resolved and not recurred following 2 more doses of gel

before it was reported to the study team at Visit 5. As there were neither any on-going symptoms nor any signs on examination, the CI decided that there was no clinical reason to suspend dosing in this participant.

As a result of feeling unwell and going to bed early, MAB014 omitted 1 dose of study gel (Dose 3). However, by the time she attended for Visit 5, 2 days later, and reported her illness, she was feeling better and had already used the next dose of gel. Thus, again, the decision was taken that there was no clinical reason to omit any further doses.

4.1.1.5 Follow up of AEs Continuing Beyond Visit 8

There was no requirement in the study protocol to follow up any AE beyond Visit 8 unless it had been recorded as an SAE, in which case it would be followed up by the study team/CI until resolution. Likewise, any pregnancies in participants reported during the study period would have been followed up in accordance with the current North and East Yorkshire R & D Alliance procedure. All of the AEs which continued beyond Visit 8 were classified as mild (grade 1) and most were related to mildly abnormal blood parameters detected on samples taken at visit 8. In all cases, participants were informed of these results verbally by the SP and their GPs informed in the completion of study letter. Since none of these results were in the range likely to reflect significant pathology, it was left to the discretion of GPs as to whether they were followed up or repeated. GPs and participants were asked to notify us of any ongoing problems.

4.1.1.6 Genital AEs

AEs affecting the vulva, vagina and cervix are commonly seen in trials of vaginal products, such as microbicides, and their accurate recording and analysis is extremely important in assessing the safety and acceptability of the product. Hence, as discussed in section 2.8.3, genital AEs were specifically solicited through questioning at each visit and at telephone calls, and examination performed with the use of a colposcope to allow visualisation of the mucosa at higher magnification. Genital AEs are categorised in Table 4-1 according to the signs/symptoms presented in the study grading criteria for genital reactions and other commonly reported symptoms e.g. discharge. Discomfort includes descriptions of itching (pruritis), warmth/burning, dysuria, soreness and irritation. Where a single AE combines 2

of the above e.g. soreness and redness (i.e. a clinical sign and a symptom) the AE is listed under the sign only so as not to alter the total number of AEs from that recorded (only 1 participant MAB027 had both). Non-menstrual per vaginal (PV) bleeding includes any bleeding or suggestion of previous bleeding, e.g. a brown discharge, reported by a participant or seen on genital examination. Other descriptions of discharge, including reports of stickiness, are included in the discharge category.

There were 9 genital AEs in the high dose Mabgel arm (all at least possibly attributable to gel use), 13 in the low dose Mabgel arm (all at least possibly attributable to gel use) and 19 in the placebo arm (17 at least possibly attributable to gel use). The 2 non-study gel related AEs in the placebo arm were folliculitis in participant MAB006 due to an in-growing hair and cervical trauma caused by the volumetric aspirator in MAB010. Both of these are discussed below in sections 4.1.1.10 and 4.1.1.11.

The most common genital AE reported was non-menstrual vaginal bleeding, affecting 13 of the 28 participants (46%) (2 in high dose, 5 in low dose, 6 in placebo arms). 2 of these cases (1 in low dose, 1 in placebo) were recorded as moderate AEs, based on duration or volume of bleeding, and are discussed above (section 4.1.1.2). Except for the case of MAB004, discussed in section 4.1.1.3, the remainder were clearly mild AEs: - PV spotting/brown discharge or slight bleeding from the cervical os, reported by participants and/or seen on examination, which lasted < 48 hours.

Overall, there were 10 reports (4 in high dose, 3 in low dose, 3 in placebo arms) of unusual vaginal discharge in 7 women, with 3 women, 1 in each arm, (MAB016, MAB023, MAB024) reporting 2 episodes. The discharge reported varied in nature. 3 women (MAB001, MA008, MAB023) reported a white or pinkish white discharge that differed in appearance to what they usually experienced, but was not described as being heavy or thick. 2 participants described a thicker, heavier discharge than usual (MAB017, MAB023). The remaining 4 reports (MAB011, MAB024 (high dose) MAB016 x 2 (low dose)) were of stickiness and/or gel being noticed in underwear or coming out as discharge during the dosing period. The latter was also described by several participants in the interviews conducted at the end of the trial, with 7 women stating that they wore knickers, or pads when they normally would not to prevent gel leaking out on to bed clothes or underwear.

In total 9 participants complained of discomfort (soreness, itching, irritation, warmth/burning); (1 in high dose arm, 2 in low dose arm, 6 in placebo arm). Only 1 participant who complained of discomfort (MAB027), had erythema on examination. Interestingly, she was one of 3 women to have positive baseline candida cultures (see section 4.1.1.12).

Findings suggestive of possible mucosal inflammation were seen on examination in 5 participants: erythema - none in high dose, 3 in low dose, 1 in placebo arm; superficial epithelial disruption - 2 areas < 1 swab tip in diameter just inside the introitus in 1 participant in placebo arm (MAB010). In all women, except MAB006 (see section 4.1.1.2) symptoms were easily tolerated and signs were mild and localised. All were self-limiting resolving within 4 days except for MAB021 who experienced intermittent small patches of dry skin that were mildly red but caused no discomfort between visit 6 and 8 (post gel dosing). There were no cases of visible swelling or mucosal oedema.

Very few AEs were seen on colposcopy that would not have been detected by participant report or visual examination with the naked eye alone. MAB002 had a small area of erythema on her posterior vaginal wall that was not seen with the naked eye. The epithelial disruption in MAB010 was just visible with the naked eye but better delineated using the colposcope. Both of these findings were detected at visit 3 (8 hours post 1st dose) but had resolved by visit 4 (24 hours post 1st dose). Neither were visible at subsequent evaluations, although both were attributed as possibly related to use of the study gel.

4.1.1.7 Other Clinical AEs

Signs/symptoms that were reported by participants or detected on clinical examination that related to organ systems outside of the genital tract were designated as other clinical AEs. In general, the AEs reported were conditions that are seen commonly in the general population. Only 1 non-genital clinical AE was designated as moderate (grade 2) (see section 4.1.1.2), all the rest were mild (grade 1).

For the most part, unless the AE was reported prior to the 1st dose of gel being administered, it was assigned as being at least possibly related to the study gel. The one

exception was a headache reported by MAB010 (placebo arm) during the dosing period that occurred on a morning following her having drunk an excessive amount of alcohol at a party. The participant described this as being exactly like a 'hangover' and felt better after taking ibuprofen. It was thus felt that it was more likely to be alcohol-related than caused by the study gel. Later in the dosing period, the same participant experienced another mild headache which was generalised and relieved by ibuprofen as before, but this time she had not drunk any alcohol. Thus, the second episode was attributed as being possibly related to the study gel.

Headaches were in fact one of the most common AEs, reported by 8 participants (1 in high dose, 3 in low dose, 4 in placebo arm). All were mild and resolved spontaneously within 24 hours or with stat doses of non-prescription analgesics. Other frequently reported AEs were related to the gastro-intestinal tract. These included 4 cases of generalised abdominal pain (MAB012, MAB015, MAB016, MAB023), 1 case of diarrhoea and vomiting (MAB028), bloating and loose stools (MAB027), nausea (MAB004) and upper GI intolerance (indigestion/churning/heartburn) (MAB001). All of these symptoms resolved within 48 hours except for 1 case of lower abdominal pain and constipation (MAB011), which was reported just prior to the end of study follow up, and relieved by non-prescription suppositories after visit 8. Hence this was recorded as continuing beyond the end of the study. Other AEs seen included back pain (MAB021, MAB022) and rash (MAB015, localised to thigh, relieved with non-prescription antihistamine).

4.1.1.8 Laboratory AEs

In total there were 16 laboratory AEs recorded in the 28 participants enrolled: 6 in high dose, 4 in low dose and 6 in placebo arms. All laboratory AEs were mild.

Most laboratory AEs recorded were results of tests of blood parameters that met the criteria for being an AE (as defined in the study AE grading tables). These are listed below. In addition, there were 3 baseline positive candida cultures, taken just prior to the first gel dosing at Visit 2, that were not associated with any clinical signs or symptoms at the time the swab was taken. These are discussed in section 4.1.1.12.

The blood results recorded as AEs were as follows:

- MAB003 (High dose Mabgel arm): raised bilirubin (27 µmol/l; ULN = 19 µmol/l) in mild AE range (> 1.25 ≤ 2 X ULN) first detected at Visit 6 and resolved on re-checking at visit 8. No elevations in other liver enzymes (alanine aminotransferase (ALT) or alkaline phosphatase (ALP)) were seen.
- MAB014 (Low dose Mabgel arm): raised serum creatinine (108 µmol/l; ULN = 90 µmol/l) in mild AE range (> 1 < 1.5 x ULN). Detected at visit 8. Participant was contacted and returned for repeat 5 days later at which point it had returned to be within the normal range (64 µmol/l).

These were the only two AEs involving biochemical abnormalities. The remainder involved haematological abnormalities of white blood cell counts (WBC), either total WBC or neutrophils alone, or a prolonged prothrombin time (PT).

- MAB006 (placebo arm): low total WBC count (3.2 10^9/L) in mild AE range (<3.5 >3.0 x 10^9/L) detected at visit 6, resolved by visit 8.
- MAB007 (Low dose Mabgel arm): Low neutrophil count (1.4 x10^9/L) in mild AE range (≤ 1.5 > 1 x10^9/L) was detected at visit 6 but had returned to normal range when re-checked at visit 8.
- MAB018 (high dose Mabgel arm)- low total WBC count (3.2 x10^9/L) and low neutrophils (1.1 x10^9/L) Detected on blood tests at Visit 8. Bloods rechecked 30 days later (participant unable to attend sooner as on holiday) and had returned to within normal range for both parameters.
- MAB021 (placebo arm): Low neutrophil count (1.5 x10^9/L) detected at visit 6, resolved by visit 8.

All of the following had mildly elevated PT results (> 1 ≤ 1.25 x ULN) (at varying time-points. Those with abnormalities remaining at visit 8 required no treatment but their GP was informed in the end of study letter.

- MAB006 (placebo arm): Detected at Visit 6 and Visit 8.

- MAB008 (high dose Mabgel arm): Detected at Visit 3 (8 hours post 1st dose) and on subsequent blood tests including Visit 8. No treatment was required but the participant's GP was informed of the ongoing mild blood abnormalities detected.
- MAB012 (placebo arm): Detected at Visit 8.
- MAB017 (placebo arm): First detected on Visit 2 pre-dose sample (but not increased on 8 hours post 1st dose sample) and at Visit 6, resolved by Visit 8.
- MAB020 (high dose Mabgel arm): Elevated on Visit 2 pre- 1st dose and Visit 8 samples but not in between. Considered to be unlikely to be related to study gel use by the CI as only elevated before and several weeks after mAb exposure.
- MAB024 (high dose Mabgel arm): Raised on 8 hour post 1st dose sample but resolved on visit 6 sample.

All of the above blood abnormalities (apart from MAB020 raised PT) were assigned as being possibly attributed to gel use during the trial and remained so for the statistical analyses. However, it is now felt unlikely that any of the non-genital laboratory AEs were caused by the use of the study gel given that there was no evidence of systemic absorption of the mAbs and no blood abnormalities have been associated with the use of other HEC-based placebo gels in other trials [399,400,415].

4.1.1.9 Coagulation Assays and Anticardiolipin Titres

The PT and APTT measure the extrinsic, and combined intrinsic (contact) and common pathways of blood coagulation respectively. The PT measures the activity of clotting factors produced by the liver (II, V, VII, and X) which require vitamin K for their manufacture and is often significantly elevated in people with vitamin K deficiency, e.g. due to poor diet or malabsorption, or in the presence of inhibitors, such as warfarin. It is also a measure of general liver synthetic function. The APTT, on the other hand, is much less specific, being elevated in deficiencies of all coagulation factors except VII and XIII. Apart from cases of hereditary isolated factor VII deficiency and early liver failure, it is unusual to have an elevated PT without accompanying derangement in the APTT. The APTT, on the other hand, is affected independently of the PT in most hereditary haemophilias and in the presence of heparin or anti phospholipid antibodies [715].

In a previous human trial, transient elevations in APTT were temporally associated with the intravenous infusion of high doses of the mAbs [506]. In the 4 subjects tested, all of which had a normal APTT and PT at baseline, infusion of 5g of each of mAb in combination led to a mean increase in the APTT of 9.6 +/- 3.7 seconds at 30 min post infusion. This led to APTTs at peak serum mAb concentrations that were either still within the normal range or in the range which constituted a mild AE in that trial (>1.1 ≤ 1.25 x ULN). In contrast, there were minimal or no alterations to PT results and all remained within the normal range. *In-vitro* studies and retrospective analysis of samples from clinical trials in which participants received C2F5 and C2G12 or C4E10 alone, has determined that, unlike the other 2 mAbs, C4E10 displays cross-reactivity to the phospholipid cardiolipin (CL), with detectable elevations in serum aCL titres (105.7 +/- 7.9) and it is this activity that increases the APTT.

In our trial, we saw no elevations of APTT or any detectable rise in IgG anti-cardiolipin titres in any participants during the dosing or follow up periods. However, there were a number of women with elevated PT results recorded as mild AEs (range > 1 ≤ 1.25 x ULN). 3 of these women were in the high dose Mabgel arm and 3 in the placebo arm, with none in the low dose Mabgel arm. There was no indication clinically or from results of other blood tests that any of the participants were significantly malnourished or had liver disease and no participants were taking warfarin or other anticoagulants. Thus, it is likely that the PT results were elevated due to another reason.

There seems to be no clear pattern to the PT results that were above the normal range in relation to time point or study group. Two of three women in the high dose Mabgel arm who had elevations in PT first had this detected at Visit 3 (8 hours post 1st dose) which was when it was felt that peak systemic absorption of the mAbs might occur. However, in one of these women the PT results continued to be raised for the remainder of the trial whereas in the other they had fallen back to baseline when next measured at Visit 6 (12 hours post 12th dose). In the third high dose participant, the elevated PT levels were deemed by the CI to be not related to gel use as they occurred on samples taken prior to use of the 1st dose of the gel and several weeks after (Visit 8), being normal in the dosing period. Three women who received placebo also had documented high PT results, and in

these participants they were again seen at pre-1st dose in one participant and at Visit 8 alone in another, and none were at the 8 hours post 1st dose visit.

Given the fact that there is no evidence of any systemic absorption of the mAbs, no rise in APTT (usually accompanying a PT rise) and PT values outside of normal range in blood samples taken before any doses of gel were given, and in the placebo arm, it would suggest that the elevated results may have arisen due to variables intrinsic to the blood collection process or to the PT assay itself. PT is measured on plasma from blood drawn into a tube containing citrate, which prevents clotting by binding calcium. For accurate measurement, the proportion of blood to citrate has to be fixed at a ratio of 9:1, and in York, as in most hospital laboratories, they will only process blood samples in tubes which look, on visual inspection, to have been filled to the correct level. In the laboratory, a known excess volume of calcium is added to the sample, together with a fixed amount of tissue factor (coagulation factor III) and the time taken for the sample to clot at 37 °C is assessed either by optical or mechanical means, depending on the exact methodology used. In most clinical laboratories, including York, this process is automated. It is well recognised that variations in the purity of tissue factor between different manufacturers, or even batches from the same manufacturer, or in the measuring equipment used can lead to variability in results [716]. Indeed, International Normalised Ratio (INR) was introduced by the World Health Organization (WHO) and the International Committee on Thrombosis and Hemostasis to try to standardise measurements between laboratories [717]. The INR is generated by comparing a patient's sample with that of a normal control processed in the same way, with a correction factor applied reflecting the purity and source of tissue factor supplied by a particular manufacturer [716]. The INR is very useful in allowing the standardisation of the measurement of the anticoagulant effect of warfarin, as unlike the actual measurements and normal ranges for PT, it should remain relatively constant between laboratories. However, there is evidence to suggest, that this may not be the case in practice [718,719]. In our study, because 1) all of the participants were seen in the same centre with samples processed by the same laboratory, and 2) the DAIDS coagulation gradings are for PT (and APTT) rather than INR (and expressed as > ULN for the laboratory being used), it was decided to use the PT. Whether the INR would have resulted in fewer out of normal range results or variability between participants and

visits is felt unlikely, since the control PT time is laboratory specific and only altered if there is a change in equipment or processing procedures.

It is interesting to note that the York Hospital laboratory changed their methodology for analysing PT and APTT samples on 27th April 2010. This was a planned upgrade to replace a Trinity Biotech MDA II optical analyser with a more modern IL ACL TOP500 model and also required a change in the thromboplastin used for PT analysis from Simplastin HTF (BioMerieux) to Recombiplastin 2G (Beckman Coulter). Although the reference ranges for APTT and PT changed as a result of these modifications, these were clearly stated on the written laboratory reports for each result and graded in relation to the ULN as previously. Of the above, outside of normal range PT results detected, the Visit 8 sample for MAB020 and all samples for MAB024 were processed using the new methods. Since the new methodology was only used for the last 3 months of the trial and in a relatively small number of enrolled and potential participants it was not possible to assess formally whether it had resulted in a change in the proportion of out of range PT values being reported. There were no AEs resulting from > ULN APTT values either before or after the change.

4.1.1.10 AEs Not Related to Gel Use

Of the 11 AEs assigned as being unrelated to use of the study gel, 7 arose in the time after consent was provided at the screening visit but before the first dose of study gel was administered and did not increase in severity with gel use (i.e. they were recorded as AEs in accordance with the North and East Yorkshire R&D Alliance SOP on Adverse Event Reporting, as required by the Sponsor, but were to be excluded from main analyses). The other 4 were assigned as being unrelated to gel use either because they were a recurrence of a mildly abnormal blood result detected at visit 2 (pre-1st dose), but not during the dosing period (e.g. MAB020, visit 8 PT result) or because another medical cause of the sign or symptom was felt to be more likely (e.g. MAB006, folliculitis related to an in-growing pubic hair; MAB010, headache related to drinking excess alcohol the night before).

It is acknowledged that assigning causality can be subjective; thus, on the advice of Professor Bland, it was decided to statistically analyse the number of documented AEs per

study arm both including and excluding those AEs assigned as being not/unlikely to be related to gel use i.e. all reported AEs vs only those AEs possibly, probably or definitely attributed to gel use (see section 4.2).

Two occurrences that were recorded as AEs not related to gel use deserve further discussion. These are: 1) AEs related to use of volumetric aspirator, and 2) positive baseline (visit 2 pre-dose) candida cultures.

4.1.1.11 AEs related to use of the Volumetric Aspirator

As detailed in section 2.8.3.1, genital examination, including evaluation by colposcopy, was generally performed prior to any sampling procedures, as advised in the CONRAD/WHO Manual for the Standardization of Colposcopy for the Evaluation of Vaginal Products (2004) [574]. This was to minimise the risk of epithelial abrasions and other injuries caused by swab taking being erroneously attributed to use of the study gel. In the course of the study, there was only 1 incidence where trauma was documented to have arisen as the direct result of a sampling procedure. This occurred in MAB010 (Visit 4) where no abnormalities were visible on initial colposcopic examination, but a circular indentation was visible on the cervix after performance of self-sampling using the volumetric aspirator. The participant experienced no pain during the procedure, and no epithelial disruption or disruption to blood vessels (e.g. echymosis or petechiae) was visible following a repeat colposcopic examination. The findings had resolved on review at Visit 5, which took place the following day.

There were no other reports of pain or trauma arising from use of the aspirators during visits. However, as evidenced by the paucity of analysable data (section 3.6.1.3) and comments provided in the qualitative interviews (see Chapter 5), women found them difficult and uncomfortable to use.

4.1.1.12 Positive Baseline Candida Cultures

As discussed in section 2.7.2.6 baseline swabs for candida culture and speciation were performed on all participants at Visit 2, during the pre-1st dose evaluation. When the initial study protocol was written, in Summer 2008, there was no capacity at York Hospital to perform quantitative candida culture and speciation, thus, in line with the procedure

for processing similar samples from clinical settings in York, our study swabs were to be sent to the Mycology Reference Laboratory in Leeds. Since the primary intention was to examine for the presence of *Candida* species in asymptomatic women that could potentially impact on mAb pharmacokinetic results, the fact that there would be a delay in receiving the results of these swabs of around 10 days was not thought to pose any problem. Women were to be assessed clinically at each study visit, with the swab results analysed after the dosing period to aid interpretation of mAb and AE data.

However, by the time the study was initiated, in September 2009, York Hospital Microbiology Laboratory had begun to perform in-house testing for *Candida* species. Thus, it was no longer necessary to forward the samples to Leeds and results became available to the study team within 3 to 4 days. A potential dilemma was therefore created as to how to action any positive results that were received during the dosing period whilst minimising any impact on the conduct of the study and protecting participant's well-being. A decision was made by the SP, in conjunction with the CI, that any positive results would be dealt with on a case by case basis, with treatment being administered according to presence of signs/symptoms that could be attributed to candidiasis or participant wishes. Since the application of topical treatments to the vagina such as pessaries and creams could affect the pharmacokinetics of the mAbs, oral treatment with fluconazole 150mg po stat was preferred. Women would be discontinued from dosing only in the event that they had signs/symptoms graded as 2 (moderate) or above in AE severity, in keeping with the study protocol.

The management of participants with positive baseline candida results was discussed with the DSMC. It was deemed, by Professor George Kinghorn, that the above management was reasonable and would not impact on the integrity of study data or the safety of participants.

No women seen at Visit 2 had any clinical signs of symptoms of genital tract inflammation or infection on the pre-dose evaluation, thus all women proceeded to receiving the 1st dose of study gel that day. 3 out of 28 women had positive baseline candida cultures (MAB014; MAB019; MAB027). All 3 of these cultures was positive for *C. albicans* and all at heavy growth (+++). All 3 of the women were randomised to the low dose Mabgel arm. Results were available for all 3 women in time for Visit 5 (day 5 to 7 of dosing) and were

communicated to them by the SP at this time. MAB019 reported symptoms of vaginal dryness and mild irritation lasting 3 days in total, but had no visible erythema or discharge on examination. She was offered treatment but her symptoms may just as easily have been caused by the study gel so were attributed as possibly related to gel. Neither of the other women displayed signs or symptoms associated with candida at this visit, but both were offered treatment as prophylaxis. MAB014 and MAB019 accepted treatment with fluconazole 150mg po stat when initially offered but MAB027 declined. However, she was eventually treated at Visit 6 after developing mild vulval soreness and redness. This was first noticed by the participant after 10 doses of gel, but not reported to the study team until Visit 6, and could have been related to candida or to the study gel.

4.2 Statistical Analysis of Main Safety Data

As with the pharmacokinetic data, all statistical analyses were performed in conjunction with Professor Martin Bland using STATA version SE 10.1, Stata Corp., College Station, Texas, released 1st October 2009. All analyses were performed on an intention-to treat basis unless specified otherwise.

As described in section 4.1.1, AEs were recorded for nearly all participants. The number of AEs per woman was therefore deemed to be much more important and relevant than their presence or absence for a woman. It was therefore decided that it would be more meaningful to compare the safety data for each group in relation to the average number of AEs per participant, using the negative binomial distribution, as opposed to the proportion of women experiencing an AE, using Fisher's exact test

Analyses were performed both for AEs in their entirety and excluding those felt to be not/unlikely to be related to gel use.

4.2.1 Analyses of All AEs Regardless of Attribution to Study Gel Use

Table 4-2 Severity of All AEs by Study Arm

AE severity	**Study Arm**			**Total**
	High Dose Mabgel	**Low Dose Mabgel**	**Placebo**	
Mild (grade1)	24	26	34	84
Moderate (grade 2)	0	2	2	4
Severe (grade 3)	0	0	0	0

The average numbers of AEs (including all AEs even if assigned as being not related to study gel use) reported in the three arms are displayed below

Table 4-3 Mean and Standard Deviation of All AEs Per Study Arm

Treatment group	**Number in group**	**Mean number of events**	**Standard deviation**
Placebo	9	4.00	1.94
Low dose Mabgel	9	3.11	1.76
High dose Mabgel	10	2.40	1.17

The difference in reported AEs between study arms was not statistically significant, $P = 0.1$ (negative binomial regression).

4.2.2 Analyses of AEs Attributed as Being At Least Possibly Related to Study Gel Use

Table 4-4 Attribution of Likely Causality of AEs By Study Arm

Relationship to Study Gel Use	Study Arm			Total
	High Dose Mabgel	Low Dose Mabgel	Placebo	
Not Related	3	3	5	11
Unlikely to Be Related	0	0	0	0
Possibly Related	19	23	31	73
Probably Related	2	2	0	4
Definitely Related	0	0	0	0
Any	24	28	36	88

Table 4-5 Severity of AEs Attributed as Being At Least Possibly Related to Study Gel Use by Study Arm

AE severity	Study Arm			Total
	High Dose Mabgel	Low Dose Mabgel	Placebo	
Mild (grade1)	21	23	29	73
Moderate (grade 2)	0	2	2	4
Severe (grade 3)	0	0	0	0

Table 4-6 Mean and Standard Deviation of Number of AEs Attributed As Being At Least Possibly Related to Use of Study Gel Per Study Arm

Treatment group	Number in group	Mean number of events	Standard deviation
Placebo	9	2.67	2.24
Low dose Mabgel	9	2.11	1.69
High dose Mabgel	10	1.80	1.40

The difference in reported AEs between study arms was not statistically significant, P = 0.6 (negative binomial regression). There was nothing to suggest that recorded AEs were related to the application of mAbs.

4.2.3 Summary and Discussion of Main Safety Results

In general, daily vaginal application of up to 50g of each mAb over 12 days appeared safe. Although AEs were reported by all but 1 participant, 95 % were mild, none were serious and only 4 were moderate. There was no statistically significant difference in the number of AEs reported per participant between the 3 study arms, and this was true for all AEs

and those considered to be related to use of the study gel (i.e ARs). Thus, there was no evidence that the addition of either 10mg/g or 20mg/g of the mAb combination to the HEC gel vehicle resulted in increased toxicity. However, since there was no observation only group, i.e. all participants received either an 'active' or a placebo gel product, it is not possible to determine whether the reported AEs were related to use of the HEC gel vehicle or other causes.

Clinical AEs not involving the genital tract were fairly evenly distributed between study arms and although attributed to the gel, likely reflect background morbidities. As is typical for vaginal microbicide studies, over half (51 %) of reported ARs involved the genital tract. Non-menstrual bleeding (NMB) is a frequently reported event and, as in our trial, is often at least as common in those using placebo as in users of active product [720]. However, for reasons which are not fully understood, rates are highly variable even between (and within) studies of the same product [721]. It has been suggested that variation in the processes for eliciting AEs, lack of uniformity in documentation, and differences in study populations, in terms of their background rates of factors known to increase the incidence of (NMB) e.g. hormonal contraceptive use, sexually transmitted infections and willingness to report symptoms, may impact on results [721,722]. In our study, the relatively large number of pelvic examinations and an all-female clinical team [723] may have increased the number of recorded events. In all but 2 cases the bleeding was very mild; spotting/brown discharge reported by participants and/ or visible fresh or old blood coming from the cervical os which resolved within 4 days even with continued gel use. Of note, 1 woman with moderate bleeding admitted missing a dose of her low-oestrogen COCP, which could have triggered or contributed to the episode. Although a number of mildly elevated PT results were detected, none was temporally associated with a bleeding event. In view of the lack of evidence of mAb systemic absorption, the absence of a concomitant rise in aPTT or aCL, and lack of a biologically plausible explanation, these were felt to be most likely related to intrinsic variability in the laboratory PT assay rather than an indication of toxicity.

In addition to PV bleeding, incidences of vaginal discharge (10 reports, in 7 (25 %) of participants) and genital discomfort (reported by 9 (32%) of participants) were also relatively common. However, apart from one report of severe vaginal itching > 2 hours,

these were mild and easily tolerated. Reports were fairly equally distributed across the 3 study arms, except for symptoms of discomfort which were more common in placebo-users. Examination findings suggestive of possible mucosal inflammation were seen in 5 participants. There were 4 cases of erythema (none in high dose, 3 in low dose, 1 in placebo arm) and 1 case of superficial epithelial disruption (< 1 swab tip) (placebo arm); all were mild and localised. One of the cases of erythema, associated with soreness, was attributed to *C. albicans* and treated with fluconazole; all others were self-limiting.

Interestingly only 2 abnormalities were detected on colposcopy that would not have been seen with the naked eye. This contrasts with results from a meta-analysis of 9 phase 1 microbicide trials, where 43 % of abnormalities were only seen on colposcopy [724]. It is possible that some abnormalities may have gone undetected by the author, who is a trained but relatively inexperienced colposcopist. Although no new lesions were discovered on review of stored digital images, only two images were captured using the colposcope camera per visit, thus it was not possible for the CI to visualise the entire ectocervical and vaginal mucosae. Some abnormalities may also have been difficult to detect from a digital photograph. However, the utility of colposcopy in assessing microbicide safety has recently been questioned. The significance of findings, other than deep epithelial disruption (which was not seen in any of our participants), in relation to HIV transmission is unclear and, in a prospective evaluation, colposcopy was found to be no better than naked-eye examination alone at detecting toxicity related to use of N9 vs HEC placebo [725].

Overall, as was highlighted in section 4.2.3, the current process of identifying and recording AEs is largely subjective, being reliant on the accuracy of participants' reports of symptoms and the interviewing, examination and interpretation skills of study clinicians. As discussed in section 6.2.2, efforts are being made to develop more objective methods of evaluating microbicide toxicity which can be validated directly against HIV transmission risk. In the meantime, it is important to be aware of the limitations of current clinical safety assessments, which may be poorly predictive of 'real-world' effects.

4.3 Results and Analyses From the Vaginal Flora Sub-Study

4.3.1 Data Relating to Quantitative PCR for *Lactobacillus* Species, Nugent Scores and Vaginal pH

Data regarding *Lactobacillus* Species concentrations and Nugent scores were provided by Dr Vicky Jespers, Institute of Tropical Medicine, Antwerp, Belgium. Vaginal pH was measured and recorded on the HYMS EMU.

4.3.2 Evaluation and Statistical Analysis of Lactobacillus PCR Results

The primary objective of the vaginal flora sub-study was to identify whether there were any differences between the study arms with regards to changes in concentrations of specific *Lactobacillus* species, detected by real-time, quantitative PCR.

Statistical testing was performed in conjunction with Professor Martin Bland using STATA (version SE 10.1, Stata Corp., College Station, Texas, released 1st October 2009).

4.3.2.1 Analysis of *Lactobacillus* species according to presence or absence in participants within each study arm at each visit

Table 4-7 shows the % of women in each study arm at each visit who had detectable *Lactobacillus* species by PCR. P values given in Table 4-7 in were obtained using Fisher's Exact test, which compares the number of participants with and without each *Lactobacillus* species in each study arm at each visit. This test was chosen over the Chi-square test in view of the small sample sizes in each group.

Lactobacilli were detected in all women at all visits using the generic PCR primers. In general, the presence or absence of a particular *Lactobacillus* species appeared to remain constant throughout the study. At the screening visit, *L. crispatus* was detectable in 56, 89 and 70 % of participants in the placebo, low dose and high dose arms respectively. At Visit 7, the respective percentages were 67, 89 and 70 and at Visit 8 they were 67, 89 and 80. A similar picture was seen for *L. jensenii*, which was found in 78, 78 and 70 % of participants in placebo, low dose and high dose arms respectively at both screening and Visit7, and 78, 78 and 60 % at Visit 8.

For *L. iners*, the percentages of participants with detectable counts at screening was 78, 22 and 50 % for placebo, low dose and high dose arms respectively. For placebo, the percentage reduced slightly to 67 % for both Visits 7 and 8, however, for both Mabgel arms, the percentage was the same at all visits.

The *Lactobacillus* species present in the fewest participants overall was *L. gasserii*. This was present in 33, 44 and 10 % of women in placebo, low dose and high dose arms at screening, 44, 44 and 30 % in the same groups at Visit 7 and 44, 33 and 30 at Visit 8. This is broadly consistent from findings of other studies, which suggest it may be less abundant than the other strains. However, the percentage of healthy women found to be colonized by each species varies with technique used and population sampled [541, 726,727].

Overall, there was no evidence to suggest that the presence or absence of any *Lactobacillus* species was affected by the application of study gel, and no statistically significant differences were seen across the groups. The lower percentage detection of *L. iners* in the low dose arm was present from screening, where it was of borderline statistical significance, thus was independent of study gel use.

Table 4-7 Number (%) of women with specific *Lactobacillus* species by visit and study arm

P-values were obtained using Fisher's Exact test

Visit and Study Arm		*Lactobacillus* Species				
	N	*L. crispatus*	*L. iners*	*L. jensenii*	*L. gasseri*	*Lactobacillus (generic)*
Screening						
Placebo	9	5 (56%)	7 (78%)	7 (78%)	4 (44%)	9 (100%)
Low dose	9	8 (89%)	2 (22%)	7 (78%)	3 (33%)	9 (100%)
High dose	10	7 (70%)	5 (50%)	7 (70%)	1 (10%)	10 (100%)
P-value		0.343	0.080	1.00	0.256	
Visit 7						
Placebo	9	6 (67%)	6 (67%)	7 (78%)	4(44%)	9 (100%)
Low dose	9	8 (89%)	2 (22%)	7 (78%)	4 (44%)	9 (100%)
High dose	10	7 (70%)	5 (50%)	7 (70%)	3 (30%)	10 (100%)
P-value		0.639	0.183	1.00	0.793	
Visit 8						
Placebo	9	6 (67%)	6 (67%)	7 (78%)	3 (33%)	9 (100%)
Low dose	9	8 (89%)	2 (22%)	7 (78%)	4 (44%)	9 (100%)
High dose	10	8 (80%)	5 (50%)	6 (60%)	3 (30%)	10 (100%)
P-value		0.639	0.161	0.664	0.887	

4.3.2.2 Analysis of quantitative Lactobacillus species counts by study arm and visit

Table 4-8 shows the average counts detected by PCR of each *Lactobacillus* species. Both the arithmetic mean and the median have been given. The data are highly skewed, reflecting the large range of values, so the latter is likely to be more representative of a

central value. In view of the large number of zero values, it was felt inappropriate to perform a log transformation of the data or calculate the geometric mean.

Values > 100 are given to zero decimal places, values > 10 < 100 to 1 decimal place, and those < 10 to 2 decimal places.

P-values were obtained using the Kruskal Wallis rank test, allowing for ties, comparing ranked data across all 3 study arms.

In keeping with the findings from the previous analysis (see section 4.3.2.1), the only time point and *Lactobacillus* species where there was a significant difference in counts detected between the study arms was at screening for *L. iners.* However, since this was seen prior to the application of the 1st dose of study gel it is unrelated to gel use. There was no evidence of any adverse effect of the Mabgel on *Lactobacillus* species counts.

Analysis was not performed for the counts recorded using the lactobacilli generic primers as it was apparent that the counts they detected were lower than the total counts found using the species-specific primers. It therefore appears that their amplification efficiency is lower than those of the species-specific primers at detecting individual species and it is unclear whether these primers amplify all species with equal efficiency. It was felt that formally analysing these counts would add little to the overall conclusions from the sub-study and could even be potentially misleading.

Lactobacillus species counts by study visit and arm are represented graphically in Figure 4-1 to Figure 4-4. Note: the bottom and top of each box represent the 25th and 75th percentile (the lower and upper quartiles, respectively), and the band near the middle of the box represents the median (50th percentile). In most cases, the ends of the whiskers represent the minimum and maximum of all the data. However, where there is a single outlier this has been excluded and is represented by a dot.

Table 4-8 Average (mean and median) *Lactobacillus* species counts per ml of elution buffer by study arm and visit

Visit and Study Arm	N	*Lactobacillus* Species (x 10^6/ml)							
		L. crispatus		***L. iners***		***L. jensenii***		***L. gasseri***	
		Mean	Med	Mean	Med	Mean	Med	Mean	Med
Screening									
Placebo	9	161	97.9	191	16.9	5.97	1.81	0.43	0.00
Low dose	9	213	243	3.52	0.00	1.99	0.02	0.01	0.00
High dose	10	99.8	35.7	49.1	1.61	2.24	0.12	0.01	0.00
P-value		0.45		0.04		0.54		0.26	
Visit 7									
Placebo	9	121	126	31.4	0.69	49.2	22.9	0.07	0.00
Low dose	9	125	109	36.7	0.00	22.9	0.37	5.22	0.00
High dose	10	150	71.7	30.3	0.17	2.13	0.49	0.00	0.00
P-value		0.96		0.21		0.21		0.64	
Visit 8									
Placebo	9	201	42.2	88.0	24.6	8.15	2.04	0.01	0.00
Low dose	9	190	139	243	0.00	7.76	1.21	0.15	0.00
High dose	10	204	144	48.1	2.00	2.13	0.43	0.09	0.00
P-value		0.86		0.43		0.23		0.69	

Figure 4-1 Box Plot of *L. crispatus* Counts By Study Arm and Visit

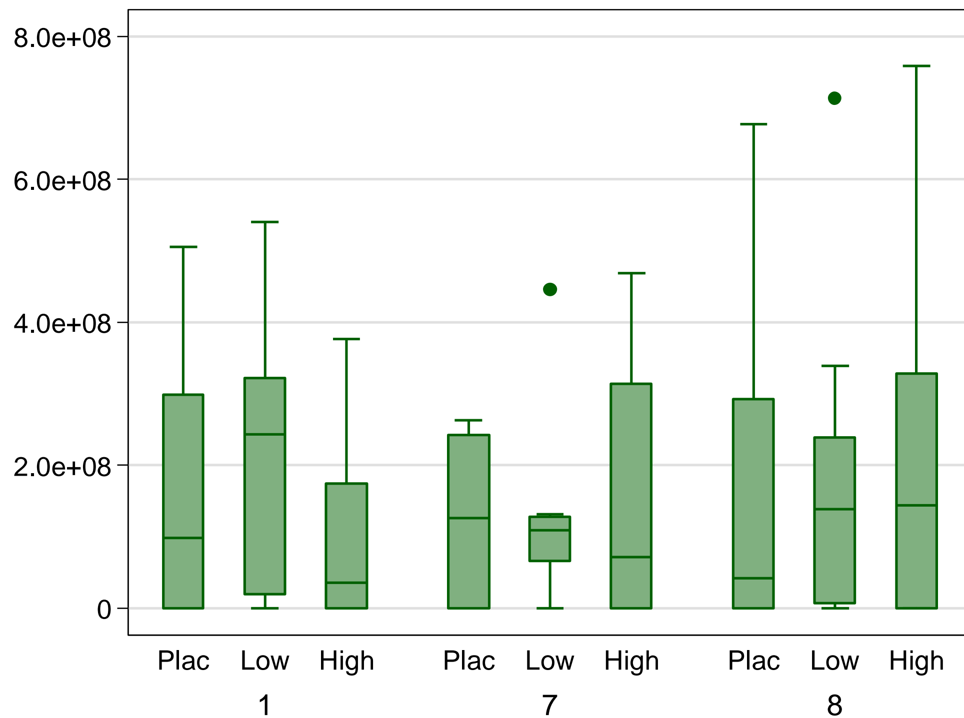


Figure 4-2 Box Plot of *L. iners* Counts By Study Arm and Visit

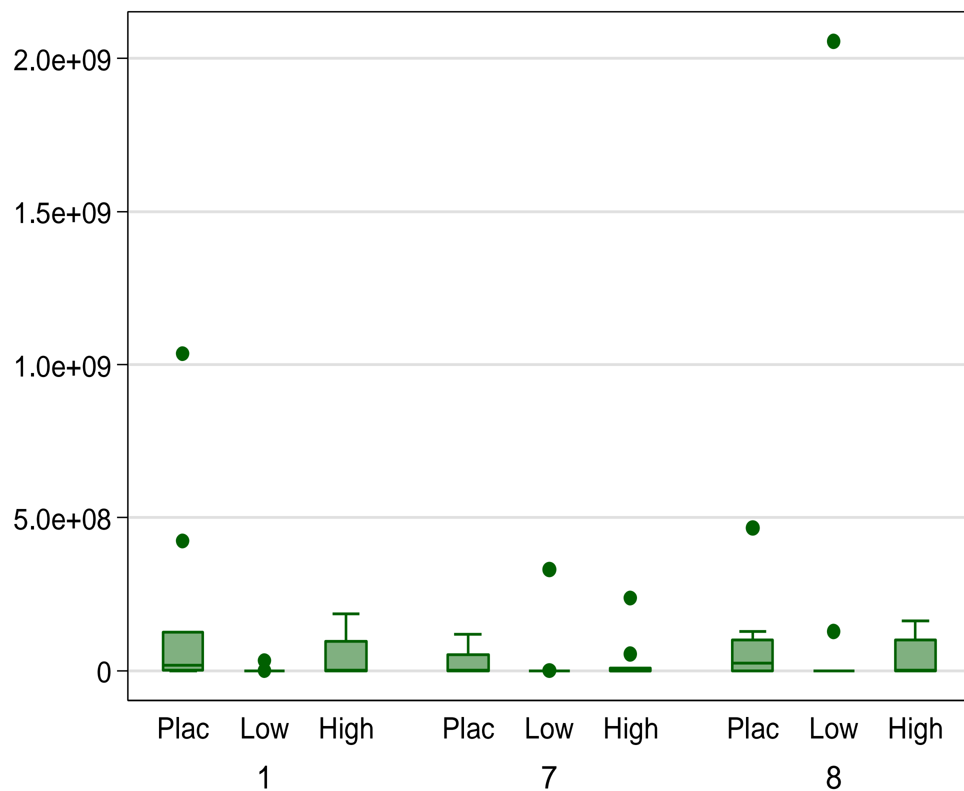


Figure 4-3 Box Plot of *L. jensenii* Counts By Study Arm and Visit

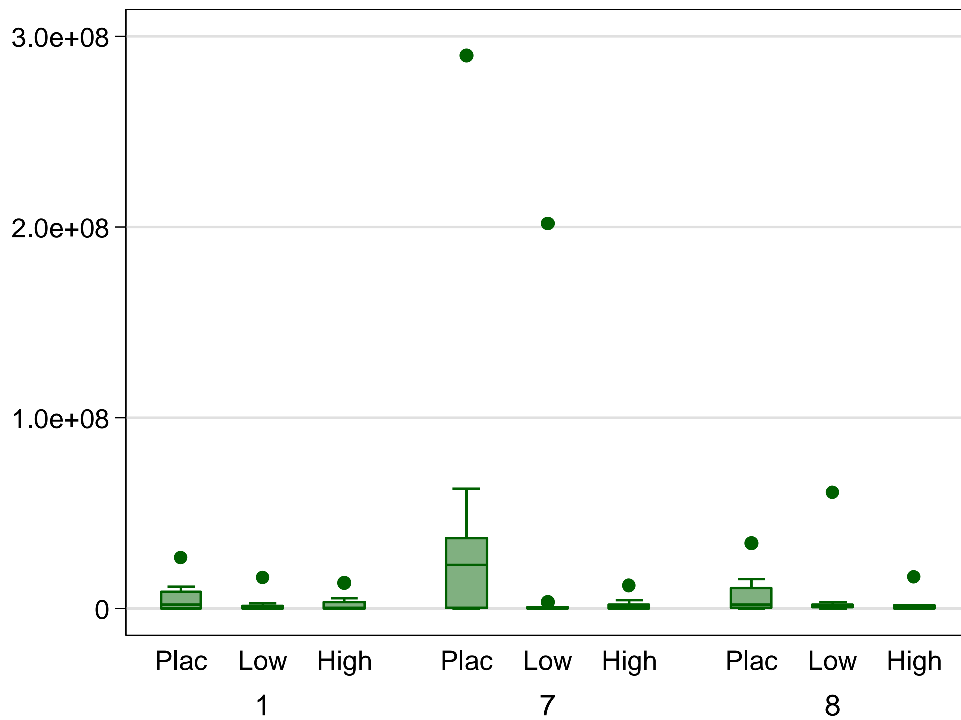
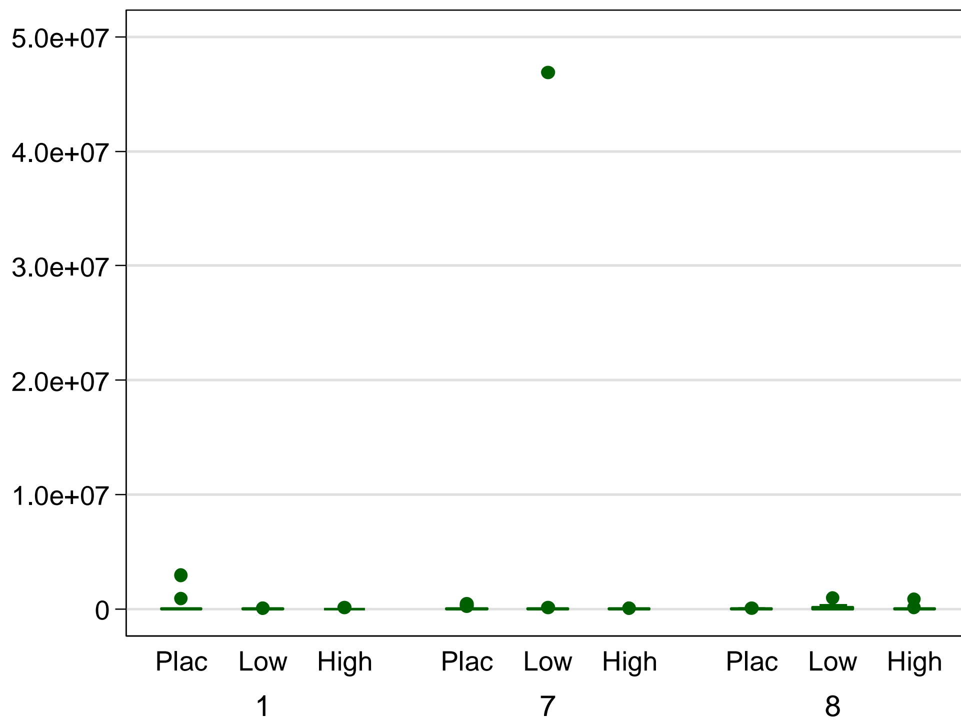


Figure 4-4 Box Plot of *L. gasseri* Counts By Study Arm and Visit



4.3.3 Discussion of Methods Used to Assess Vaginal Flora

In 1983, Amsel *et al.* proposed clinical criteria for the diagnosis of BV. The presence of three of the following four criteria is considered to be consistent with the presence of BV: vaginal pH of > 4.5, clue cells on saline wet mount, release of a fishy amine odour on addition of 10% potassium hydroxide to a drop of vaginal discharge, and a characteristic thin, homogenous vaginal discharge [728]. Of these, vaginal pH has been shown to be the most sensitive of the individual criteria, and amine odour the most specific [729]. Unfortunately, however, it is often difficult to perform a 'whiff test' in practice due to the need to apply the potassium hydroxide rapidly before the sample dries and health and safety issues preventing its use in the consultation room.

A healthy, normal vaginal pH is generally accepted as being < 4.5, however, studies have demonstrated that a pH of > 5.0 provides a more specific indicator of BV [729,730] and a cut-off of ≥ 4.7 is employed in some clinical and research settings [714,731]. Many factors, other than the absence of lactobacilli/presence of BV associated organisms, influence the pH of vaginal fluid. These include presence of other infections, in particular *Trichomonas vaginalis*, hormonal status, size of cervical ectopy and menstruation [732,733,734]. Cervical secretions have a higher pH (around 8.0) than those in the vagina thus it is imperative to take pH readings from the lateral vaginal wall [735]. Presence of alkaline seminal fluid and vaginal products, including lubricating gel, can also alter the pH of the vagina. In our trial, no participants admitted to having had vaginal intercourse during the study, and mimimal aqueous lubricant was used for speculum insertion. The study gels were buffered to pH 5.5 in their formulation hence it is possible that if some residual were present in the vagina it may increase the recorded pH. The latter is unlikely, however, given the timings of pH and vaginal flora evaluations in relation to dosing (screening, 36 hours post 12th dose, next menstrual cycle post gel use).

In 1991, Nugent *et al.* described a system for scoring Gram-stained vaginal smears to diagnose BV (see section 2.11.4.2 for methodology) [561]. In contrast to clinical methods, and the earlier Gram-stain method devised by Spiegel *et al.* the Nugent scoring system allows assessment of vaginal flora on a continuous scale, providing more information than just a positive or negative BV diagnosis. Although still commonly used to diagnose BV in

clinical practice, the reliability of the Amsel criteria is dependent on the clinical expertise of the examiner and has been shown to have reduced sensitivity and inter-observer reproducibility compared to the Nugent score [558, 736,737,738,739]. In addition, Gram-stain methods have the advantage of providing a specimen, in the form of a slide, that can be archived for subsequent verification. The Nugent score has thus become the gold standard method for diagnosing BV in research studies. However, like all Gram-stain methods, Nugent scoring is highly dependent on the quality of slide preparation and factors such as small specimen volume and poor gram-stain uptake can greatly influence interpretation. In our study, despite all specimens being taken by a single clinician and Gram-stained by experienced personnel at a single centre, there were unfortunately a few slides which could not be read due to inadequate preparation.

Alternatives to Gram-stain and clinical methods for assessing vaginal flora include both quantitative culture and PCR techniques. The former has been used to demonstrate increases in $\log_{10}$ counts of the BV associated bacteria *Gardnerella vaginalis* and *Mycoplasma hominis* and decreases in *Lactobacillus* species in women diagnosed as having BV by Nugent score [740,741]. One advantage of culture is that *Lactobacillus* colonies can be directly assessed for hydrogen peroxide production using tetramethylbenzidine agar plates [742].

The advent of molecular techniques has enabled accurate differentiation and identification of lactobacilli to the individual species level. Studies using qPCR to quantify differences in particular *Lactobacillus* species have yielded interesting and sometimes conflicting results, suggesting that the changes associated with BV are far more complicated than a simple reduction in numbers. As mentioned in section 2.4.2, *L. crispatus* and to a lesser extent, *L. jensenii* are the most commonly detected species in women with normal flora, but *L. iners* is most abundant in women with BV and *L. gasseri* in women with intermediate flora [542, 543]. Interestingly, nearly all strains of *L. crispatus* and *L. jensenii* have been reported to produce hydrogen peroxide, whereas this is true for only 9% of the strains of *L. iners* [541]. Until recently, *L. iners* was an underappreciated member of the vaginal microbiota, since it does not grow on the Rogosa agar traditionally used to isolate lactobacilli [743]. However, there is now evidence that it may be the most abundant *Lactobacillus* species overall, being isolated from 2/3 of black and Caucasion

women in one US study, and dominating the vaginal flora in 1/3 of women from a range of ethnic backgrounds [726,727].

Indeed, what is classed as a 'normal' flora has itself been called into question. Verhelst *et al.*, on the basis of Gram stain, terminal restriction fragment length polymorphism analysis of the 16S rRNA gene (T-RFLP) and molecular identification (tDNA-PCR) of cultured isolates, have suggested a new classification of the normal vaginal microflora [744]. Referring to the Ison-Hay grading system, a simplification of the Nugent scoring method used in clinical settings, whereby Gram-stained slides are classified according to the estimated proportions of various bacterial morphotypes rather than actual counts [745], they suggested that the normal, lactobacillus-predominant (grade I) microflora could be split up into four categories, designated grade Ia, Ib, Iab and I-like. Grade Ia was shown to contain predominantly *L. crispatus*, grade Ib predominantly *L. gasseri* and *L. iners*, and grade Iab a mixture of these three species. Surprisingly, grade I-like flora was shown to consist of *Bifidobacterium* species rather than *Lactobacillus* species. Grade I-like flora has been suggested to represent an unrecognized type of disturbed vaginal microflora that has previously not been distinguished from a healthy vaginal microflora [746].

Support for the notion of a limited range of background vaginal microbiota has come from recent studies which have used high-throughput molecular methods based on pyrosequencing of barcoded 16S rRNA genes. A US study of 396 assymptomatic American women identified five clusters of vaginal microflora; four of which were dominated by *Lactobacillus iners*, *L. crispatus*, *L. gasseri,* or *L. jensenii*, while the fifth had lower proportions of lactic acid bacteria and higher proportions of strict and facultative anaerobes [727]. This low-*Lactobacillus* group accounted for about 25% of the women sampled and was over-represented in Hispanic (34.3%) and black (38.9%) women as compared with Asian (17.6%) and white (9.3%) women. In addition, Zhou *et al.* have shown that microbial communities dominated by > 1 species of *Lactobacillus* are less common in black women than those of white or Japanese ethnicity, perhaps rendering them more susceptible to BV and other sexually transmitted infections, including HIV [726,747].

4.3.4 Comparison Between Clinical Assessment, Nugent Score and PCR Methodologies in Our Trial

Although the primary method for evaluating vaginal flora in our study was qPCR, clinical assessments (appearance of discharge and vaginal pH) and Gram-stained slides were also performed at the same time-points. Consistent with the PCR data, overall, there was no microscopic evidence from the Nugent scores to suggest that either dose of the Mabgel or the placebo caused a detrimental change in vaginal flora. The majority of all vaginal smears were Nugent scored as 0, i.e. there was a preponderance of lactobacillus morphotypes and no other gradable organisms. Of the 4 smears which were scored higher than 0, 2 were obtained at the screening visit, none at visit 7 and 2 at visit 8.

MAB003 (high dose Mabgel arm) had a grade 4 flora at screening (intermediate between normal and BV), with a high pH of 6.4. Clinically, there was no evidence of any heavy or malodorous discharge at this visit, thus she met eligibility criteria for study enrolment, having neither microscopic nor clinically defined BV. Unfortunately her Visit 7 slide was judged to be inadequate for interpretation. However, her flora had returned to normal at Visit 8, with a slightly high pH of 4.9. Interestingly, PCR results showed that *L. iners* dominated this participant's flora at all 3 visits and *L. gasseri* was not detected.

These data are in contrast to the findings of de Backer *et al.* but support those of others, in indicating a possible 'role' for *L. iners* as a marker of transition in the vaginal flora [542,748]. They also highlight the paucity of published data on how quickly changes in the composition of the vaginal microflora occur and how much represents 'natural' flux rather than pathology. Until recently, research assessing vaginal flora by PCR techniques has mostly taken a 'snap shot' view, evaluating the microbiome at a single moment in time. In a recent small study, fluctuations in the levels of *Lactobacillus* species and BV associated organisms were evaluated over the menstrual cycle and following antibiotic treatment in women diagnosed clinically with BV [749]. Levels of *L. crispatus* and *L. jensenii* declined with onset of menstruation, whereas those of *G. vaginalis* and *L. iners* increased. Spontaneous reversal beginning at the end of menses occurred in most individuals subsequently. In women with BV, in contrast to most BV associated bacteria, *L. iners* levels did not decline post treatment, but remained constant or increased, and

some women became re-colonised with other *Lactobacillus* species. These findings concord with observations from studies using Gram-stain methods, which detected transient changes in vaginal flora at the start of the menstrual cycle and rapid resolution of BV, within 3 days in around half of episodes, even without treatment [750,751].

MAB007 (low dose Mabgel arm) had a grade 8 (BV) flora at screening, as judged by Dr Jespers, thus would not have been eligible for the study based on this evaluation. However, on the screening slide reviewed by Dr Morris at EMU she was judged as having 2+ short lactobacillus morphotypes and 1+ gram-variable rods per high powered field and was given a score of 3. Clinically she had only a scanty, inoffensive white discharge with a pH of 4.3, thus was not judged to have BV in York. Subsequent slides at Visits 7 and 8 were both graded as showing normal flora.

This example demonstrates the fact that, although more reliable than clinical criteria, inter-observer disagreement over Nugent gradings can still exist. Indeed, in their original study, Nugent *et al*. found an inter-centre correlation of 0.82, which although better than with the previously used Spiegel criteria, indicates an element of subjectivity in slide interpretation [561]. Molecular methods, such as PCR, offer a more objective approach. PCR results for this participant showed that her vaginal flora was dominated by *L. iners,* which increased 10 fold with each subsequent visit. *L. jensenii* and *L. gasseri* were also present, increasing from being barely detectable at screening, to a peak at visit 7. *L. iners* has been noted to cause confounding on microscopy as it often stains Gram-negative and is coccobacillar in appearance in contrast to the longer, Gram-positive appearance of the classical lactobacillus morphotype [743]. This may explain the discrepant Nugent scores assigned on the screening visit smears.

MAB017 (placebo) had a grade 4 flora at visit 8. This lady had a normal vaginal pH (4.3) and no clinically apparent discharge at this visit. As with MAB003 discussed above, this lady had a pre-dominance of *L. iners* and a relative paucity of other *Lactobacillus* species in her flora.

MAB014 (low dose Mabgel) was given a Nugent score of 7 on her vaginal flora smear from Visit 8. Clinically, this lady had quite a heavy, homogenous white discharge and an elevated vaginal pH of 6.4 at this visit. A diagnostic slide was not performed, as this was

outside of the study protocol. However, the putative BV diagnosis was discussed with the patient and an offer made of evaluation and treatment at the York GUM clinic. Interestingly, unlike the other participants with abnormal Nugent scores, this lady had an absence on *L. iners* on PCR and her flora was dominated by *L. crispatus*. However, her Visit 8 sample had the lowest levels of *L. crispatus* and highest detectable *L. gasseri* levels of her 3 visits.

In our study, excluding the 2 occasions where there was no pH value recorded, 50/74 (67.5 %) of vaginal smears given a Nugent score of 0 had an accompanying pH < 4.5. Of those > 4.5, 17/24 (70.8 %) had a pH of 4.6 and 7/24 (29.2%) a pH in the range 4.9 to 5.9. Interestingly the highest pH readings (5.8, 5.9 and 4.9) were seen in MAB021, in whom neither *L. crispatus* nor *L jensenii* were detected on PCR, but in whom there were high levels of *L. iners*.

MAB008 also had high pH readings (5.2 x2 and 4.6) with an absence of a vaginal discharge consistent with BV at any visit. This participant is intriguing as slides from all 3 visits were graded as Nugent 0 (indicating that 4+ lactobacilli morphotypes were visible microscopically) but she had the lowest detectable *Lactobacilli* counts on PCR of any participant. Of note, only the non-specific *Lactobacillus* genus primers produced detectable counts suggesting that any *Lactobacilli* present were likely to have been from a species other than those detectable with the species-specific primers. The generic primers were designed based on sequences conserved between *L. Jensenii* and *L. crispatus* [560] and it is possible that they may not detect all *Lactobacillus* species that exist with equal efficiency. Another possibility is that this participant had a microflora dominated by *Bifidobacteria* or another organism that can resemble lactobacilli microscopically [744].

Although our study was not designed to directly compare methodologies for assessing the vaginal flora, it is reassuring to note that in general there was agreement between clinical, microscopic and PCR assessments and this increases the validity of our findings. However, it should be noted that since we currently only have PCR data from our study related to *Lactobacillus* species and not BV associated organisms, we only have a partial analysis of the flora by molecular methods. Results of further analyses by Dr Jespers are awaited.

As we have found, PCR is a useful and reliable tool for assessing changes in the vaginal flora in the context of microbicide safety studies and It is likely to be increasingly used to detect subtle changes and provide additional information to microscopy in other areas of research. However, it is unlikely that it will replace clinical and Gram stain methods for initial participant screening or evaluation of vaginal discharge due to cost and time needed to process samples.

5. QUALITATIVE INTERVIEW SUB-STUDY

5.1 Rationale behind the qualitative interview sub-study

The initial planning for the interview sub-study was carried out by Sarah Woodhall, non-clinical Research Fellow, HYMS with advice from Dr Yvonne Birks, Senior Lecturer, Department of Health Sciences, University of York and Dr Susan Rosenthal, Assistant Professor of Pediatrics, Columbia University, New York, USA.

The overall aim of the sub-study was to evaluate participants' experience of taking part in the MABGEL 1 trial.

As with many Phase 1 trials, MABGEL 1 participants were required to attend for several visits and undergo multiple examinations. The proposed purpose of Mabgel, however, meant that product use and examinations were of a far more intimate nature than would be experienced in many studies. Women were also required to abstain from sexual intercourse and use devices such as the gel applicator and vaginal self-sampling aspirator, which may have incurred difficulties.

Since MABGEL 1 was the first study to be conducted on the HYMS EMU, it was felt that feedback from participants, regarding their motivation for volunteering and their interactions with the research team, would be useful for optimising recruitment and retention in future clinical trials. In addition, it was considered important to obtain participants' views of the conduct of the study: to identify any procedures and/or features of the IMP that could be altered to improve acceptability.

5.2 Research Objectives

The following were identified as being the main objectives of the sub-study:

1. To determine participants' motivation for volunteering

2. To ascertain whether participants' rights and dignity were being respected whilst taking part in the clinical trial

3. To identify any areas of study conduct which could be improved in future trials

4. To determine the utility and acceptability of the self-sampling aspirator

5. To identify any features of Mabgel which should be altered as part of product development

5.3 Methodology

5.3.1 General conduct of the interviews

A semi-structured interview was carried out with each of the 28 Mabgel trial participants at their final study visit (Visit 8). In order to create a relaxed and open environment, in which the participant felt that they were able to give honest answers, it was important that the interviewer was not someone they had met previously during the trial. Because some of the questions being asked were of an intimate nature e.g. pertaining to sexual activity, application of gel, vaginal examinations, and to better enable the interviewer to establish a rapport with the participant, it was decided that the interviewer should be female. It was initially envisaged that the interviews would be conducted by Sarah Woodhall, however, she was no longer working in York by the time the study took place. The interviews were instead conducted by three female members of clinical research staff, none of whom were otherwise involved in the MABGEL 1 Trial. These were Dr Victoria Ledgar, F2 Doctor on a 4 month placement with Professor Lacey's research group, Sarah Russell-Sharpe, Senior Research Nurse and Sarah Douglas, Clinical Trials Assistant and MSc Student in Heath Sciences. Interviews took place in the consulting rooms of the EMU. Guidance and training on conducting the interviews was provided by Dr Birks.

Participants were informed of the interviews in the PIS that they were asked to read before consenting to take part in the trial. They were permitted to decline to take part in the sub-study whilst still being able to take part in the main trial if they wished. Written consent was sought and obtained for the sub-study from all participants at the screening

visit. Prior to commencing each interview, the interviewer reaffirmed verbally that the participant remained happy to consent to being interviewed and that they agreed to the interview being audio-recorded. Each participant was given a short briefing at the start of the interview explaining the general aims and nature of the questions. All interviews were digitally recorded and transcribed verbatim by a trained Medical Secretary.

Participants were reassured that their comments would be treated confidentially. All interviews were conducted and documented anonymously; no personal identifiable information was included on the recording or transcript. Instead, each interviewee was given a new interview ID independent from their subject number for the main trial. All interview transcripts have been archived in the Trial Master File (TMF).

5.3.2 Interview topic guide

A topic guide was produced by Sarah Woodhall and Susan Rosenthal to aid the interviewer in focusing the discussion on relevant areas. This was loosely based on a guide used by Dr Rosenthal in a previous study [752].

The following topic areas were listed:

1. Motivation for taking part in the trial
2. Adequacy and suitability of information provided before and during the trial
3. Experience of clinical trial visits and procedures
4. Compliance with study procedures
5. Experience of using the gel and self-sampling device

In addition, some sample questions were included. These were meant to be used as guidance only and there was no requirement to use the exact wording or order given in the guide. It was envisaged that all interviews would be different and scope remained for further issues to be raised by either the interviewer or the participant during the conversation.

For example:

Opening questions

- Where did you find out about the trial?
- How long did you take to decide whether or not you wanted to take part?
- If a friend asked you what it was like to take part in the study, what would you tell them?

Motivation for taking part in the trial

- Why did you decide to take part in the study? / What interested you in taking part in the study?

Adequacy and suitability of information provided before and during the trial

- What did you think about the information you received about the trial before you decided to take part?
    - Initial advertisement
    - Information leaflet
    - Consent form
    - Website
    - Did you read the information leaflet?- If so, was it easy to understand? Any suggested changes?
- Did the information you received at the start of the trial give you a good idea of what the trial involved?
    - What was different to expectations? Any surprises?
    - Did anything make you feel uncomfortable?
    - Anything you were unsure about?
    - Any questions at start of the trial? - if so, what were they?
    - Were your questions answered adequately at the start of the trial?

Experience of clinical trial visits, procedures and use of self-sampling device

- What was the hardest part of the study for you?
    - Anything you disliked?
    - Any concerns about taking part?
- What was your experience of attending for the trial visits?
    - Ease of fitting in round own life
    - Samples and investigations
    - Blood tests, vaginal examination, general examinations
    - Use of self-sampling device
        - Easy or hard to use?
        - Would you prefer to take your own samples or have a doctor/nurse do it?

Compliance with study procedures

- Did you find it easy or hard to follow the trial procedures (self-administering the study gel, remaining sexually abstinent)?
    - Did you use all doses of the study gel?
        - When did you use the gel?
        - What was it like to use?
        - Any problems using it?
    - Understanding of what was required during the trial
        - When to use gel
        - When to come in for visits
        - When allowed to have sex
        - Completing the diary card
- Did you remain sexually abstinent during the dosing period of the study?
    - Was this easy or hard to do?
    - Did you inform the study nurse?

Experience of using the gel

- What was your experience of using the gel?
    - Feel
    - Leakage
    - Appearance
    - Ease of use

5.3.3 Data Analysis

Transcripts were analysed by the author and Sarah Douglas with advice and guidance from Dr Yvonne Birks and Dr Catherine Montgomery, Postdoctoral Research Fellow, Department of Sociology, University of York. Data were analysed using a 'framework' approach similar to that described by Ritchie and Spencer [753].

Transcripts were read several times and initial themes identified by consensus. Unsurprisingly, given the semi-structured nature of the interviews, these broadly matched the topic areas suggested in the topic guide. Further themes and sub-themes emerged as transcripts were coded and coding schemes were refined as the process developed. Analysis was carried out manually as there had been no plans to use a software package e.g. NVivo, and access to such programmes was unavailable in York Hospital or the Monkgate Clinic, which was where the research team were based.

The purpose of the analysis was to try to identify recurring concepts to develop and explain hypotheses but efforts were made to ensure the diversity of view-points were represented, especially any that were contradictory.

Since the author had been involved in the co-ordination and clinical aspects of the trial, there was the potential for this to have had an influence on the interpretation of the data, particularly with regards to comments relating to study conduct. For this reason, the results and conclusions generated were reviewed by Dr Montgomery, together with the transcripts, to verify that they accurately reflected the content and breadth of the data.

Quotes from the original transcribed interviews have been used to try to support the development of theories and findings. Quotation marks have been used to indicate when a participant has been quoted directly otherwise the author has used reported speech, aiming to keep as close to the original speech as possible.

5.4 Results

Interviews were completed and recorded for all 28 participants. Interviews ranged in duration from 10 minutes to 20 minutes 20 seconds. The mean duration was 13 minutes 27 seconds.

5.4.1 Trial Recruitment

5.4.1.1 Awareness of the existence of the study

All participants were asked how they first found out about the trial. 13 of the 28 participants (46%) reported that they became aware of the trial through word of mouth. Interviewee 20 said that she knew a member of staff from the EMU and Interviewee 26 confirmed that her friend had already participated in the trial and had told her all about it. 2 of the 28 participants stated that they found out about the trial through York Hospital's intranet site therefore indicating that they were themselves NHS staff. 4 participants said that there was information about it on the University of York's Intranet, implying that they worked or studied there. 4 of the participants mentioned Local Link magazine which is a free magazine that is delivered around the York area and an additional participant referred to a local magazine but did not provide the name of it. This again may have been the Local Link magazine, since this was the only magazine in which adverts were placed. 1 woman said that there was an advert in her local newspaper (the York Press) and the remaining 2 participants had seen posters displayed at York Hospital that were advertising the trial.

5.4.1.2 Motivation for taking part in the study

Participants were asked what it was that interested them in the trial in the first place and made them want to take part. 3 of the 28 participants stated that their sole motivation

for taking part in the trial was the financial remuneration. A further 15 participants said that the money had played a part but in addition they had other reasons for wanting to take part. 20 of the participants saw value in the research being undertaken either because they were particularly interested in HIV or because they had an interest in research in general. Interviewee 10 commented that she was interested in the concept of using a gel as a preventative measure against HIV. Interviewee 16 expressed a desire to help others as she said that she was a nurse herself. In addition to the financial motivation and being able to see value in the research 3 participants highlighted a different reason for wanting to be a part of the trial. Interviewee 13 reported that it was the opportunity of having a *'full sexually transmitted infection check-up'* that appealed to her. Interviewee 26 also stated that she thought that through the screening tests, blood tests and examinations that would be performed she would also be getting something out of the trial. A unique comment was made by Interviewee 19, whose interest in the trial was partly due to the time commitment of it. She told the interviewer that her hours had been cut at work and her son was at school so she saw the trial as something to do part-time to fill her day.

5.4.2 Discussing the trial with others

It was clear from some participants' responses as to how they had become aware of the study that some of the women who were already enrolled in the trial had talked about it to their friends, family or work colleagues and this had sparked further interest in the trial. All participants who were asked if their decision to take part in the trial had been influenced by any additional factors such as friends or family stated that the decision was ultimately their own, however a number referred to discussions they had had with others which had played a role in their decision making.

Interviewee 10 and Interviewee 26 had friends who were already enrolled on the trial, therefore their friends' experiences had partly influenced them. Interviewee 10 said that her friend '*was quite positive about it (the trial)'* and commented that '*if she had said anything particularly negative about it, it probably would have put me off but she didn't have anything bad to say'*.

5 participants mentioned that they had discussed the trial with their partners first before signing up. Interviewee 13 said that this was due to the need for sexual abstinence.

One woman (Interviewee 9) expressed an initially negative or apprehensive view of discussing her participation with others. *'I kept it quite quiet because I didn't know how people would react'.* However, in her experience, this was unfounded: *'after I'd signed up and I was accepted then I started to tell people and nobody was negative or worried about it so everyone was quite like "Tell me a bit more about it" '*.

Several participants found it awkward discussing the trial in view of the 'intimate' nature of the product and procedures. Interviewee 6 told the interviewer that there was one occasion when her partner had had some friends round and she did not want to explain to them about the trial and so found it a bit awkward *'disappearing'* to use the gel. Interviewee 26 found it slightly uncomfortable telling her friends what the gel was when she was on holiday with them. Similarly, Interviewee 25 had to explain what the gels were to her housemate as she lives in a shared house and had to store them in a communal fridge.

The question *'If a friend asked you what it was like to take part in the study what would you tell them?'* was also posed. All of the responses to this question were predominantly positive: *'Everyone was going out of their way to help you'* (Interviewee 3); Interviewee 19 described the trial as *'fantastic'.* Interviewee 11 however advised that the trial probably would not be suitable for women who hate having smear tests performed. Similarly, Interviewee 12 said that she thought that the trial would be better suited to women in their 30s or 40s who are used to smear tests or who have already had children as they would find the examinations and procedures less embarrassing. 9 out of the 28 participants reported that either they would recommend the trial to their friends, family or colleagues or that they had already told others about it.

5.4.3 Adequacy and suitability of information provided before and during the trial

The question *'What did you think about the information that you received before you started the trial?'* was posed to participants. The discussions around this topic chiefly

focused on the written information provided in the PIS which all participants read before consenting to be involved in the MABGEL1 trial. The majority of participants were very positive about the information they had read. Interviewee 1 responded with *'Everything that I wanted to know was there'*. Other participants described the information as *'thorough'* (Interviewee 4, Interviewee 2). Interviewee 20 said that she was a nurse but thought that the information could be understood by anyone. Similarly, Interviewee 15 described the information as being written in *'lay terms'* and *'not too medical'*. Interviewee 18 mentioned that she found the chart of what happened and when during the trial particularly useful. Interviewee 10 liked that there was so much information and said *'You can really get a picture before having to speak to anyone'*. She thought this took away the feeling of being under pressure to take part in the trial just because one of the team had taken so much time to explain all about it to you. In contrast Interviewee 2 was unsure if potential participants would read all the information due to the sheer volume of it and did comment that she did not understand some of the technical terms used in the PIS such as *'colposcopy'* and thought a bit more explanation of such terms was needed in the literature. Interviewee 16 commented that the information was *'initially quite boggling'* but then said that she found it easier to understand once a member of the research team had talked her through it. Similarly, Interviewee 11 stated that the verbal information that had been disseminated from the team and her friend made the written information easier to digest. Interviewee 17 had a more negative evaluation of the information and said that she found it *'slightly difficult to understand'*.

During the discussion the participants were asked if they remembered having any questions or concerns about the trial after having read all the information that was available to them. 4 of the participants recalled expressing concern over the possible side effects associated with using the gel, for example, Interviewee 3 '*did not remember the issue of fertility being raised at any point and if involvement in the trial could affect it in any way*.' 2 participants had questions relating to the availability of parking at the hospital and the costs of this. Other concerns raised were participants who were anxious about being able to fit in the appointments around their working life. Interviewee 28 said that she would have liked to have known in advance how long each visit would last. Interviewee 13 was worried that the trial might be '*too invasive'*.

An additional question posed was *'Did you (the participant) feel that anything unexpected happened in the trial that had not been mentioned in the PIS?* or *'Could you suggest anything which might have been added or changed to improve the PIS? '*. Interviewee 11 suggested that advising participants to ensure they had had something to eat or drink before coming in for an early morning appointment would make giving blood easier. 2 participants said that they had not been aware that they would have to take some of their own samples during the trial. Interviewee 23 reported that she had not expected the gel to be so cold and said that it had not been suggested to her that she take the gel out of the fridge for a short while first to allow it to get up to room temperature.

In contrast, Interviewee 19 said *'When I'd actually been and done it it's a lot easier than it looks on paper'*. Similarly, Interviewee 20 said there were fewer visits than she first thought. She had expected that she would have had to come in for visits everyday.

5.4.4 Experience of Clinical Trial Visits and Procedures

When participants were asked *'what was the hardest part of the trial for you?'* the responses were quite varied. Several of the participants identified more than one specific aspect of the trial as being difficult. Most of the difficulties surrounded genital examinations, sampling, and fitting study visits and gel use into their normal routine.

Interviewee 1 recalled being daunted at first by the prospect of a speculum being used during the vaginal examinations but she stated that it became easier after the first time. She was also apprehensive about a camera being used to take photographs during these examinations but she did say that she was asked if she wanted to see the screen or not. 2 additional participants also felt uncomfortable about the camera being used. 4 participants identified the self-sampling procedure as being the most challenging part of the trial. 3 participants found it difficult fitting trial visits in around either their family or working life. Remembering to put the gel in everyday at roughly the same time was described as being problematic for 3 of the participants. Interviewee 17 said that using the gel in general was the hardest part for her as she was conscious of the gel inside her all the time she was using it and had to wear knickers to bed which she would not normally do. A couple of the participants were uneasy about having blood tests, finding

them uncomfortable and having concerns regarding the volume of blood taken. 1 participant stated that she had not found anything difficult during her time in the trial.

Some of these aspects are explored further below.

5.4.4.1 Ease of Attending Study Visits

Participants were asked specifically to comment on how easily they fitted trial visits in around their normal life. None of the women reported any major problems with regards to this. However, Interviewee 2 said that it was a struggle when there were 3 visits that were very close together. Similarly, Interviewee 16 described her first day of dosing as being *'quite intense'*. She scheduled this specifically for her day off but said she was doubtful that she would have managed to fit in both of the visits if she had been at work. Interviewee 12 had also scheduled the first day of dosing to occur on her day off. Interviewee 5 commented that she did have to change her routine a bit while she was involved in the trial. 4 of the participants worked flexi-time and reported that they found it very easy to attend for the visits. There were 9 references made to the flexibility of the research team which participants stated made it easier for them to fit in the visits and to not have to change their routines too much. Interviewee 1 suggested that it would be good if the research team could offer early evening appointments for those people that finish work at around 4 or 5pm. Interviewee 25 said that she did not get the impression that there were appointments available outside of normal 9am-5pm office hours but she commented that there were no issues for her coming in for appointments within that time frame.

5.4.4.2 Experience of undergoing intimate examinations

Only one of the participants recalled any really negative experiences relating to the intimate examinations which were performed as part of the trial. Interviewee 17 said that the research staff had presumed that because she had already taken part in a similar clinical trial that she would be familiar with all the procedures that would be performed. She commented that *'I prefer being more looked after'*, suggesting that she believed her quality of care could have been higher. In contrast, Interviewee 9 reported that *'the doctor and nurses always talked through all of the procedures with her before they did*

them'. Interviewee 24 commented that the examinations were *'sometimes quite uncomfortable' but* highlighted that the research staff had made her feel as comfortable as possible. Several further references were made to the quality of care provided by the research staff. Interviewee 1 said that the staff were always very considerate during examinations and procedures and Interviewee 9 stated that the staff always respected her privacy. Interviewee 5 had never had an examination using a speculum before and found the procedure uncomfortable at first but then got used to it. Interviewee 8 commented that she was *'well used to intimate examinations'* as she had had 4 children and therefore had no issues with the examinations being performed. As previously stated, 3 of the participants were slightly uncomfortable with photographs being taken during some of the internal examinations however Interviewee 26 said that she was actually really interested in seeing the images that were taken.

5.4.4.3 Experience of using the self-sampling vaginal aspirator

Participants' experiences of the self-sampling device were also discussed specifically during the interview. 8 of the participants described the procedure as either *'easy'* or *'fine'*. Interviewee 2 said that she found the device simple to use but stated that by doing the procedure herself she was unsure if she was collecting an adequate sample. A further 3 participants reported that they had doubts regarding whether they had managed to collect a good enough sample or if they were performing the procedure correctly. Interviewee 8 said that the device became easier to use once she had had a couple of goes at using it. Similarly, Interviewee 13 stated that she found the procedure *'strange'* the first time but then she felt comfortable with using the device after that. 2 of the participants compared the self-sampling device to like using a tampon. Interviewee 9 said that the device was easy to use but slightly uncomfortable. A further 7 participants reported that they had experienced some kind of discomfort during the procedure, most commonly the device catching on the vaginal wall. As stated previously, 4 of the participants identified this procedure as being the most challenging aspect of the trial for them. The most negative experience of self-sampling seems to have been for Interviewee 27 who described the procedure as *'horrible'* and she stated *'I would never want to do that again'.*

Following on from the discussion about the self-sampling device participants were then asked which sampling method they preferred: having their samples taken by a clinician or taking the samples themselves. 10 out of the 28 participants stated that they preferred taking the samples themselves. Some of the reasons given were: '*it was less intrusive'* (Interviewee 23); *'it did not involve using a speculum'* (Interviewee 2); '*it was less embarrassing*' (Interviewee 25). Interviewee 3 said that by doing the samples herself she felt more involved in the trial and it gave her a sense of being in control. 11 of the participants reported that they preferred having their genital samples taken by the trial's doctor. Of those, Interviewee 9 and Interviewee 1 said that it made more sense for the clinician to take the samples since they were already performing other examinations. A further 3 said that they found it less painful when the clinician took samples. One interviewee stated '*I did 2 self samples and the first one it was quite painful. The second one wasn't painful but because the first one had been I was really dubious when I was doing it. That first experience of it just put me off really'.*

6 participants commented that the doctor was more knowledgeable about the procedure and could see more clearly what they were doing and would therefore obtain better samples. Interviewee 22 expressed a strong preference for the doctor taking samples so that she would not have to see what was happening herself. When she was able to see she stated *'I felt quite disgusted really'*. 2 of the participants had no preference for either of the 2 sampling methods however Interviewee 26 reported that if it meant that she didn't have to come in for examinations then being able to take the samples herself would be nicer.

5.4.4.4 Experience of venepuncture

When the subject of blood tests arose, 11 of the participants shared some negative experiences with the interviewer. 6 of those 11 reported that they had had to go to Phlebotomy at least once during the trial as the research team were unable to obtain a blood sample. Interviewee 5 experienced some bruising after having given blood and stated that the first time was quite painful. Interviewee 13 also reported that she had bruised once. However, as previously stated only 2 participants identified the blood tests as being the most difficult part of the trial for them. None of the participants suggested

that their venepuncture problems were in any way caused by the research staff and seemed to view their difficulties as just being unfortunate or even expected.

5.4.5 Compliance with study procedures

5.4.5.1 Compliance with gel usage

Participants were asked if they had successfully managed to use all of the doses of the study gel. Interviewee 12 reported that she had missed one dose as she had been unwell. Interviewee 15 also said that she had missed one dose. She explained that on one occasion she had returned home after midnight and she thought that she should not use the gel because she said that the team had been quite specific about the timings of when to use the gel and it was after that time frame. Interviewee 23 stated that she had one gel left at the end of the trial but was unsure why this was the case as she claimed she was certain that she had used one every day. Interviewee 8 commented that if the gel had had to be used at a specific time each day then she would have struggled complying with the trial but because there was some flexibility she managed to successfully complete the dosing schedule.

No participants reported having spilt any of the gel. However, 3 women commented that they had found transferring the gel from the syringe to the applicator “a bit fiddly” and that transfer would have been made easier if the fit between the end of the syringe and the applicator had been tighter.

5.4.5.2 Completion of the diary card

The diary card that participants had to complete during the dosing period of the study was also discussed during the interview. The majority of participants reported that the diary card was very easy to fill in. Interviewee 5 and Interviewee 15 both said that they found it a useful way of keeping track that they had actually used the gels. Interviewee 3 suggested that a column could be added so that participants could list any problems or side effects that they had experienced as otherwise she said it can be sometimes difficult to remember specific things that have happened. Interviewee 2 thought that the importance of the diary card could have been emphasised more as she thought she recalled being told that it was not an important part of the trial at the start. 2 of the

participants stated that they were unsure if they had to fill in the first box on the diary card themselves or if the research staff should have completed this as their first dosage was at York Hospital.

5.4.5.3 Sexual abstinence

The issue of remaining abstinent during the dosing period of the trial was also raised. No participant stated that they had had oral or vaginal sex during the dosing period of the study. 24 of the participants reported that they had not had any problems with remaining abstinent. Interviewee 12 commented that she found it easy to remain abstinent as she had been married for a while. Interviewee 28 stated that she did not have a boyfriend so had no problems with the abstinence side of the trial. Interviewee 1 however commented that it may have become an issue if the period she had had to remain abstinent was any longer. Interviewee 10 also was not in a relationship but reported that had she been in one then not being able to have sex maybe would have been an issue. Interviewee 13 said the abstinence was not challenging for her as she was in a relationship and had talked it through with her boyfriend but she imagined that remaining abstinent may have been a problem for women who were not in a serious relationship, especially if they went out and got drunk.

The remaining 4 participants did find the abstinence side of the trial quite difficult. Interviewee 2 reported that she found it '*a bit frustrating towards the end of the trial'*. Interviewee 20 described it as *'tough'*. Interviewee 17 told the interviewer that she met up with a former partner during the dosing period of the trial and was unsure whether she could have sex or not. She stated that she did not have intercourse with him but that her reasons for not doing so were not due to the trial and that being in the trial probably would not have stopped her from having sex had she wanted to.

5.4.6 Experience of using the gel

Women were asked to comment on the appearance and consistency of the gel. None of the participants expressed any strong feelings on this subject. Interviewee 9 stated *'It was a clear gel; it was absolutely fine. It was easy to use; it wasn't sticky or problematic'.* Interviewee 3 stated *'it didn't look like anything particularly scary'.*

Participants shared their experiences of using the gel and were specifically asked if they had had any problems with leakage. 4 participants reported that they had not experienced any leakage when they used the gel. 2 of those 4 stated that they had expected there to be some leakage and were surprised when there was not any. 5 of the participants mentioned that they had used either sanitary pads or panty liners whilst using the gel, and 2 that they wore knickers at night when they wouldn't normally. Interviewee 24 said that she felt uncomfortable at having to wear panty liners as she would not usually use them. Interviewee 20 had not experienced any leakage whilst using the gel but reported some bleeding between doses and therefore had had to wear a sanitary pad as the trial rules stated that she could not use tampons. She commented that she did not like having to use a pad. Interviewee 8 told the interviewer that she had been given a pad to wear on her first visit but that she had not needed to wear one after that. 3 participants compared the sensation of using the gel to being like *'as if you'd just come on your period'* (Interviewee 1, Interviewee 5, Interviewee 18). 5 of the participants mentioned that the gel leaked out when they were standing up but that they had experienced no leakage when they were lying down. Interviewee 16 was one of those participants and commented that '*I think sometimes it depends where you put it as to how long it stays there'*. Interviewee 9 said that one night she had used the gel before going to the pub and had felt like the gel was trickling down her leg whilst she was there. Interviewee 5 and Interviewee 17 reported some itchiness whilst using the gel. Interviewee 23 told the interviewer that a couple of times during the dosing period she had had a pale pink discharge but she said that she had been assured by the research staff that this was not harmful. Interviewee 27 reported that she found the gel a bit cold when she used it but in contrast Interviewee 13 said that she experienced a warming sensation after applying it. Interviewee 4 stated that she had to take the gel out of the fridge an hour before using it otherwise it was too cold to use.

5.4.7 Closing comments and suggestions to improve future trials

At the end of the interview all participants were asked if there were any other points which they would like to raise about taking part in the trial. At this point 8 of the participants were complimentary towards the research staff. Interviewee 3 said that the research team were a really nice group of people who helped her to relax. Interviewee 1

stated that she was made to feel welcome by the staff. Interviewee 22 described the staff as *'very respectable'* and *'very considerate'* and she commented that *'they put my mind at rest'*. Interviewee 27 liked that she saw the same nurses and doctor at her visits and was able to build a rapport with them. Interviewee 8 mentioned that the staff always made sure that she knew exactly what was happening at each stage of the trial. Interviewee 10 commented that the trial was not the easiest of things to do as it was *'quite invasive*' but she said that once she was used to the procedures and examinations it became more tolerable. Interviewee 4 described the first few days of the visits as *'intense'* as there were lots of examinations and blood tests and she mentioned that she had developed some bruising as a result.

A few suggestions about what could be done to improve future trials were proposed. Interviewee 3 suggested that a radio or television playing in the background would help participants to relax more and suggested that gowns (rather than paper sheets) could be provided to participants so that they would feel less exposed during the examinations. Interviewee 18 reported that she did not think that any changes needed to be made to the trial except for the self-sampling part. Interviewee 20 suggested that panty liners could be provided to the participants as she said *'I got through them like nobody's business'*. Interviewee 10 suggested that 'some sort of pre-filled (application) device or better fitting ends between the syringe and applicator would have made it easier to apply the gel'. Interviewee 14 was unsure about why she had to keep the syringes from the trial and was asked to bring them back. She would have liked this to have been explained to her.

5.4.8 Discussion and conclusions

Overall, participants gave a very positive account of their experience of taking part in the MABGEL 1 trial. Women were generally satisfied with the information they had received before they decided to take part, and most found it informative and easy to understand. The participants were complimentary towards the research team, reporting that staff were friendly and helped put them at ease.

Although the intimate nature of the examinations and the photographic images were uncomfortable for a minority of women, overall, these were well-tolerated. The self-

sampling procedure was identified by 12 participants as being problematic, however, partly because some of the women found it painful to use but also because others worried that they were not obtaining an adequate sample. However, 10 participants said that if given the choice between taking samples themselves or having the clinician taking them they would prefer to take their own samples.

Fitting in study visits and adhering to study procedures/ requirements did not appear to pose any difficulties for the majority of participants. Being single or in a long-standing relationship and discussing the study with partners initially, before deciding to enrol, were identified as being helpful in facilitating sexual abstinence.

There was some suggestion that the gel consistency was too runny, with 5 participants reporting that they had experienced the gel leaking out when they were standing up, and 7 women stating that they wore pads, panty-liners or underwear to avoid the gel leaking onto sheets or clothing. However, it is possible that the requirement for sexual abstinence contributed to any leakage through not enabling the product to be spread throughout the vagina as it would if used prior to penile intercourse [594]. If the product were to be developed further then attention would need to be given to ensuring that the gel was the optimum consistency to maximise delivery and retention of the mAbs and that any leakage was acceptable to potential users.

Trying to ensure that trial participants have as good an experience as possible is clearly very important. Not only should clinicians act in the best interests of individuals already enrolled in a study, but they should also have a view to future recruitment. One of the clearest findings from the MABGEL 1 interviews is the importance of word of mouth. Some of the women who were already taking part in the trial talked about it to their friends, family and work colleagues and this led to further recruitment. Indeed, almost half of all MABGEL 1 participants found out about the trial this way.

Although not enquired about specifically, none of the participants reported experiencing any social stigma or undue resistance from friends or partners towards their taking part in the study. Only one participant reported feeling apprehensive about discussing the study with others through fear of a negative response, although several expressed awkwardness due to the intimate nature of the product. Importantly, 3 of the participants

explicitly stated that they would be keen to take part in another clinical trial if a similar study was recruiting in the future.

Our study has several limitations. Firstly, the sample was restricted to women enrolled in a Phase 1, pharmacokinetic and safety study who were all considered to be at low risk of acquiring HIV infection. In addition, the participants were of majority white British ethnicity (28/29). In these respects, they were a different population from that which would be likely to use a microbicide if one became available. The setting of product use was also artificial, with participants being required to abstain from sexual intercourse. Although there has been debate about the appropriateness of incorporating research surrounding acceptability into clinical trials in which participants or settings are unlikely to be representative of 'real life' use [754], participants in Phase 1 clinical trials are the first to experience daily use of a product and can offer important early insights into its consistency, feel and smell [755]. In addition, as we have found, integration of qualitative and quantitative approaches can facilitate fuller evaluation and interpretation of safety and pharmacokinetic data. In our study, participants' opinions and experience with regards to using the gel were only a small part of what we wished to ascertain. Much of the interview focused on areas of specific relevance to the conduct of clinical trials, both in helping to assess the utility and acceptability of self-taken sampling and in determining the effectiveness of particular recruitment strategies and study information. Overall, the aim was to gauge an over-view of participants' experience in taking part in the trial and identify any areas which should be modified or improved. Since the MABGEL 1 trial was the first study to take place on a newly opened research facility, such information will be invaluable in the planning and conduct of future studies.

6. SUMMARY, OVERALL CONCLUSIONS, AND FUTURE DIRECTIONS

6.1 Summary

As described in chapter 1, significant advances have recently been made in the development and evaluation of biomedical HIV prevention strategies. Although there is now clear evidence that treating those already infected with HIV prevents onward transmission, particularly in motivated, stable sero-discordant partnerships [310], additional interventions are still required for use by HIV negative individuals. Young women are especially vulnerable to acquiring HIV infection and may lack control over their partners' behaviour [4,16]. Thus, interventions that women can use themselves, such as oral or topical PrEP, would be potentially beneficial.

However, implementing current ARV-based prevention approaches will be a challenge, even in richer nations, especially in view of recent reductions in global funding for HIV treatment for individuals' health [756,336,339]. In addition, over-reliance on the same classes of agent for both prevention and treatment is not ideal, particularly with the emergence of increasing ARV resistance in low income countries [454,455]. Use of protective, non-ARV based anti-HIV agents may therefore be advantageous.

One potential strategy is the topical use of anti-HIV-1 mAbs. 2F5, 4E10 and 2G12 were among the 1st generation of broadly neutralizing mAbs to be identified in the sera of HIV positive individuals, and have been shown both to have potential to prevent HIV-1 transmission in NHP SHIV challenge studies [486,487,488,489,490,491,492,494, 495,496,497,498,499] and be safe when given IV to humans [501,502,503,504,505,506]. They were therefore considered to be suitable candidates for a prototype, combination mAb-based microbicide.

MABGEL 1 was a Phase 1 clinical trial involving 28 healthy, female volunteers. It was both the first study of intravaginally administered mAbs in humans and the first to be

conducted in the HYMS EMU. It was designed, primarily, to assess the pharmacokinetics of C2F5, C2G12, and C4E10 (produced in CHO-cells) when applied to the vagina in a HEC-based gel (Mabgel). In addition, data was obtained on a number of secondary outcomes: local and systemic toxicity of the Mabgel, including effects on the vaginal flora; utility of using Weck-Cels and a self taken vaginal aspirator for sampling genital secretions; and participants' experiences of taking part in the trial. Study conduct and results have been presented and discussed in Chapters 2 to 5.

This final chapter will evaluate the potential viability of the concept of using combination HIV-1 NAbs as a global microbicide, discuss the contribution our research has made to the microbicide development field and propose ideas for future investigation.

6.2 Mabgel: a feasible microbicide for the 'real-world'?

To be successful at preventing HIV transmission in the 'real-world' a microbicide formulation would need to be stable, both at high ambient temperatures and in the presence of male and female genital secretions, safe, efficacious, acceptable and cheap to produce [388,389]. At present, whether a combination NAb-based product could fulfil all of these criteria is yet to be determined. Studies of C2F5, C4E10, C2G12, and the existing Mabgel, have provided important early insights, including identification of several pitfalls. However, as will be discussed in sections 6.2.4 and 6.2.6, some current problems may potentially be overcome through the use of novel NAbs and technologies.

6.2.1 Stability

At time of writing, stability of the mAbs in product buffer has now been established for over 2 years at 37 °C and 1 year at 45°C, with no evidence of significant mAb degradation (B Vcelar, personal communication). Thus, it would appear that the Mabgel would be suitable for use in regions with high ambient temperatures, with no reliable access to refrigeration. However, as discussed in 3.8.9.6, there is evidence to suggest that the atypical structure of C2G12 may render it susceptible to rapid enzymatic and/or acid induced cleavage on exposure to cervico-vaginal secretions, accounting, at least in part, for its more rapid elimination after vaginal application. Although generally possessing

more typical Ig conformations, based on these findings, we would recommend all future NAbs be evaluated in the presence of both female genital secretions and semen prior to animal or clinical studies.

6.2.2 Safety

As reported in chapter 4, there was little to indicate from our study that the mAbs or gel vehicle were toxic to the cervico-vaginal mucosa or disturbed the normal bacterial flora. That said, there is now a general consensus that the relatively crude and subjective assessments undertaken in early phase microbicide safety trials to date are inadequate at predicting potential enhancement of HIV-1 transmission, and that most studies, involving 2 weeks or less of product exposure, are too short to elucidate toxicities that develop slowly or with cumulative exposure [418]. Indeed, as Poynten *et al.* point out, we have reached a stage at which HIV-1 incidence in Phase III trials has become the ultimate safety end-point as well as the only end-point that can be used to assess effectiveness of potential microbicide. Recommendations have been made for improvements to safety evaluations (at both preclinical and clinical stages) to facillitate the earlier detection of more subtle pro-inflammatory effects [392]. Suggestions include the use of new imaging techniques, e.g. optical coherence tomography [757,758,759], the measurement of cytokines and innate antimicrobial peptides in addition to perturbations of vaginal flora [760] and performance of biopsies (vaginal, cervical +/- endometrial (with lavage)) pre and post microbicide exposure [761,762]. However, the complexities of the female genital mucosal environment, including the effects of hormones, infections and semen, and variation in sampling methods between studies, have thus far impeded attempts at identifying any specific biomarkers predictive of clinically relevant toxicity [99,265,763].

Although the data we have obtained to date regarding the safety of Mabgel are reassuring, it is clear that if the product were to be taken into further development, additional safety assessments would be needed in line with current recommendations [391,392]. These would include evaluation of cytokine generation, both in explants and in animal studies, teratogenicity assessments in rodents and extended safety studies in women, incorporating mucosal biopsies and measurement of antimicrobial and

inflammatory mediators. Safety trials involving sexually active and HIV positive populations and male penile tolerance studies should also be conducted [764].

6.2.3 Effectiveness

Assuming that a microbicide is safe, to be efficacious it would need to deliver: 1) an agent capable of protecting against all circulating HIV-1 strains; 2) to the right anatomical site; 3) in a high enough concentration; and 4) for an adequate duration to mediate preventative effects [765]. In deciding whether to continue to develop the Mabgel in its current form, its potential to achieve these should be considered.

6.2.3.1 Breadth of protection

As mentioned in chapter 1, the mAbs differ in the breadth of their *in-vitro* neutralization capabilities, with 4E10 mediating cross-clade neutralization of virtually all isolates tested to date, with more limited coverage by 2F5 and, in particular, 2G12 [484,485,486,766]. The neutralization breadth mediated by 4E10 is highly unusual and has not been equalled by any other HIV-1 NAb discovered to date (see section 6.2.6). The 4E10 MPER epitope appears to be highly conserved whereas glycan residues recognised by 2G12 are often absent, particularly from viruses belonging to Clades A, C and CRF01 AE [484]. Unfortunately, Clade C viruses are also neutralized poorly by 2F5 [484,766]. Given that Clade C isolates account for > 50 % of HIV-1 infections world-wide and are the most prevalent viruses in Southern Africa, India and China [24], the fact that they are neutralized well by only one of the mAbs in the current microbicide is not ideal and may compromise its global effectiveness.

6.2.3.2 Site and mechanism of action

Even if theoretically capable of neutralizing the HIV strains present in the donor inoculum (i.e. they can prevent infection of target cells, such as TZM-bl cells *in-vitro*) the success of the mAbs *in-vivo* will depend on complex pharmacodynamic interactions which are more difficult to evaluate.

To inhibit the establishment of HIV infection in the FGT, an anti-HIV agent must either prevent active virions from crossing the epithelium, or interfere with their entry into, replication or early spread within target cells. The exact site(s) and speed of onset of activity of the agent depend on its mode of action. Those which inactivate virions or interfere non-specifically with binding to cells, such as surfactants and polyanions, are predicted to act rapidly, and mainly in the lumen/mucosal surface, whereas those which block an intermediary stage in viral entry, viral integration or replication, e.g. CCR5 inhibitors, NRTIs/NtRTIs, integrase inhibitors, protease inhibitors, and/ or mediate destruction of infected cells, act within the mucosa after a short delay. However, in the case of NAbs, which can have a range of effects, the situation may be complicated [767].

In general, antibody-mediated neutralization of HIV-1 has been attributed to binding of antibody to sites on Env trimers and interfering with virion attachment to host cell receptors and/or fusion and cell entry. However, with a few exceptions, the precise molecular mechanisms through which individual antibodies mediate their effects have been poorly understood [768]. Recently, important mechanistic differences have been identified between the MPER mAbs, 2G12 and b12 which could impact on their *in-vivo* efficacy. It has long been recognised that maximal *in-vitro* neutralization of susceptible isolates by 2G12 occurs rapidly (within 1 hour) whereas that mediated by b12 and the MPER mAbs develops over a number of hours [484]. This is partly due to differences in epitope accessibility; the 2G12 binding site consists of glycated residues on the external surface of gp120, whereas the CD4bs epitope of b12 is deeply recessed and the MPER is partially buried in the viral membrane [472,473,476,477]. Resistance to neutralization by b12, 2F5 and 4E10 can arise even in the presence of their cognate amino acid residues, due to variations in the 3-dimensional structure of the Env trimer which preclude access to their epitopes [769,770]. In addition, whereas the primary mechanism of virus neutralization by 2G12 appears to be competitive inhibition of virion-host cell attachment (by binding V3 in a way which blocks conformational changes required for efficient binding of CD4 and CCR5) [475] that for the MPER mAbs is much more complex. Evidence to date suggests that whilst 2F5 and 4E10 can bind to their epitopes, within their lipid environment, on free virions prior to target cell engagement (and this enhances neutralization), they interact with gp41 more strongly once it forms a hairpin intermediate on binding CD4 [479,481,482,483,770]. This action likely prevents formation

of the fusion peptide hence blocks virus entry. Recently, Trkola *et al.* have shown that the MPER mAbs and b12, but not 2G12, can also induce shedding of Env from the surface of virions resulting in irreversible neutralization [771]. In contrast, although much more rapid, neutralization mediated through 2G12 binding was shown to be reversible; viable virions being released once excess antibody was removed [475,771]. However, Env shedding is a slow process, requiring prolonged incubation of the mAbs with free virions or the continued presence of antibody at the time of contact with target cells. For the MPER mAbs, high concordance between the degree of observed shedding and neutralization, and similarities in the kinetics of the two processes, suggest they are functionally linked. In contrast, the primary mechanism of b12 neutralization appears to be independent of shedding. The exact time required for 100 % shedding was variable, dependent on both mAb concentration and temperature, but took a minimum of 16 hours using 100µg/ml MPER mAb (sufficient to neutralize 70% of challenge JR-FL pseudovirus stock at 20 hours) at 37 °C [771].

In addition to direct neutralization, both NAbs and non-NAbs can mediate other activities in the mucosa which may contribute to HIV clearance [146,147,345]. These include binding of virions to prevent trancytosis, and Fc mediated functions, e.g. phagocytosis, ADCC and ADCVI of infected cells and complement activation. Large, multimeric antibodies (IgM and IgA) can aggregate virions, and impede their passage through epithelial layers [345]. However, although most antibodies (isolated mainly from highly-exposed HIV-1 unifected individuals) that have been shown to block transcytosis *in-vitro* have been of the IgA class [772,773,774,775], HIV-1 binding to syndecans, which is dependent on a conserved arginine in gp120 [260, 776], can be blocked by the IgG1 NAb b12, inhibiting its uptake by epithelial cells [777].

For b12, there is *in-vitro* and *in-vivo* evidence to suggest that Fc-mediated effector functions, but not complement activation, play a role in its protective effects when it is given IV [778,779]. To date, the ability of other anti-HIV NAbs to perform non-neutralizing activities has been studied less extensively and has been confined to *in-vitro* analyses. In contrast to b12, both 2G12 and the MPER mAbs have been shown to be inefficient when assessed using antibody-dependent cell-mediated viral inhibition assays (ADCVI) [499, 778, 780]. However, antibodies may differ in their individual abilities to elicit particular

cell-mediated effector functions. Both ADCVI and ADCC depend on interactions between the antibody, FcR-bearing effector cells and an infected target cell. However, whereas ADCVI assays measure the quantity of HIV virions released by infected target cells (which may be influenced by a number of both cytotoxic and noncytotoxic inhibitory mechanisms), those for ADCC detect cell lysis [148]. When focusing only on ADCC, 2F5 has been shown to have potent activity, acting in an FcγRI-dependent manner [781]. Interestingly, FcγRI interactions have also been reported to increase the neutralization potency of both 2F5 and 4E10, but not b12 and 2G12, when expressed on TZM-bl target cells [782]. In contrast, for both b12 and 2G12, ADCC activity can be boosted *in-vitro* through mutations which enhance Fc binding to FcγRIIIa [783,784]. In general, antibody concentrations needed to elicit Fc-mediated effector functions are much higher than those required for neutralization [499,779,781].

The implications of the above findings for *in-vivo* protection are poorly understood. FcγRI binds monomeric IgG with high affinity; thus, given the high concentration of IgG in serum and genital secretions, is thought to be saturated under physiological conditions. In contrast, FcγRIIa, FcγRIIb, and FcγRIIIa bind monomeric IgG with low affinity and probably require the formation of immune complexes for efficient IgG binding; the latter being more compatible with a role in pathogen clearance and immunoregulation [143,147,784]. However, although antibody-FcγRIIIa mediated effector functions have been correlated with protection from HIV and SIV in vaccine studies [785,786,787], and with enhanced VL control in infected individuals [788,789,790], a direct relationship has not been proven. Despite displaying enhanced affinity for human and macaque FcγRIIIa, and demonstrating significantly (at least 10-fold) greater ADCVI, ADCC and NK activation abilities *in-vitro,* a non-fucosylated variant of b12 (administered IV) produced no difference in protection or post infection VL in macaques, following repeated low-dose vaginal $SHIV_{162P3}$ challenge, compared to wild-type b12 [791]. Thus, it may be that other Fc-mediated activities were behind the previously observed increased efficacy of wild-type b12 compared with Fc-deficient (LALA mutant) b12 [778,779] or that enhancement of FcγIIIa binding and NK stimulation, beyond that which occurs naturally, has little observable impact in the context of the antibody, SHIV challenge dose/strain and small numbers of animals studied.

Overall, with respect to anti-CD4bs antibodies, mAb concentration and ability to neutralize SHIV challenge virus (i.e. to both bind virus and inhibit virus-target cell attachment) have been shown to be the critical determinants of protection in macaques (following vaginal exposure), with non-neutralizing activities offering additional, but limited, benefits [500,778,779,791,792]. This appears to be true both for IV and topically administered antibodies. Whether such findings can be generalised to other mAbs and to human/HIV-1 contexts, has not yet been definitively established. However, they are consistent with the inability of IV administered polyclonal anti-HIV Ig preparations (containing non-neutralizing anti-Env antibodies) to protect macaques against challenge with several different SHIVs [490,491] and with the lack of efficacy demonstrated by recombinant monomeric HIV-1 gp120 vaccines in phase III trials, despite them frequently eliciting non-neutralizing, gp120-binding antibodies in serum [343,344 ,352]. That said, as highlighted by a recent vaccine study in macaques [793], antibody responses and effects in blood may differ from those at the mucosal interface.

A current important question for microbicide research is whether intracellularly acting anti-HIV agents, e.g. tenofovir, can target HIV once it has been endocytosed by mDCs or if this is a potential route for HIV to escape and enable dissemination and T cell infection beyond the FGT [248]. Trans-infection has also been proposed as a means for pathogens to evade immune system recognition and avoid neutralization by antibody. However, in the context of the T cell-T cell VS, HIV transfer has been shown to be sensitive to blockade by a range of entry inhibitors, including b12, CCR5 inhibitors, PRO 2000 and T20 [279]. Whether this is also true for mDC-T cell transmission is uncertain but SDF production by mDCs has been shown to inhibit R4 using virus transfer by VS and may help explain the preferential transmission of R5 virus [794]. Given that they would likely have sufficient time to act, mAbs which can neutralize irreversibly at low concentrations, inhibit cell-cell spread and mediate destruction of infected cells (via Fc mediated effects) could clearly be of benefit.

Enhancements in knowledge regarding the mechanism of action of 2F5, 4E10 and 2G12 have raised a number of questions regarding their potential efficacy as a vaginal microbicide.

1. Could virions be bound rapidly enough by 2G12 in the vaginal lumen to become trapped in mucus or lost in vaginal secretions before they either pass beyond reach of the antibody (e.g. into the submucosa or upper FGT) or antibody concentrations fall to levels where they became viable again?
2. Do 2F5 and 4E10 concentrations remain high enough for long enough on the mucosal surface to irreversibly neutralize virions through Env shedding?
3. Are sufficient mAbs taken up into the mucosa to a) prevent binding of virions to initial target cells, b) inhibit transinfection and c) elicit Fc-mediated effects?

Such questions will not be easy to answer. Pharmacokinetic data obtained from the Mabgel trial (see Chapter 3) provides a direct indication of the concentration of each mAb present on the vaginal surface over time, following gel application, including dilutional effects of vaginal secretions. Thus, it would be possible to see if corresponding mAb concentrations were capable of neutralizing a range of viral isolates at likely physiological concentrations *in-vitro.* Ideally, such pharmacodynamic assessments would be conducted using mAbs obtained in neat vaginal secretions, and if possible, semen, to try to mimic the physiological situation. Unfortunately, due to the paucity of time-points where self-aspiration was performed and the difficulties encountered with the sampling device, such experiments were not possible using the Mabgel samples. However, if future pharmacokinetic studies were conducted, neat samples, perhaps obtained using an INSTEAD® Soft Cup (Evofem, San Diego, California, USA) [536], could be collected at multiple time-points. Such studies could also include biopsies, which would enable mAbs to be detected in the mucosa, and also provide tissue for explant challenge. An *ex-vivo* technique has recently been developed whereby tissue biopsies, performed in humans at fixed time-points post application of a microbicide, are infected *in-vitro* with low-dose HIV-1 isolates and compared with those from untreated controls through the measurement of levels of p24 and integrated provirus [386]. Adaptation of such models may eventually enable the study of more detailed mAb-cellular interactions, although mimicking the exact virus: mAb concentrations and complex pharmacodynamic interactions that occur in natural infection would be extremely challenging.

6.2.3.3 mAb concentrations required for protection *in-vivo*

In the absence of any direct human pharmacodynamic and efficacy data, an indication of whether, and for how long post application, the Mabgel could offer protection from HIV-1 can be gained by comparing the vaginal mAb concentrations (from Weck-Cels) in our trial with those shown to block SHIV vaginal transmission in NHP studies. Comparisons can be made with vaginal concentrations of (a) transudative mAbs present after IV infusion, or (b) directly vaginally administered mAbs; the latter being more directly relevant to our study and proposed vaginal microbicide development.

Unfortunately, for the initial high dose (≥ 300 $TCID_{50}$) vaginal SHIV NHP challenge studies (using IV administered mAbs) [491,492,493], limited data was published regarding antibody titres in vaginal secretions at the time of challenge. In addition, comparison between studies was complicated by differences in sampling methodologies and lack of adjustment for dilution factors [492,792]. Like other early mucosal challenge studies (see Table 1-8), being primarily designed to inform development of vaccines, they were largely concerned with identifying systemic, serological correlates of protection. Although the exact mAb concentrations vary, (depending on the antibody, neutralization assay, route of challenge, and SHIV subtype used) the majority of data suggest that sterilizing protective effects *in-vivo* require serum mAb levels in the challenged animal 30 to >100 times higher than those needed to produce 90% neutralization in standard *in-vitro* peripheral blood mononuclear cell assays (i.e. the PBMC IC_{90}) [493,795]. The exception to this is 2G12, which has been shown to mediate significant protection at serum levels close to the PBMC IC_{90} (although relatively high 2G12 concentrations are needed to attain IC_{90}) [491,780].

Encouragingly for the 'real-world' setting, recent NHP studies suggest that protection from multiple low dose (3 to 10 $TCID_{50}$ SHIV) vaginal exposures may be achieved with serum and mucosal antibody levels around 20-fold lower than needed to prevent transmission from a single high dose challenge [779, 784]. Using 1mg/kg of b12 IV, in a multiple bi-weekly 10 $TCID_{50}$ vaginal $SHIV_{162P3}$ challenge model, rhesus macaques were protected in 104 out of 108 challenges, with median vaginal concentrations at challenge time points of approximately 20 and 5 µg/ml [784].

Unfortunately, no data is yet available regarding the vaginal levels of 2G12, 2F5 or 4E10 that might be capable of protecting from such ‘physiological’ viral inoculae. However, for 2F5, an idea of the concentrations needed to protect from a single high dose challenge is known. In a study conducted by Veasey *et al*, 5 macaques received a 300 $TCID_{50}$ vaginal challenge of $SHIV_{162P3}$ following a 25mg/kg IV infusion of 2F5. The challenge inoculum was administered at the time of peak vaginal 2F5 concentration, which was found to be 350 to 500 µg/ml at 6 hours post infusion. No animals became infected (R Veasey and R Shattock, personal communication). As these researchers used the same dilution factor correction and ELISA methodology as we did, extrapolating to the corrected concentrations found in our study would suggest that the median levels of 2F5 detected in both the high and low dose Mabgel arms at 8 hours (post 1st dose) and 12 hours (post 12th dose) would be sufficient to offer protection. However, such extrapolations should be treated with caution. There was a wide range of mAb levels detected at all time-points in our study, even within each of the high and low dose groups, so all women might not be equally protected. In addition, mAbs detected from Weck-Cels applied to the vaginal wall following systemic administration reflect not only those antibodies present in the vagina but also those in the submucosa, i.e. the systemic to mucosal compartment gradient. Since it is likely that the mAbs would be distributed more widely within the FGT following systemic rather than topical application it is possible that they would have a broader range of effects and/or a longer duration of action for the same measured luminal concentration.

A more direct reflection of whether the vaginal mAb levels detected in our trial would offer protection from some HIV-1 strains can be gained from comparing our data with that from NHP challenge studies conducted by the CEA. In a study designed to investigate the prophylactic efficacy of 20mg/g Mabgel (2ml), 22 macaques in 4 groups (untreated controls, placebo-treatment 1 hour before challenge, Mabgel 1 hour before challenge and Mabgel 4 hours before challenge) were exposed intravaginally to 3 AID_{50} (50 % animal infectious doses) of $SHIV_{162P3}$ in 50 % human seminal plasma following treatment with DMPA. 5/6 untreated control animals were infected, as were 4/5 animals who received placebo. Of the animals who received Mabgel, 1/5 became infected when challenged at 1 hour post application and 2/6 animals at 4 hours post application [Dereuddre-Bosquet and R Le-Grande personal communication]. As mAb concentrations detected in our study

were similar in magnitude over time to those found in the macaques (see Table 3-10), there is potential for a similar protective effect to also be shown in women. That said, physiological differences between women and macaques, and the 'atypical' nature of the challenge ($SHIV_{162P3}$ is relatively susceptible to neutralization by all 3 mAbs and 3 AID_{50} is a relatively low dose challenge stock for a single exposure study) mean it is difficult to predict with any certainty. In addition, as with most microbicide candidates to date, all animal studies have been conducted using free virions; potential efficacy against cell-associated viral transmission is thus as yet unexplored. Advances in the development of even more 'realistic' repeated low-dose SHIV challenge models [233,234], and in the establishment of reliable NHP cell-associated transmission systems [796] will hopefully improve the predictive accuracy of pre-clinical efficacy studies in future.

6.2.4 Production Costs

One disadvantage of using biological molecules produced in mammalian cells as therapeutic agents is their high manufacturing cost. At present, the estimated cost of producing C2F5, C4E10 and C2G12 using CHO cells is 50 to 100 Euros/g of mAb (B. Vcelar, personal communication). Thus, each single 2.5g dose of 20mg/g Mabgel, containing a total of 150mg of mAbs, would cost between 7.5 and 15 Euros to manufacture, not including the cost of the gel vehicle. In addition, the quantities of mAbs that can be produced using mamalian cell-culture systems are relatively small (3 to 75 pg/cell/day with high-producing clones), due to the limited transcriptional and translational capacities of the cells [797]. Although feasible for generating therapeutic mAbs, for use in small numbers of individuals in wealthy nations, CHO-cell based production systems are too expensive and constrained for viable scale-up of a global microbicide. Alternative manufacturing strategies would therefore need to be found. One potential option that is currently being explored is production of mAbs in plants ('plantibodies')[798, 799]. Viral vector constructs have been generated that achieve transient, rapid, high-yield expression of mAbs in tobacco (*Nicotiana benthamiana*) [800,801]. Entire mature plants are infiltrated with a diluted Agrobacterium suspension containing T-DNAs encoding viral replicons with IgG light and/or heavy chain gene inserts. mAbs can be harvested from leaves within 4 to 10 days (0.5 mg of mAb per g leaf fresh weight), with production being directly scaleable to the volume of biomass infected [800,801,802]. Since no genes are

incorporated into the plant genome (unlike with transgenic plants) and no replication-competent virus is produced, there is no risk of the transgene being spread via pollen, seeds, or virus [802].

2G12, 2F5 and 4E10 have all been produced successfully in tobacco plants [803, 804, 805] and 2G12 has also been produced in transgenic maize [806,807]. Such mAbs have been shown *in-vitro* to demonstrate antigen recognition, FcγR binding, and HIV-1 neutralization capabilities that are at least as good as their CHO- cell produced counterparts, despite differences in glycosylation [804, 805,806, 807, 808]. IgG has an oligosaccharide covalently attached to a single asparagine residue (Asn 297) on the CH2 domain of each of its heavy chains which appears to be integral to maintaining the correct structural conformation of Fc [809]. Additional glycans may also be added to the Fab region. The nature and range of sugar moieties added varies between species, with CHO cell-derived glycans being more similar to those found in humans than those derived from plants [810,811]. However, both CHO and plant –specific oligosaccharides can potentially be immunogenic in humans and can influence IgG pharmacokinetics and effector functions [810,811, 812]. In view of their molecular differences, and the potential for contamination with herbicides and pesticides, plantibodies require separate evaluation and regulatory approval to those produced in CHO cells [798,799]. A phase 1 clinical trial of the safety and pharmacokinetics of *N. Benthamiana* – derived, GMP grade 2G12 as a vaginal microbicide is due to be conducted at the University of Surry (CI Professor David Lewis) in 2012 as part of the EU funded Planta-Pharma project [813].

6.2.5 Acceptability

Predicting whether an efficacious microbicide will be used in the 'real-world' is a developing but inexact science. Historically, two approaches have been used to try to understand the factors that influence 'acceptability.' One method is to use surrogate products (those with similar formulation and application characteristics to candidate microbicides being developed) and ask women whether they would use 'a product like this one' in the future. The second method is to assess acceptability during clinical trials. However, as in the MABGEL1 trial, volunteers who take part in acceptibility studies or early phase trials are not always representative of the eventual target group for the

product and its evaluation is independent of sexual activity. In contrast, assessments of acceptability performed during efficacy studies, involving sexually active women from high risk populations, have greater applicability to the 'real-life' setting. That said, it is important not to equate product adherence with acceptibility, since the two are imperfectly related [765].

A woman's decision to use a microbicide has been found to be influenced by multiple factors, related to herself, the product and the context [765]. User-related characteristics which may have an impact include ethnicity, socioeconomic status, and prior experience of using prevention products [814]. Contextual factors, include nature and dynamics of the sexual partnership, perception of HIV risk and socio-cultural influences [815,816]. Features of the product which may impact on its acceptibility include colour, smell, consistency, propensity for leakage, timing and frequency of use in relation to coitus and overall effect on sexual pleasure [720,817,818].

Whilst the colourless and odourless nature of the Mabgel attracted no negative comments, in common with other semi-solid gels, it was prone to leakage. However, as has also been found with other products, reports and experience of leakage varied markedly between women (see section 5.4.6). In acceptability studies for cellulose sulphate vaginal gel and KY lubricant, 20% of participants reported gel leakage to an extent that it would discourage them from future use [818,819]. In research addressing the application of BufferGel, a significant minority of women described leakage, messiness and an overall negative experience [817]. When used in the context of sex, women's perceptions of a product's characteristics are often different to when used during sexual abstinence [817,818]. In an acceptability study of cellulose sulphate gel involving sexually active women, while 40% of participants stated that the product leaked out during sex, 100% reported that their vagina felt 'more wet' which was considered a positive feature [720]. Experiences of South African women enrolled in the MDP 301 trial of PRO2000 were also positive; up to 60% reported that sex became 'wetter' and more enjoyable when using the gel and only 1% felt it had a negative impact [820].

Coital-dependent gel use may not be convenient for women in all parts of the world. Women may have little warning that sex is going to take place, or live in regions where 'dry sex' is the norm [821]. In addition, frequent insertion of gel or applicators may cause

trauma or be associated with adverse events. In the MABGEL 1 trial, intermenstrual bleeding was experienced by almost half of all participants across the three study arms (see section 4.1.1.6). Although it is difficult to determine whether this was related to background incidence, or caused / increased by the gel vehicle, gel insertion or examination, it was perceived to be related to product use by participants. Even if not felt to be a sign of toxicity, such common AEs can be problematic, since they may deter women from using the product.

As mentioned in section 1.6.6, intravaginal rings (IVR) are an alternative dosing form which offer long-term coitally-independent release of active agents and avoid the leakage, and other problems, associated with gels. They have been shown to be acceptable in several studies [822,823]. However, until recently, they were only suitable for releasing small molecule, hydrophobic agents, such as contraceptive hormones or the NNRTI Dapivirine [440]. A new ring device (the insert vaginal ring (InVR®)) has now been developed that is capable of releasing mAbs and other proteins over an extended period, thus opening up possibilities for using this method in the future [824].

An even more novel approach is the use of adeno-associated viruses (AAVs) to transfer genes encoding "minibodies" to cells to enable them to secrete NAbs constitutively. A "minibody" is a single-chain variable fragment: Fc fusion molecule which dimerizes to form a protein which is shorter than a full-length IgG, but with similar neutralizing capabilities. To date, this strategy has been assessed in mice and NHPs, where systemic b12 minibodies (mice) and SIV-specific immunoadhesins (i.e. anti-SIV Fabs) (macaques) were produced continuously for > 1 year through transduction of muscle tissue [825,826]. However, although the latter afforded some protection from IV SIV challenge, this was hampered by transgene immunogenicity (i.e. antibodies elicited against the immunoadhesins). Recently, the ability of AAVs to transduce cervico-vaginal epithelial stem cells has been demonstrated *in-vitro*, with the resulting b12 minibodies able to inhibit HIV-1 infectivity and transfer of cell-free virus through VEC tissues [827]. Further research is needed to examine the effectiveness of such a 'self-generating microbicide' in NHPs before testing in humans.

6.2.6 Novel anti-HIV-1 NAbs

A growing number of novel, cross-clade HIV-1 neutralizing mAbs are being identified through efficient, high-throughput screening of sera from a range of HIV-1 infected individuals [158,485,828,829,830]. Among the first of these 'new generation' broadly-neutralizing mAbs to be discovered , PG9 and PG16 bind conserved, glycosylated epitopes on the V2 and V3 loops of 'native' trimeric gp120 or uncleaved gp160 [485]. VRCO1 and PGV04, recognise the gp120 CD4 binding-site [828,830]. These 4 mAbs display an overall greater breadth of neutralization than 2F5, 2G12 and b12, particularly to Clades A and C, and can inhibit most isolates at concentrations around 10 fold lower than 2F5, 2G12, b12 and 4E10. Thus, although none are as broadly neutralizing as 4E10 at high concentrations (i.e. VRC01 and PGV04 neutralize approximately 90 % of tested isolates, PG9 and 16 around 70 % and 4E10 > 95% at IC_{50} < 50 μg ml^{-1}) at low concentrations they can neutralize a larger range of global HIV-1 strains than any of the four 1st generation mAbs [485, 828]. Recent *in-vitro* data has confirmed the potential for additive coverage if used in combination [831].

Recently, a range of anti-HIV-1mAbs (PGT 121-123, 125-128 130-131, 135-137 and 141-145) have been isolated with potencies which are 10 times greater still (i.e. the median antibody concentrations required to inhibit HIV activity by 50% or 90% (IC_{50} and IC_{90} values) are 100-fold lower than for the original broadly neutralizing mAbs)[828]. Several of these, including the broadest (PGT 128), recognise mannose ($Man_{8/9}$) epitopes on the gp120 glycan shield and, unlike 2G12, may mediate cross-linking of adjacent Env trimers, accelerating decay in viral infectivity [832]. Although individually having less broad maximal coverage than earlier NAbs (e.g. PGT 128 neutralized 73 % of strains at IC_{50} < 50 μg ml^{-1}), if present at the time of exposure, certain combinations have the potential to provide protection from the majority of circulating HIV-1 isolates at very low concentrations (e.g. PGT 128 + PGT 145 could neutralize 63 % of viruses at IC_{50} < 0.1 μg ml^{-1}, compared with 1% for 4E10) [829]. Thus, their induction through vaccination or application as a microbicide could be highly efficacious. However, further research is needed to confirm their activity and safety *in-vivo* and fully understand their mechanism (s) of neutralization.

6.2.7 Conclusion

Unfortunately, given its relatively limited coverage of clade C isolates, high manufacturing costs, and possible need for re-formulation, the Mabgel is unlikely to be a viable global HIV microbicide in its current form. However, in the light of advances in antibody production in plants and the ongoing discovery of novel, increasingly potent, cross-clade HIV-1 NAbs, the general concept of using combinations of mAbs for HIV prevention looks increasingly promising. For example, a microbicide containing high concentrations of 4E10 as a 'backbone' combined with 2 of the newer, more potent gp120 binding NAbs may have potential. Combining plantibodies with other biopharmaceuticals, e.g. lectins, is another way of potentially enhancing coverage breadth and efficacy [833]. The concept of combining microbicides with mucosally-delivered vaccines is also being explored [834].

Although the existing Mabgel is unlikely to be progressed further, data regarding its pharmacokinetics and safety will be useful in the development of future mAb-based products. As calculated from the MABGEL 1 trial, the vaginal residence t ½ of the MPER mAbs would be compatible with their application as a coitally-dependent microbicide, thus demonstrating 'proof of principle'. However, the more rapid decay of 2G12, has highlighted potential differences between the vaginal environment of macaques and humans and provided a caveat to investigate the effects of genital secretions early in future product development. Results from MABGEL 1 have been considered by the MHRA in granting permission to conduct the 1st trial of tobacco-derived 2G12 in women (T Cole, personal communication, 2011). Experience gained from the trial has also influenced the design and conduct of a subsequent HIV vaccine study conducted on the EMU, including its recruitment strategy and the choice of the INSTEAD® Soft Cup, rather than an aspirator, for self-sampling vaginal secretions. In addition, having had its utility demonstrated both in our trial, and subsequently in a trial of a dapivirine-releasing IVR [835], the use of PCR in the analysis of vaginal flora is gaining acceptance and has been incorporated into a number of planned microbicide studies.

In spite of recent progress, we are still a long way from controlling the HIV-1 pandemic. Given the current economic down-turn it is now even more important that we continue to strive to limit the number of new HIV infections and reduce the global cost of

healthcare and treatment. However, it is vital that only the most promising microbicide, PrEP and vaccine candidates are advanced to avoid squandering time and resources. Although of crucial importance, efficacious biomedical interventions will be insufficient on their own to reduce the sexual transmission of HIV. Ideally, they should be used as an adjunct to behavioural strategies, aimed at reducing numbers and concurrency of partners, encouraging condom use and increasing HIV testing [17]. Structural changes will also be required at both national and community levels to provide the financial and infrastructural support needed for successful implementation of behavioural and biomedical interventions and address policies and societial norms that pose barriers to HIV prevention [17,295]. A "combination prevention" approach (using multiple, co-ordinated, evidence-based interventions operating at individual, relationship, community and national levels) which is tailored to the specific needs of each particular population, is generally considered to be the only route likely to achieve a large and sustainable reduction in global HIV incidence [836].

REFERENCES

---

1 Centers for Disease Control (CDC). Pneumocystis pneumonia—Los Angeles. MMWR Morb Mortal Wkly Rep 1981;30:250-252.

2 Centers for Disease Control (CDC). Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men in New York City and California. MMWR Morb Mortal Wkly Rep. 1981; 30:305-308.

3 AIDS at 30: Nations at the crossroads. Geneva, Switzerland: Joint United Nations Programme on HIV/AIDS (UNAIDS); 2011. Available at: http://www.unaids.org/unaids_resources/aidsat30/aids-at-30.pdf. Accessed September 1 2011.

4 Together we will end AIDS. Geneva, Switzerland: Joint United Nations Programme on HIV/AIDS (UNAIDS); July 2012. Available at: http://www.unaids.org/en/media/unaids/contentassets/documents/unaidspublication/2010/20101123_globalreport_en.pdf. Accessed August 17, 2012.

5 Gallo RC, Montagnier L. The discovery of HIV as the cause of AIDS. N Engl J Med 2003; 349: 2283-2285.

6 Dieffenbach CW, Fauci AS. Thirty years of HIV and AIDS: future challenges and opportunities. Ann Intern Med 2011;154; 766-771.

7 MacPherson P, Chawla A, Jones K, Coffey E, Spain V, Harrison I *et al*. Feasibility and acceptability of point of care HIV testing in community outreach and GUM drop-in services in the North West of England: A programmatic evaluation. BMC Public Health 2011; 11**:**419 doi:10.1186/1471-2458-11-419**.**

8 D Asboe, C Aitken, M Boffito, C Booth, P Cane, A Fakoya *et al.* British HIV Association guidelines for the routine investigation and monitoring of adult HIV-1-infected individuals 2011. HIV Med 2012; 13: 1-44.

9 DHHS Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents. Bethesda, Maryland, USA: Department of Health and Human Services; 2011. Available at: http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf. Accessed September 1, 2011.

10 Antiretroviral Therapy Cohort Collaboration. Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. Lancet 2008;372:293-299.

11 World Health Organization. Antiretroviral Therapy for HIV Infection in Adults and Adolescents: Recommendations for a Public Health Approach. 2010 Revision. Geneva,

Switzerland: World Health Organization; 2010. Available at: http://whqlibdoc.who.int/publications/2010/9789241599764_eng.pdf. Accessed September 24, 2011.

12 Potts M and Walsh J. Tackling India's HIV epidemic: lessons from Africa. BMJ 2003; 326:1389-1392.

13 Getting to zero. UNAIDS 2011-2015 strategy. Geneva: Joint United Nations Programme on HIV/AIDS (UNAIDS); 2010. Available at: http://www.unaids.org/en/media/unaids/contentassets/documents/unaidspublication/2010/jc2034_unaids_strategy_en.pdf Accessed September 1, 2011.

14 Myer L, Kuhn L, Stein ZA, Wright TC Jr, Denny L. Intravaginal practices, bacterial vaginosis, and women's susceptibility to HIV infection: epidemiological evidence and biological mechanisms. Lancet Infect Dis 2005; 5:786-794.

15 Hel Z, Stringer E, Mestecky J. Sex steroid hormones, hormonal contraception and the immunobiology of human immunodeficiency virus-1 infection. Endocrine Reviews 2010; 31: 79-97.

16 Opportunity in Crisis: Preventing HIV from early adolescence to young adulthood New York, USA: United Nations Children's Fund (UNICEF); 2011. Available at http://www.unicef.org/publications/index_58708.html. Accessed September 1, 2011.

17 Padian NS, McCoy SI, Karim SS, Hasen N, Kim J, Bartos M *et al.* HIV prevention transformed: the new prevention research agenda. Lancet 2011;378:269-278.

18 Barré-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J *et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science. 1983: 20;220: 868-871.

19 Gallo RC, Sarin PS, Gelmann EP, Robert-Guroff M, Richardson E, Kalyanaraman VS, Mann D, Sidhu GD, Stahl RE, Zolla-Pazner S, Leibowitch J, Popovic M. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). Science 1983; 220:865-867.

20 Clavel F, Guyader M, Guétard D, Sallé M, Montagnier L, Alizon M. Molecular cloning and polymorphism of the human immune deficiency virus type 2. Nature1986;324:691-695.

21 Peeters M, Honoré C, Huet T, Bedjabaga L, Ossari S, Bussi P, Cooper RW, Delaporte E. Isolation and partial characterization of an HIV-related virus occurring naturally in chimpanzees in Gabon. AIDS 1989 ;3:625-630.

22 Campbell-Yesufu OT, Gandhi RT. HIV/AIDS: Update on Human Immunodeficiency Virus (HIV)-2 Infection. Clin Infect Dis 2011; 52: 780-787.

23 Eberle J, Gürtler L. HIV types, groups, subtypes and recombinant forms: errors in replication, selection pressure and quasispecies. Intervirology 2012;55:79-83.

24 Hemelaar J, Gouws E, Ghys PD, Osmanov S, WHO-AIDS Network for HIV Isolation and Characterisation. Global trends in molecular epidemiology of HIV-1 during 2000–2007. AIDS 2011; 25:679–689.

25 Sierra S, Kupfer B, Kaiser R. Basics of the virology of HIV-1 and its replication. J Clin Virol 2005;34:233-244.

26 Frankel AD, Young JAT. Fifteen proteins and an RNA. Annu Rev Biochem. 1998:67:1-25.

27 Pollard VW, Malim MH. The HIV-1 Rev protein. Annu Rev Microbiol 1998; 52:491–532.

28 Harrich D, Hooker B. Mechanistic aspects of HIV-1 reverse transcription initiation. Rev Med Virol 2002, 12: 31–45.

29 Malim MH, Emerman M. HIV-1 accessory proteins–ensuring viral survival in a hostile environment. Cell Host Microbe 2008;3: 388–398.

30 Kirchhoff F. Immune evasion and counteraction of restriction factors by HIV-1 and other primate lentiviruses. Cell Host Microbe 2010; 8:55-67.

31 Marin M, Rose KM, Kozak SL, Kabat D. HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation Nat Med 2003;9:1398–1403.

32 Boyer R. Interactive concepts in biochemistry. Second edition. Hoboken, New Jersey, USA: Wiley; 2002.

33 Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, Pomerantz RJ. The challenge of finding a cure for HIV infection. Science 2009;323:1304-1307.

34 Pantophlet R, Burton DR. GP120: target for HIV-1 neutralizing antibodies. Annu Rev Immunol 2006; 24: 739-769.

35 Prabakaran P, Dimitrov AS, Fouts TR, Dimitrov DS. Structure and function of the HIV envelope glycoprotein as entry mediator, vaccine immunogen, and target for inhibitors. Adv Pharmacol 2007;55:33-97.

36 Zhu P, Liu J, Bess J, Chertova E, Lifson J, Grisé H *et al*. Distribution and Three-Dimensional Structure of AIDS Virus Envelope Spikes. Nature 2006; 441:847-852.

37 Morrison SJ, Weissman IL. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity 1994; 1: 661–673.

38 Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. Structure of an HIV gp120 envelope protein in complex with the CD4 receptor and a neutralizing human antibody. Nature 1998; 393:648-659.

39 Miyauchi K, Kim Y, Latinovic O, Morozov V, Melikyan GB. HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. Cell 2009; 137:433-444.

40 Moore JP, Kitchen SG, Pugach P, Zack JA. The CCR5 and CXCR4 coreceptors--central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS Res Hum Retroviruses 2004;20:111-126.

41 Goodenow MM, Collman RG. HIV-1 coreceptor preference is distinct from target cell tropism: a dual-parameter nomenclature to define viral phenotypes. J Leukoc Biol 2006; 80:965-972.

42 Bleul CC, Wu L, Hoxie JA, Springer TA, Mackay CR. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc Natl Acad Sci USA 1997; 94:1925–1930.

43 Viardot A, Kronenwett R, Deichmann M, Haas R. The human immunodeficiency virus (HIV)-type 1 coreceptor CXCR-4 (fusin) is preferentially expressed on the more immature CD34+ hematopoietic stem cells. Ann Hematol; 1998: 77:193–197.

44 Ishii T, Nishihara M, Ma F, Ebihara Y, Tsuji K, Asano S, Nakahata T, Maekawa T. Expression of stromal cell-derived factor-1/pre-B cell growth-stimulating factor receptor, CXC chemokine receptor 4, on CD34+ human bone marrow cells is a phenotypic alteration for committed lymphoid progenitors. J Immunol 1999; 163: 3612–3620.

45 Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR. Change in coreceptor use correlates with disease progression in HIV-1-infected individuals. J Exp Med 1997; 185: 621–628.

46 Shepherd JC, Jacobson LP, Qiao W, Jamieson BD, Phair JP, Piazza P, Quinn TC, Margolick JB. Emergence and persistence of CXCR4-tropic HIV-1 in a population of men from the multicenter AIDS cohort study. J Infect Dis 2008;198: 1104–1112.

47 Weiser B, Philpott S, Klimkait T, Burger H, Kitchen C, Bürgisser P *et al.* Swiss HIV Cohort Study. HIV-1 coreceptor usage and CXCR4-specific viral load predict clinical disease progression during combination antiretroviral therapy. AIDS 2008;22: 469–479.

48 Bebenek K, Abbotts J, Roberts JD, Wilson SH, Kunkel TA. Specificity and mechanism of error-prone replication by human immunodeficiency virus-1 reverse transcriptase. 1989 The Journal of Biological Chemistry 1989; 264: 16948-16956.

49 Lewis TLT, Chamberlain GVP. Gynaecological anatomy and physiology. In: Lewis TLT, Chamberlain GVP, editors. Gynaecology by Ten Teachers. 15th Edition. London, England: Hodder Arnold; 1990; p. 1-31.

50 Stenchever MA, Droegemueller W, Herbst AL. Comprehensive Gynecology. 4th edition. St. Louis, Missouri, USA: Mosby; 2002; p 40 – 44.

51 Barnhart KT, Izquierdo A, Pretorius ES, Shera DM, Shabbout M, Shaunik A. Baseline dimensions of the human vagina. Hum Reprod 2006;21: 1618 – 1622.

52 Fawcett DW, Raviola E. The female reproductive system. In: Bloom JR, Fawcett DW, editors. A Textbook of Histology. 12th edition. New York, USA: Chapman & Hall; 1994; p 857 – 858.

53 Owen DH and Katz DF. A vaginal fluid simulant. Contraception 1999; 59: 91-95.

54 Iwasaki A. Antiviral immune responses in the genital tract: clues for vaccines. Nat Rev Immunol 2010; 10:699-711.

55 Papanicolau GN. The sexual cycle in the human female as revealed by vaginal smears. American Journal of Anatomy 1933; 52 (supplement 1); 519-637.

56 Eschenbach DA, Thwin SS, Patton DL, Hooton TM, Stapleton AE, Agnew K *et al*. Influence of the Normal Menstrual Cycle on Vaginal Tissue, Discharge and Microflora. CID 2000;30:901–907.

57 Patton DL, Thwin SS, Meier A , Hooton TM, Stapleton AE, Eschenbach DA. Epithelial cell layer thickness and immune cell populations in the normal human vagina at different stages of the menstrual cycle. Am J Obstet Gynecol 2000; 183: 967 – 973.

58 World Contraceptive Use 2010. Geneva, Switzerland: United Nations, Department of Economic and Social Affairs, Population Division; 2011. Available at: http://www.un.org/esa/population/publications/wcu2010/WCP_2010/Data.html. Accessed 30th June 2011.

59 Bhathena RK. The long-acting progestogen-only contraceptive injection: an update. Br J Obstet Gynaecol 2001; 108: 3–8.

60 Makarainen L, van Beek A, Tuomivaara L, Asplund B, Bennink HC. Ovarian function during the use of a single contraceptive implant: Implanon compared with Norplant. Fertil Steril 1998;69: 714–721.

61 Shu-Rong Z, Huai-Mei Z, Shao-Zhen Q, Guo-Wei S, Kaper RF. A long-term study of the efficacy and acceptability of a single rod hormonal contraceptive implant (Implanon) in healthy women in China. Eur J Contracept Reprod Health Care 1999;4: 85–93.

62 Rice CF, Killick SR, Dieben TOM, Coelingh Bennink H. A comparison of the inhibition of ovulation achieved by desogestrel 75 micrograms and levonorgestrel 30 micrograms daily. Hum Reprod 1999; 14: 982–985.

63 Kurunäki H, Toivonen J, Lähteenmäki PLA, Luukkainen T. Pituitary and ovarian function and clinical performance during the use of a levonorgestrel-releasing intracervical contraceptive device. Contraception 1984; 29: 31–43.

64 Nilsson CG, Lähteenmäki PLA, Luukkainen T. Ovarian function in amenorrheic and menstruating users of a levonorgestrel-releasing intrauterine device. Fertil Steril 1984;41: 52–55.

65 Curtis KM, Chrisman CE, Mohllajee AP, Peterson HB. Effective use of hormonal contraceptives. Part I: Combined oral contraceptive pills. Contraception 2006; 73: 115–124.

66 The Management of unscheduled bleeding in women using hormonal contraception. London, England: Faculty of Sexual and Reproductive Healthcare in collaboration with the Royal College of Obstetricians and Gynaecologists; 2009. Available at: http://www.fsrh.org/pdfs/UnscheduledBleedingMay09.pdf . Accessed 10 July 2011.

67 Hild-Petito S, Veazey RS, Larner JM, Reel JR, Blye RP. Effects of two progestin-only contraceptives, Depo-Provera and Norplant-II, on the vaginal epithelium of rhesus monkeys. AIDS Res Hum Retroviruses 1998;14 Suppl 1:S125-30.

68 Poonia B, Walter L, Dufour J, Harrison R, Marx PA, Veasey RS. Cyclic changes in the vaginal epithelium of normal rhesus macaques. J Endocrinol 2006;190:829-835.

69 Mauck CK, Callahan MM, Baker J, Arbogast K, Veazey R, Stock R *et al.* The effect of one injection of Depo-Provera on the human vaginal epithelium and cervical ectopy. Contraception. 1999;60:15-24.

70 Miller L, Patton DL, Meier A, Thwin SS, Hooton TM, Eschenbach DA. Depomedroxyprogesterone-induced hypoestrogenism and changes in vaginal flora and epithelium. Obstet Gynecol. 2000;96:431-439.

71 Ildgruben AK, Sjoberg IM, Hammarstrom ML. Influence of hormonal contraceptives on the immune cells and thickness of human vaginal epithelium. Obstet Gynecol 2003;102: 571 – 582.

72 Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in female genital tract: cellular responses and interactions. Immunol Rev 2005; 206: 306-335.

73 Murphy KM. Janeway's Immunobiology. 8th Edition. London, England: Garland Science; 2011.

74 Collins KB, Patterson BK, Naus GJ, Landers DV, Gupta P. Development of an in vitro organ culture model to study transmission of HIV-1 in the female genital tract. Nat Med 2000; 6:475–479.

75 Chancey CJ, Khanna KV, Seegers JF, Zhang GW, Hildreth J, Langan A, Markham RB. Lactobacilli-expressed single-chain variable fragment (scFv) specific for intercellular adhesion molecule 1 (ICAM-1) blocks cell-associated HIV-1 transmission across a cervical epithelial monolayer. J Immunol 2006; 176:5627–5636.

76 Van Herrewege Y, Michiels J, Waeytens A, De Boeck G, Salden E, Heyndrickx L *et al*. A dual chamber model of female cervical mucosa for the study of HIV transmission and for the evaluation of candidate HIV microbicides. Antiviral Res 2007; 74:111–124.

77 Anderson DJ, Politch JA, Nadolski AM, Blaskewicz CD, Pudney J, Mayer KH. Targeting Trojan Horse leukocytes for HIV prevention. AIDS 2010;24:163-187.

78 Girard M, Mahoney J, Wei Q, van der Ryst E, Muchmore E, Barre-Sinoussi F, Fultz PN. Genital infection of female chimpanzees with human immunodeficiency virus type 1. AIDS Res Hum Retroviruses 1998; 14:1357–1367.

79 Miller CJ, Alexander NJ, Sutjipto S, Lackner AA, Gettie A, Hendrickx AG *et al*. Genital mucosal transmission of simian immunodeficiency virus: animal model for heterosexual transmission of human immunodeficiency virus. J Virol 1989; 63: 4277–4284 .

80 Weiler AM, Li Q, Duan L, Kaizu M, Weisgrau KL, Friedrich TC *et al*. Genital ulcers facilitate rapid viral entry and dissemination following intravaginal inoculation with cell-associated simian immunodeficiency virus SIVmac239. J Virol 2008; 82:4154–4158.

81 Kaizu M, Weiler AM, Weisgrau KL, Vielhuber KA, May G, Piaskowski SM *et al*. Repeated intravaginal inoculation with cell-associated simian immunodeficiency virus results in persistent infection of nonhuman primates. J Infect Dis 2006; 194:912–916.

82 Ball JK, Curran R, Irving WL, Dearden AA. HIV-1 in semen: determination of proviral and viral titres compared to blood, and quantification of semen leukocyte populations. J Med Virol 1999; 59:356–363.

83 Tachet A, Dulioust E, Salmon D, De Almeida M, Rivalland S, Finkielsztejn L *et al.* Detection and quantification of HIV-1 in semen: identification of a subpopulation of men at high potential risk of viral sexual transmission. AIDS 1999; 13:823–831.

84 Hickey DK, Patel MV, Fahey JV, Wira CR. Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. J Reprod Immunol 2011;88:185-194.

85 Gipson IK. Mucins of the human endocervix. Front. Biosci 2001; 6: D1245–1255.

86 Khanvilkar K, Donovan M D, Flanagan D R. Drug transfer through mucus. Advanced Drug Delivery Reviews 2001; 48 : 173–193.

87 Cone RA. Barrier Properties of Mucus. Advanced Drug Delivery Reviews 2009; 61:75–85.

88 Lai SK, Hida K, Shukair S, Wang YY, Figueiredo A, Cone R *et al*. Human immunodeficiency virus type 1 is trapped by acidic but not neutralized human cervicovaginal mucus. J Virol 2009;83:11196-11200.

89 Habte HH, Mall AS, de Beer C Lotz ZE, Kahn D. The role of crude human saliva and purified salivary MUC5B and MUC7 mucins in the inhibition of human immunodeficiency virus type 1 in an inhibition assay. Virol J 2006;3:99. doi:10.1186/1743-422X-3-99.

90 Domino SE, Hurd EA, Thomsson KA, Karnak DM, Holmén Larsson JM, Thomsson E *et al*. Cervical mucins carry alpha(1,2)fucosylated glycans that partly protect from experimental vaginal candidiasis. Glycoconj J 2009;26: 1125–1134.

91 Harmsen MC, Swart PJ, De Bethune MP, Pauwels R, De Clercq E, The TH, Meijer DK. Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication in vitro. J Infect Dis 1995; 172:380–388.

92 Quinones-Mateu ME, Lederman MM, Feng Z, Chakraborty B, Weber J, Rangel HR *et al.* Human epithelial beta defensins 2 and 3 inhibit HIV-1 replication. AIDS 2003; 17: F39-F48.

93 Chang TL, Vargas J Jr, DelPortillo A, Klotman ME. Dual role of alpha-defensin-1 in anti-HIV-1 innate immunity. J Clin Invest 2005; 115:765-773.

94 Bergman P, Walter-Jallow L, Broliden K Agerberth B, Söderlund J. The antimicrobial peptide LL-37 inhibits HIV-1 replication. Curr HIV Res 2007; 5: 410-415.

95 McNeely TB, Dealy M, Dripps DJ, Orenstein JM, Eisenberg SP, Wahl SM. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. J Clin Invest 1995; 96:456–464.

96 Ghosh M , Shen Z, Fahey JV, Cu-Uvin S, Mayer K, Wira CR. Trappin-2/Elafin: a novel innate anti-human immunodeficiency virus-1 molecule of the human female reproductive tract.Immunology 2010;129:207-219.

97 Cole AM, Cole AL. Antimicrobial Polypeptides are Key Anti-HIV-1 Effector Molecules of Cervicovaginal Host Defense. Am J Reprod Immunol 2008; 59:27-34.

98 Klotman ME, Chang TL. Defensins in innate antiviral immunity. Nature Reviews Immunol 2006; 6: 447-456.

99 Wira CR, Patel MV, Ghosh M, Mukura L, Fahey JV. Innate immunity in the human female reproductive tract: endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections. Am J Reprod Immunol 2011; 65: 196–211.

100 Wira CR, Ghosh M, Smith JM, Shen L, Connor RI, Sundstrom P *et al*. Epithelial cell secretions from the human female reproductive tract inhibit sexually transmitted pathogens and Candida albicans but not Lactobacillus. Mucosal Immunol. 2011;4:335-342.

101 Hill GB, Eschenbach DA, Holmes KK. Bacteriology of the vagina. Scand J Urol Nephrol 1984; 86(Suppl):23–39.

102 Klebanoff SJ, Hillier SL, Eschenbach DA, Waltersdorph AM. Control of the microbial flora of the vagina by H2O2 generating lactobacilli. J Infect Dis 1991;164:94–100.

103 Hawes SE, Hillier SL, Benedetti J*, et al.* Hydrogen peroxide–producing lactobacilli and acquisition of vaginal infections. J Infect Dis 1996; 174:1058–1063.

104 Olmsted SS, Khanna KV, Ng EM, Whitten ST, Johnson ON 3rd, Markham RB *et al*. Low pH immobilizes and kills human leukocytes and prevents transmission of cell-associated HIV in a mouse model. BMC Infect Dis 2005; 5:79. doi:10.1186/1471-2334-5-79.

105 Klebanoff SL, Coombs RW. Viricidal effect of *Lactobacillus acidophilus* on human immunodeficiency virus type 1: possible role in heterosexual transmission.J Exp Med 1991;174:289–292.

106 Bolton M, van der Straten A , Cohen CR. Probiotics: potential to prevent HIV and sexually transmitted infections in women. Sex Transm Dis 2008; 35:214–225.

107 Lagenaur L A, Berger EA. An anti-HIV microbicide comes alive. Proc Natl Acad Sci USA 2005;102:12294–12295.

108 Vangelista L, Secchi M, Liu X, Bachi A, Jia L, Xu Q , Lusso P. Engineering of Lactobacillus jensenii To Secrete RANTES and a CCR5 Antagonist Analogue as Live HIV-1 Blockers. Antimicrob Agents Chemother 2010; 54: 2994–3001.

109 Langbein L Grund C, Kuhn C, Praetzel S, Kartenbeck J, Brandner JM *et al.* Tight junctions and compositionally related junctional structures in mammalian stratified epithelia and cell cultures derived there from. Eur J Cell Biol 2002. 81:419–435.

110 Kayisli UA, Mahutte NG, Arici A. Uterine chemokines in reproductive physiology and pathology. Am J Reprod Immunol 2002; 47: 213–221.

111 Shoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, Rice CM. A diverse range of gene products are effectors of the type 1 interferon antiviral response. Nature 2011;472:481-485.

112 Kawai T, Akira S. The role of pattern recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 2010; 11:363-365.

113 Schaefer TM, Desouza K, Fahey JV, Beagley KW, Wira CR. Toll-like receptor (TLR) expression and TLR-mediated cytokine ⁄ chemokine production by human uterine epithelial cells. Immunology 2004;112:428–436.

114 Pioli PA, Amiel E, Schaefer TM, Connolly JE, Wira CR, Guyre PM. Differential expression of Toll-like receptors 2 and 4 in tissues of the human female reproductive tract. Infect Immun 2004; 72: 5799-5806.

115 Fazeli A, Bruce C, Anumba DO. Characterization of Toll-like receptors in the female reproductive tract in humans. Hum Reprod. 2005;2:1372-1378.

116 Nazli A, Yao XD, Smieja M, Rosenthal KL, Ashkar AA, Kaushic C. Differential induction of innate anti-viral responses by TLR ligands against Herpes simplex virus, type 2, infection in primary genital epithelium of women. Antiviral Res 2009; 81:103–112.

117 Hart KM, Murphy AJ, Barrett KT, Wira CR, Guyre PM, Pioli PA. Functional expression of pattern recognition receptors in tissues of the human female reproductive tract. J Reprod Immunol 2009; 80:33–40.

118 Biron Ca, Brossay L. NK cells and NKT cells in innate defense against viral infections. Curr Opin Immunol 2001;13:458-464.

119 Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, Yokoyama WM, Ugolini S. Innate or adaptive immunity? The example of natural killer cells. Science 2011;331:44-49.

120 Moretta A, Bottino C, Vitale M, Pende D, Biassoni R, Mingari MC, Moretta L. Receptors for HLA class-I molecules in human natural killer cells. Annu Rev Immunol 1996;14:619-648.

121 Schwartz O, Maréchal V, Le Gall S, Lemonnier F, Heard JM. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat Med 1996;2:338-342.

122 Alter G, Martin MP, Teigen N, Carr WH, Suscovich TJ, Schneidewind A *et al*. Differential natural killer cell-mediated inhibition of HIV-1 replication based on distinct KIR/HLA subtypes. J Exp Med 2007;204:3027-3036.

123 Cohen GB, Gandhi RT, Davis DM, Mandelboim O, Chen BK, Strominger JL, Baltimore D. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity 1999;10:661-671.

124 Tiemessen CT, Paximadis M, Minevich G, Winchester R, Shalekoff S, Gray GE *et al.* Natural killer cell responses to HIV-1 peptides are associated with more activating KIR genes and HLA-C genes of the C1 allotype.J Acquir Immune Defic Syndr 2011;57:181-189.

125 Martin MP, Gao X, Lee JH, Nelson GW, Detels R, Goedert JJ *et al.* Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat Genet 2002;31:429-434.

126 Boulet S, Kleyman M, Kim JY, Kamya P, Sharafi S, Simic N *et al.* A combined genotype of KIR3DL1 high expressing alleles and HLA-B*57 is associated with a reduced risk of HIV infection. AIDS 2008;22:1487-1491.

127 Jennes W, Verheyden S, Demanet C, Adjé-Touré CA, Vuylsteke B, Nkengasong JN, Kestens L. Cutting edge: resistance to HIV-1 infection among African female sex workers is associated with inhibitory KIR in the absence of their HLA ligands. J Immunol 2006;177: 6588-6592.

128 Boulet S, Sharafi S, Simic N, Bruneau J, Routy JP, Tsoukas CM, Bernard NF. Increased proportion of KIR3DS1 homozygotes in HIV-exposed uninfected individuals. AIDS 2008;22: 595-599.

129 Yeaman GR, Guyre PM, Fanger MW, Collins JE, White HD, Rathbun W, Orndorff KA, Gonzalez J, Stern JE, Wira CR. Unique CD8+ T cell-rich lymphoid aggregates in human uterine endometrium. J Leukoc Biol 1997;61:427-435.

130 White HD, Crassi KM, Givan AL, Stern JE, Gonzalez JL, Memoli VA, Green WR. CD3+ CD8+ CTL activity within the human female reproductive tract: influence of stage of the menstrual cycle and menopause. J Immunol 1997; 158: 3017–3027.

131 Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol 1994;68:6103-6110.

132 Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 1994;68:4650-4655.

133 Allen TM, O'Connor DH, Jing P, Dzuris JL, Mothé BR, Vogel TU *et al*. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature 2000;407:386-390.

134 Carrington M, O'Brien SJ. The influence of HLA genotype on AIDS. Annu Rev Med 2003;54:535-551.

135 Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA *et al*. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 1999;283:857-860.

136 Liu J, O'Brien KL, Lynch DM, Simmons NL, La Porte A, Riggs AM *et al.* Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. Nature 2009;457:87-91.

137 Goonetilleke N, Liu MK, Salazar-Gonzalez JF, Ferrari G, Giorgi E, Ganusov VV *et al.*The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. J Exp Med 2009; 206:1253-1272.

138 Liu Y, McNevin J, Rolland M, Zhao H, Deng W, Maenza J *et al.*Conserved HIV-1 epitopes continuously elicit subdominant cytotoxic T-lymphocyte responses. J Infect Dis 2009;200:1825-1833.

139 Liu Y, McNevin JP, Holte S, McElrath MJ, Mullins JI. Dynamics of viral evolution and CTL responses in HIV-1 infection. PLoS One. 2011 ;6:e15639. doi:10.1371/journal.pone.0015639.

140 Troyer RM, McNevin J, Liu Y, Zhang SC, Krizan RW, Abraha A *et al*. Variable fitness impact of HIV-1 escape mutations to cytotoxic T lymphocyte (CTL) response. PLoS Pathog. 2009;5:e1000365. doi:10.1371/journal.ppat.1000365.

141 Lewis MJ, Dagarag M, Khan B, Ali A, Yang OO. Partial Escape of HIV-1 from Cytotoxic T Lymphocytes during Chronic Infection. J Virol. 2012 ;86:7459-7463.

142 Frazer J K, Capra JD. Immunoglobulins: structure and function. In: Paul WE, editor. Fundamental Immunology 4th Edition. Philadelphia, Pennsylvania,USA: Lippincott-Raven; 1999; p37–74.

143 Nimmerjahn F, Ravetch JV. Fcgamma receptors as regulators of immune responses. Nat Rev Immunol 2008; 8:34–47.

144 Bélec L, Dupré T, Prazuck T, Tévi-Bénissan C, Kanga JM, Pathey O *et al.* Cervicovaginal overproduction of specific IgG to human immunodeficiency virus (HIV) contrasts with normal or impaired IgA local response in HIV infection. J Infect Dis 1995;172:691-697.

145 Anderson DJ, Politch JA, Tucker LD, Fichorova R, Haimovici F, Tuomala RE, Mayer KH. Quantitation of mediators of inflammation and immunity in genital tract secretions and their relevance to HIV type 1 transmission. AIDS Res Hum Retroviruses. 1998;14:(Suppl 1):S43-9.

146 Parren PW, Burton DR: The antiviral activity of antibodies in vitro and in vivo. Adv Immunol 2001;77:195-262.

147 Forthal DN, Moog C. Fc receptor-mediated antiviral antibodies. Curr Opin HIV AIDS 2009; 4:388-393.

148 Forthal DN, Landucci G, Daar ES. Antibody from patients with acute human immunodeficiency virus (HIV) infection inhibits primary strains of HIV type 1 in the presence of natural-killer effector cells. J Virol 2001; 75:6953–6961.

149 Mikell I, Sather DN, Kalams SA, Altfeld M, Alter G, Stamatatos L. Characteristics of the Earliest Cross-Neutralizing Antibody Response to HIV-1. PLoS Pathog 2011; 7: e1001251. doi:10.1371/journal.ppat.1001251.

150 Tomaras GD, Yates NL, Liu P, Qin L, Fouda GG, Chavez LL *et al.* Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp-41 antibodies with ineffective control of initial viraemia. J Virol 2008; 82:12449-12463.

151 Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X *et al*. Antibody neutralization and escape by HIV-1. Nature 2003; 422: 307-312.

152 Kwong PD, Doyle ML, Casper DJ, Cicala C, Leavitt SA, Majeed S *et al.* HIV-1 evades antibody-mediated neutralization through conformational masking of receptor binding sites. Nature 2002;420:678-682.

153 Labrijn AF, Poignard P, Raja A, Zwick MB, Delgado K, Franti M *et al.* Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. J Virol 2003;77:10557-10565.

154 Klein JS, Bjorkman PJ. Few and far between: how HIV may be evading antibody avidity. PLoS Pathog 2010;6:e1000908. doi:10.1371/journal.ppat.1000908.

155 Richman DD, Wrin T, Little SJ, Petropoulos C J. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci USA 2003; 100: 4144-4149.

156 Sather DN, Armann J, Ching LK, Mavrantoni A, Sellhorn G, Caldwell Z *et al.* Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. J Virol 2009;83: 757–769.

157 Binley JM, Lybarger E A, Crooks E T, Seaman M S, Gray E, Davis K L *et al.* Profiling the Specificity of Neutralizing Antibodies in a Large Panel of Plasmas from Patients Chronically Infected with Human Immunodeficiency Virus Type 1 Subtypes B and C. J Virol 2008; 82: 11651-11668.

158 Simek MD, Rida W, Priddy FH, Pung P, Carrow E, Laufer DS *et al*. Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high throughput neutralization assay together with an analytical selection algorithm. J Virol 2009;83: 7337–7348.

159 Doria-Rose NA, Klein RM, Daniels MG, O'Dell S, Nason M, Lapedes A *et al.* Breadth of human immunodeficiency virus-specific neutralizing activity in sera: clustering analysis and association with clinical variables. J Virol 2010;84: 1631–1636.

160 Li Y, Svehla K, Louder MK, Wycuff D, Phogat S, Miqueles SA *et al.* Analysis of neutralization specificities in polyclonal sera derived from human immunodeficiency virus type 1-infected individuals. J Virol 2009;83: 1045–1059.

161 Scheid JF, Mouquet H, Feldhahn N, Seaman MS, Velinzon K, Pietzsch J *et al*. Broad diversity of neutralizing antibodies isolated from memory B cells in HIV infected individuals. Nature 2009;458: 636–640.

162 Walker LM, Simek MD, Priddy F, Gach JS, Wagner D, Zwick MB *et al*. A Limited Number of Antibody Specificities Mediate Broad and Potent Serum Neutralization in Selected HIV-1 Infected Individuals. PLoS Pathog 2010;6: e1001028 doi:10.1371/journal.ppat.1001028.

163 Walker LM, Burton DR. Rational antibody-based HIV-1 vaccine design: Current approaches and future directions. Curr Opin Immunol 2010;22:358–366.

164 Derdeyn CA, Decker JM, Bibollet-Ruche F, Mokili JL, Muldoon M, Denham SA *et al.* Envelope-Constrained Neutralization-Sensitive HIV-1 After Heterosexual Transmission. Science 2004: 303:2019-2022.

165 Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG *et al*. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc Natl Acad Sci. USA 2008; 105:7552–7557.

166 Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H *et al.* Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. J Exp Med 2009;206:1273–1289.

167 Gosh M, Fahey JV, Shen Z, Lahey T, Cu-Uvin S, Wu Z *et al.* Anti-HIV activity in cervico-vaginal secretions from HIV-positive and –negative women correlate with innate antimicrobial levels and IgG antibodies. PLoS ONE 2010; 5: e11366. doi:10.1371/journal.pone.0011366.

168 Murphy FA. Epidemiology of Human and Animal Viral Diseases. In: Mahy BWJ, van Regenmortel MHV, editors. Encyclopedia of Virology. 3rd Edition. Oxford, England: Academic Press; 2008; p 140-148.

169 Boily MC, Baggaley RF, Wang L, Masse B, White RG, Hayes RJ, Alary M. Heterosexual risk of HIV-1 infection per sexual act: systematic review and meta-analysis of observational studies. Lancet Infect Dis 2009;9:118–129.

170 Baggaley R, White R, Boily M-C. HIV transmission riskthrough anal intercourse: systematic review, meta-analysis and implications for HIV prevention. Int J Epidemiol 2010; 39:1048–1063.

171 Jin F, Jansson J, Law M, Prestage GP, Zablotska I, Imrie JC *et al.* Per-contact probability of HIV transmission in homosexual men in Sydney in the era of HAART. AIDS 2010; 24: 907–913.

172 Halperin DT. Heterosexual anal intercourse: prevalence, cultural factors, and HIV infection and other health risks, Part I. AIDS Patient Care STDS 1999;13:717-730.

173 Baldwin JI, Baldwin JD. Heterosexual anal intercourse: an understudied, high-risk sexual behavior. Arch Sex Behav 2000; 29: 357–373.

174 Gross M, Holte SE, Marmor M, Mwatha A, Koblin BA, Mayer KH. Anal sex among HIV-seronegative women at high risk of HIV exposure. The HIVNET Vaccine Preparedness Study 2 Protocol Team. J Acquir Immune Defic Syndr 2000;24: 393–398.

175 Leichliter JS, Chandra A, Liddon N, Fenton KA, Aral SO. Prevalence andcorrelates of heterosexual anal and oral sex in adolescents and adults in the United States. J Infect Dis 2007; 196: 1852–1859.

176 Priddy FH, Wakasiaka S, Hoang TD, Smith DJ, Farah B, del Rio C, Ndinya-Achola J. Anal sex, vaginal practices and HIV incidence in female sex workers in urban Kenya. AIDS Research and Human Retroviruses 2011; 27: 1067-1072.

177 Kalichman SC, Pinkerton SD, Carey MP, Cain D, Mehlomakulu V, Simbayi LC *et al*. Heterosexual anal intercourse and HIV infection risks in the context of alcohol serving venues, Cape Town, South Africa. BMC Public Health 2011; 11:807. doi:10.1186/1471-2458-11-807.

178 Powers KA, Poole C, Pettifor AE, Cohen MS. Re-thinking the heterosexual infectivity of HIV-1: a systematic review and meta-analysis. Lancet Infect Dis 2008; 8:553-563.

179 Pilcher CD, Tien HC, Eron JJ Jr, Vernazza PL, Leu SY, Stewart PW *et al*. Quest Study; Duke-UNC-Emory Acute HIV Consortium. Brief but efficient: acute HIV infection and the sexual transmission of HIV. J Infect Dis 2004;189:1785-1792.

180 Pilcher CD, Joaki G, Hoffman IF, Martinson FE, Mapanje C, Stewart PW *et al*. Amplified transmission of HIV-1: comparison of HIV-1 concentrations in semen and blood during acute and chronic infection. AIDS 2007;21:1723-1730.

181 Quinn TC, Wawer MJ, Sewankambo NK, Serwadda D, Li C, Wabwire-Mangen F *et al.* Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. N Eng J Med 2000;342:921-929.

182 Gray RH, Wawer MJ, Brookmeyer R, Sewankambo NK, Serwadda D, Wabwire-Mangen F *et al.* Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. Lancet 2001; 357: 1149–1153.

183 Ma ZM, Stone M, Piatak M Jr, Schweighardt B, Haigwood NL, Montefiori D *et al.* High specific infectivity of plasma virus from the pre-ramp-up and ramp-up stages of acute simian immunodeficiency virus infection. J Virol 2009;83:3288-3297.

184 Wawer MJ, Gray RH, Sewankambo NK, Serwadda D, Li X, Laeyendecker O *et al*. Rates of HIV-1 transmission per coital act, by stage of HIV-1 infection, in Rakai, Uganda. J Infect Dis 2005; 191: 1403–1409.

185 Hollingsworth TD, Anderson RM, Fraser C. HIV-1- transmission, by stage of infection. J Infect Dis 2008;198: 687–693.

186 Miller WC, Rosenberg NE, Rutstein SE, Powers KA. Role of acute and early HIV infection in the sexual transmission of HIV. Curr Opin HIV AIDS. 2010;5:277-282.

187 Powers KA, Ghani AC, Miller WC, Hoffman IF, Pettifor AE, Kamanga G *et al.* The role of acute and early HIV infection in the spread of HIV and implications for transmission prevention strategies in Lilongwe, Malawi: a modelling study. Lancet 2011;378:256-268.

188 Salazar-Gonzalez JF, Bailes E, Pham KT, Salazar MG, Guffey MB, Keele BF *et al.* Deciphering human immunodeficiency virus type 1 transmission and early envelope

diversification by single-genome amplification and sequencing. J Virol 2008; 82: 3952-3970.

189 Lee HY, Giorgi EE, Keele BF, Gaschen B, Athreya GS, Salazar-Gonzalez JF *et al*. Modeling sequence evolution in acute HIV-1 infection. J Theor Biol 2009;261:341-360. Erratum in: J Theor Biol 2012;297:187.

190 Abrahams MR, Anderson JA, Giorgi EE, Seoighe C, Mlisana K, Ping LH *et al.* Quantitating the multiplicity of infection with human immunodeficiency virus type 1 subtype C reveals a non-Poisson distribution of transmitted variants. J Virol 2009;83:3556-3567. Erratum, J Virol 2009;83:6974.

191 Sagar M, Laeyendecker O, Lee S, Gamiel J, Wawer MJ, Gray RH *et al.* Selection of HIV variants with signature geneotypic characteristics during heterosexual transmission. J Infect Dis 2009; 199:580-589.

192 Li H, Bar KJ, Wang S, Decker JM, Chen Y, Sun C *et al*. High multiplicity infection by HIV-1 in men who have sex with men. PLoS Pathog 2010;6:e1000890. doi:10.1371/journal.ppat.1000890.

193 Bar KJ, Li H, Chamberland A, Tremblay C, Routy JP, Grayson T *et al*. Wide variation in the multiplicity of HIV-1 infection among injection drug users. J Virol 2010;84:6241-6247.

194 Masharsky AE, Dukhovlinova EN, Verevochkin SV, Toussova OV, Skochilov RV, Anderson JA *et al.* A substantial transmission bottleneck among newly and recently HIV-1-infected injection drug users in St Petersburg, Russia. J Infect Dis 2010;201:1697-1702.

195 Haaland RE, Hawkins PA, Salazar-Gonzalez J, Johnson A, Tichacek A, Karita E *et al.* Inflammatory genital infections mitigate a severe genetic bottleneck in heterosexual transmission of subtype A and C HIV-1. PLoS Pathog 2009;5:e1000274. doi:10.1371/journal.ppat.1000274.

196 Huang W, Toma J, Stawiski E, Fransen S, Wrin T, Parkin N *et al*. Characterisation of human immunodeficiency virus type 1 populations containing CXCR4 –using variants from recently infected individuals. AIDS Res Hum Retroviruses 2009; 25:795-802.

197 van't Wout AB, Kootstra NA, Mulder-Kampinga GA, Albrecht-van Lent N, Scherpbier HJ, Veenstra J *et al*. Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. J Clin Invest 1994; 94:2060-2067.

198 Dean M, Carrington M, Winkler C, Huttley GA, Smith MW, Allikmets R *et al*. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. Science 1996;273:1856-1862. Erratum in: Science 1996 ;274:1069.

199 Huang Y, Paxton WA, Wolinsky SM, Neumann AU, Zhang L, He T *et al*. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. Nat Med 1996;2:1240-1243.

200 Frost SD, Liu Y, Pond SL, Chappey C, Wrin T, Petropoulos CJ *et al*. Characterization of human immunodeficiency virus type 1 (HIV-1) envelope variation and neutralizing antibody responses during transmission of HIV-1 subtype B J Virol 2005;79:6523-6527.

201 Chohan B, Lang D, Sagar M, Korber B, Lavreys L, Richardson B, Overbaugh J. Selection for human immunodeficiency virus type 1 envelope glycosylation variants with shorter V1-V2 loop sequences occurs during transmission of certain genetic subtypes and may impact viral RNA levels. J Virol 2005;79:6528-6531.

202 Gnanakaran S, Bhattacharya T, Daniels M, Keele BF, Hraber PT, Lapedes AS *et al*. Recurrent Signature Patterns in HIV-1 B Clade Envelope Glycoproteins Associated with either Early or Chronic Infections. PLoS Pathog 2011;7: e1002209. doi:10.1371/journal.ppat.1002209.

203 Grivel J, Shattock R,Margolis L. Selective transmission of R5 HIV-1 variants: where is the gatekeeper?Journal of Translational Medicine 2010: 9(Suppl 1):S6. doi:10.1186/1479-5876-9-S1-S6.

204 Hughes JP, Baeten JM, Lingappa JR, Magaret AS, Wald A, de Bruyn G *et al*. Partners in Prevention HSV/HIV Transmission Study Team. Determinants of per-coital-act HIV-1 infectivity among African HIV-1-serodiscordant couples. J Infect Dis 2012;205:358-365.

205 Baeten JM, Kahle E, Lingappa JR, Coombs RW, Delany-Moretlwe S, Nakku-Joloba E *et al.* Genital HIV-1 RNA Predicts Risk of Heterosexual HIV-1 Transmission. Sci Transl Med 2011; 3: 77ra29.

206 Jakobsen MR, Ellett A, Churchill M.J, Gorry PR. Viral tropism, fitness and pathogenicity of HIV-1 subtype C. Future Virology 2010: 5: 219–231.

207 Novitsky V, Wang R, Bussmann H, Lockman S, Baum M, Shapiro R *et al*. HIV-1 Subtype C-Infected Individuals Maintaining High Viral Load as Potential Targets for the “Test-and-Treat” Approach to Reduce HIV Transmission. PLoS ONE 2010;5: e10148. doi:10.1371/journal.pone.0010148.

208 Coetzer M, Nedellec R, Cilliers T, Meyers T, Morris L, Mosier DE. Extreme genetic divergence is required for coreceptor switching in HIV-1 subtype C. J Acquir Immune Defic Syndr 2011;56:9-15.

209 Tobian A, Ssempijja V, Kigozi, Oliver AE, Serwadda D, Makumbi F *et al*. Incident HIV and herpes simplex virus type 2 infection among men in Rakai, Uganda. AIDS 2009; 23: 1589-1594.

210 Glynn JR, Biraro S, Weiss HA. Herpes Simplex Virus Type 2: a key role in HIV incidence. AIDS 2009; 23: 1595-1598.

211 van de Wijgert JH, Morrison CS, Brown J, Kwok C, Van Der Pol B, Chipato T *et al*. Disentangling contributions of reproductive tract infections to HIV acquisition in African Women. Sex Transm Dis 2009;36:357-364.

212 McClelland RS, Sangare L, Hassan WM, Lavreys L, Mandaliya K, Kiarie J *et al.* Infection with Trichomonas vaginalis increases the risk of HIV-1 acquisition. J Infect Dis 2007; 195:698–702.

213 van de Wijgert JH, Morrison CS, Cornelisse PG, Munjoma M, Moncada J, Awio P *et al.* Bacterial vaginosis and vaginal yeast, but not vaginal cleansing, increase HIV-1 acquisition in African women. J Acquir Immune Defic Syndr 2008; 48:203–210.

214 Sewankambo N, Gray RH, Wawer MJ, Paxton L, McNaim D, Wabwire-Mangen F *et al.* HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. Lancet 1997;350:546-50. Erratum in: Lancet 1997;350:1036.

215 Martin H L, Richardson BA, Nyange PM, Lavreys L, Hillier SL, Chohan B *et al*. Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. J Infect Dis 1999; 180:1863-1868.

216 Myer L, Denny LR, Telerant R, Souza M , Wright TC Jr, Kuhn L. Bacterial vaginosis and susceptibility to HIV infection in South African women: a nested case-control study. J Infect Dis 2005; 192:1372-1380.

217 Johnson LF, Lewis DA. The effect of genital tract infections on HIV-1 shedding in the genital tract: a systematic review and meta-analysis. Sex Transm Dis 2008; 35:946-959.

218 Cohen CR, Lingappa JR, Baeten JM, Ngayo MO, Spiegel CA , Hong T *et al.* Bacterial Vaginosis Associated with Increased Risk of Female-to-Male HIV-1 Transmission: A Prospective Cohort Analysis among African Couples. PLoS Med 2012; 9: e1001251. doi:10.1371/journal.pmed.1001251.

219 Al-Harthi L, Roebuck KA, Olinger GG, Landay A, Sha BE, Hashemi FB, Spear GT. Bacterial vaginosis associated microflora isolated from the female genital tract activates HIV-1 expression. J Acquir Immune Defic Syndr 1999; 21:194–202.

220 Thurman AR, Doncel GF. Innate immunity and inflammatory response to Trichomonas vaginalis and bacterial vaginosis: relationship to HIV acquisition. Am J Reprod Immunol 2011; 65: 89–98.

221 Comparison of female to male and male to female transmission of HIV in 563 stable couples. European Study Group on Heterosexual Transmission of HIV. BMJ 1992;304:809-813.

222 Aaby P, Ariyoshi K, Buckner M, Jensen H, Berry N, Wilkins A *et al.* Age of wife as a major determinant of male-to-female transmission of HIV-2 infection: a community study from rural West Africa. AIDS 1996;10:1585-1590.

223 Wira CR, Fahey JV. A new strategy to understand how HIV infects women: identification of a window of vulnerability during the menstrual cycle. AIDS 2008; 22:1909-1917.

224 Heffron R, Donnell D, Rees H, Celum C, Mugo N, Were E *et al*; Partners in Prevention HSV/HIV Transmission Study Team. Use of hormonal contraceptives and risk of HIV-1 transmission: a prospective cohort study. Lancet Infect Dis 2012;12:19-26. Erratum in: Lancet Infect Dis 2012;12:98.

225 Quinn TC, Overbaugh J. HIV/AIDS in women: an expanding epidemic. Science 2005;308:1582-1583.

226 Gray RH, Li X, Kigozi G, Serwadda D, Brahmbhatt H, Wabwire-Mangen F *et al.* Increased risk of incident HIV during pregnancy in Rakai, Uganda: a prospective study. Lancet 2005;366:1182-1188.

227 Mugo NR, Heffron R, Donnell D, Wald A, Were EO, Rees H *et al*; Partners in Prevention HSV/HIV Transmission Study Team. Increased risk of HIV-1 transmission in pregnancy: a prospective study among African HIV-1-serodiscordant couples. AIDS. 2011;25:1887-1895.

228 Rollenhagen C, Asin SN. Enhanced HIV-1 replication in ex vivo ectocervical tissues from post-menopausal women correlates with increased inflammatory responses. Mucosal Immunology 2011; 4: 671–681.

229 Haase A.Targeting early infection to prevent HIV-1 mucosal transmission. Nature 2010; 464: 217-223.

230 Miller CJ, Li Q, Abel K, Kim EY, Ma ZM, Wietgrefe S *et al.* Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus. J Virol 2005;79:9217–9227.

231 Lackner AA, Veazey RS. Current concepts in AIDS pathogenesis: insights from the SIV/macaque model. Annu Rev Med 2007;58:461-476.

232 Pilcher CD, Tien HC, Eron JJ Jr, Vernazza PL, Leu SY, Stewart PW, Goh LE, Cohen MS; Quest Study; Duke-UNC-Emory Acute HIV Consortium. Brief but efficient: acute HIV infection and the sexual transmission of HIV. J Infect Dis 2004;15;189:1785-1792.

233 Keele BF, Li H, Learn GH, Hraber P, Giorgi EE, Grayson T *et al.* Low-dose rectal inoculation of rhesus macaques by SIVsmE660 or SIVmac251 recapitulates human mucosal infection by HIV-1. J Exp Med 2009; 206:1117-1134.

234 Liu J, Keele BF, Li H, Keating S, Norris PJ, Carville A *et al.* Low-dose mucosal simian immunodeficiency virus infection restricts early replication kinetics and transmitted virus variants in rhesus monkeys. J Virol 2010; 84:10406-10412.

235 Stone M, Keele BF, Ma ZM, Bailes E, Dutra J, Hahn BH *et al.* A limited number of simian immunodeficiency virus (env) variants are transmitted to resus macaques vaginally inoculated with SIVmac251. J Virol 2010; 84:7083-7095.

236 Hatziioannou T, Ambrose Z, Chung NP, Piatak M Jr, Yuan F, Trubey CM et al. A macaque model of HIV-1 infection. Proc Natl Acad Sci U S A 2009;106:4425-4429.

237 Grivel J and Margolis L. Use of human tissue explants to study human infectious agents. Nature Protocols 2009; 4: 256-269.

238 Wilson NA, Keele BF, Reed JS, Piaskowski SM, MacNair CE, Bett AJ *et al.* Vaccine-induced cellular responses control simian immunodeficiency virus replication after heterologous challenge. J Virol 2009: 83:6508-6521.

239 Hladik F, McElrath MJ. Setting the stage: host invasion by HIV. Nat Rev Immunol 2008; 8:447-457.

240 Hladik F, Hope TJ. HIV infection of the genital mucosa in women. Curr HIV/AIDS Rep 2009; 6:20-28.

241 Blaskewicz C D, Pudney J, Anderson D J. Structure and function of intercellular junctions in human cervical and vaginal mucosal epithelia. Biol Reprod 2011; 85: 97-104.

242 Pudney J, Quayle AJ, Anderson DJ. Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone. Biol Reprod 2005;73:1253-1263.

243 Norvell MK, Benrubi GI, Thompson RJ. Investigation of microtrauma after sexual intercourse. J Reprod Med 1984; 29:269–271.

244 Kell PD, Barton SE, Edmonds DK, Boag FC. HIV Infection in a patient with Meyer-Rokitansky-Kuster-Hauser syndrome. JR Soc Med 1992;85:706-707.

245 Miller CJ, Alexander NJ, Vogel P, Anderson J, Marx PA. Mechanism of genital transmission of SIV: a hypothesis based on transmission studies and the location of SIV in the genital tract of chronically infected female rhesus macaques. J Med Primatol 1992; 21:64-68.

246 Padian NS, van der Straten A, Ramjee G, Chipato T, de Bruyn G, Blanchard K et al. MIRA Team. Diaphragm and lubricant gel for prevention of HIV acquisition in southern African women: a randomised controlled trial. Lancet 2007;370:251-261.

247 Guimaraes MD, Vlahov D, Castilho EA. Postcoital vaginal bleeding as a risk factor for transmission of the human immunodeficiency virus in a heterosexual partner study in Brazil. Rio de Janeiro Heterosexual Study Group. Arch Intern Med 1997; 157:1362–1368.

248 Hladik F, Sakchalathorn P, Ballweber L, Lentz G, Fialkow M, Eschenbach D *et al.* Initial events in establishing vaginal entry and infection by human immunodeficiency virus type -1. Immunity 2007; 26: 257-270.

249 Marchiando AM, Graham WV, Turner JR. Epithelial barriers in homeostasis and disease. Annu Rev Pathol 2010; 5: 119-144.

250 Wira CR, Fahey JV, Ghosh M, Patel MV, Hickey DK, Ochiel DO. Sex hormone regulation of innate immunity in the female reproductive tract: the role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. Am J Reprod Immunol 2010; 63:544-565.

251 Nazli A, Chan O, Dobson-Belaire WN, Ouellet M, Tremblay MJ, Gray-Owen SD *et al*. Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation PLoS Pathog 2010; 6:e1000852. doi:10.1371/journal.ppat.1000852.

252 Bomsel M: Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier. Nat Med 1997; 3:42–47.

253 Howell AL, Edkins RD, Rier SE, Yeaman GR, Stern JE, Fanger MW, Wira CR: Human immunodeficiency virus type 1 infection of cells and tissues from the upper and lower human female reproductive tract. J Virol 1997; 71:3498–3506.

254 Dezzutti CS, Guenthner PC, Cummins Jr JE, Cabrera T, Marshall JH, Dillberger A, Lal RB. Cervical and prostate primary epithelial cells are not productively infected but sequester human immunodeficiency virus type 1. J Infect Dis 2001; 183:1204–1213.

255 Wu Z, Chen Z, Phillips DM. Human genital epithelial cells capture cell-free human immunodeficiency virus type 1 and transmit the virus to CD4+ Cells: implications for mechanisms of sexual transmission. J Infect Dis 2003; 188:1473–1482.

256 Bobardt MD, Chatterji U, Selvarajah S, Van der Schueren B, David G, Kahn B, Gallay PA. Cell-free human immunodeficiency virus type 1 transcytosis through primary genital epithelial cells. J Virol 2007; 81:395–405.

257 Saidi H, Magri G, Nasreddine N, Requena M, Belec L: R5- and X4-HIV-1 use differentially the endometrial epithelial cells HEC-1A to ensure their own spread: implication for mechanisms of sexual transmission. Virology 2007; 358:55–68.

258 Bomsel M, Alfsen A. Entry of viruses through the epithelial barrier: pathogenic trickery. Nat Rev Mol Cell Biol 2003;4:57-68.

259 Yeaman GR, Asin S, Weldon S, Demian DJ, Collins JE, Gonzalez JL *et al.* Chemokine receptor expression in the human ectocervix: implications for infection by the human immunodeficiency virus-type I. Immunology 2004; 113:524–533.

260 Bobardt MD, Saphire AC, Hung HC, Yu X, Van der Schueren B *et al*. Syndecan captures, protects and transmits HIV to T lymphocytes. Immunity 2003;18:27-39.

261 Furuta Y, Eriksson K, Svennerholm B, Fredman P, Horal P, Jeansson S *et al.* Infection of vaginal and colonic epithelial cells by the human immunodeficiency virus type 1 is neutralized by antibodies raised against conserved epitopes in the envelope glycoprotein gp120. Proc Natl Acad Sci USA 1994; 91:12559–12563.

262 Stoddard E, Ni H, Cannon G, Zhou C, Kallenbach N, Malamud D, Weissman D. gp340 promotes transcytosis of human immunodeficiency virus type 1 in genital tract-derived cell lines and primary endocervical tissue. J Virol 2009; 83:8596–8603.

263 Madsen J, Mollenhauer J, Holmskov U. Review: Gp-340/DMBT1 in mucosal innate immunity. Innate Immun 2010;16: 160-167.

264 Fanibunda SE, Modi DN, Gokral JS, Bandivdekar AH. HIV gp120 Binds to Mannose Receptor on Vaginal Epithelial Cells and Induces Production of Matrix Metalloproteinases. PLoS ONE 2011; 6: e28014. doi:10.1371/journal.pone.0028014.

265 Herold BC, Mesquita PM, Madan RP, Keller MJ. Female genital tract secretions and semen impact the development of microbicides for the prevention of human immunodeficiency virus (HIV) and other sexually transmitted infections. Am J Reprod Immunol 2011; 65:325–333.

266 Politch JA, Tucker L, Bowman FP, Anderson DJ. Concentrations and significance of cytokines and other immunologic factors in semen of healthy fertile men. Hum Reprod 2007;11:2928-2935.

267 Sharkey DJ, Macpherson AM, Tremellen KP, Robertson SA. Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells. Mol Hum Reprod 2007; 13:491–501.

268 Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA. Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. J Immunol 2012;188:2445-1454.

269 Münch J, Rücker E, Ständker L, Adermann K, Goffinet C, Schindler M *et al.* Semen-derived amyloid fibrils drastically enhance HIV infection.Cell. 2007;131:1059-1071.

270 Figdor CG, van Kooyk Y, Adema GJ. C-type lectin receptors on dendritic cells and Langerhans' cells. Nature Rev Immunol 2002;2:77-84.

271 Valladeau J, Ravel O, Dezutter-Dambuyant C. Langerin: a novel C-type lectin specific to Langerhans' cell, is an endocytic receptor that induces formation of Birkbeck granules. Immunity 2000; 12:71-81.

272 Hladik F, Sakchalathorn P, Ballweber L, Lentz G, Fialkow M, Eschenbach D *et al.* Initial events in establishing vaginal entry and infection by human immunodeficiency virus type -1. Immunity 2007; 26: 257-270.

273 Ballweber L, Robinson B, Kreger A, Fialkow M, Lentz G, McElrath MJ, Hladik F. Vaginal langerhans cells nonproductively transporting HIV-1 mediate infection of T cells. J Virol 2011; 85:13443-13447.

274 McDonald D, Wu L, Bohks SM, KewalRamani VN, Unutmaz D, Hope TJ. Recruitment of HIV and its receptors to dendritic cell-T cell junctions. Science 2003; 300:1295-1297.

275 Arrighi JF, Pion M, Garcia E, Escola JM, van Kooyk Y, Geijtenbeek TB, Piguet V. DC-SIGN-mediated infectious synapse formation enhances X4 HIV-1 transmission from dendritic cells to T cells. J Exp Med 2004;200:1279-1288.

276 Mothes W, Sherer NM, Jin J, Zhong P. Virus cell-to-cell transmission. J Virol 2010; 84:8360-8368.

277 Nikolic DS, Lehmann M, Felts R, Garcia E, Blanchet FP, Subramaniam S, Piguet V. HIV-1 activates CdC-42 and induces membrane extensiuons in immature dendritic cells to facilitate cell-tocell virus propagation. Blood 2011; 118:4841-4852.

278 Felts RL, Narayan K, Estes JD, Shi D, Trubey CM, Fu J *et al*. 3D visualization of HIV transfer at the virological synapse between dendritic cells and T cells. Proc Natl Acad Sci USA 2010; 107: 13336-13341.

279 Martin N, Welsch S, Jolly C, Briggs JAG, Vaux D, Sattentau QJ. Virological synapse-mediated spread of human immunodeficiency virus type 1 between T cells is sensitive to entry inhibition. J Virol 2010; 84: 3516–3527.

280 Benlarhrech A, Patterson S. HIV-1 infection and induction of interferon alpha in plasmacytoid dendritic cells. Curr Opin HIV AIDS 2011; 6: 373-378.

281 Li Q, Estes JD, Schlievert PM, Duan L, Brosnahan AJ, Southern PJ *et al.* Glycerol monolaurate prevents mucosal SIV transmission. Nature 2009;458:1034-1038.

282 Zhang Z, Schuler T, Zupancic M, Wietgrefe S, Staskus KA, Reimann KA *et al.* Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. Science 1999;286:1353-1357. Erratum in: Science 1999; 286:2273.

283 Li Q, Duan L, Estes JD, Ma ZM, Rourke T, Wang Y *et al.* Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. Nature 2005; 434:1148-1152.

284 Schacker T, Little S, Connick E, Gebhard K, Zhang ZQ, Krieger J *et al.* Productive infection of T cells in lymphoid tissues during primary and early human immunodeficiency virus infection. J Infect Dis 2001; 183:555–562.

285 Arthos J, Cicala C, Martinelli E, Macleod K, Van Ryk D, Wei D *et al*. HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells. Nat Immunol 2008 ;9:301-309.

286 Nawaz F, Cicala C, Van Ryk D, Block KE, Jelicic K, McNally JP *et al*. The genotype of early-transmitting HIV gp120s promotes α (4) β(7)-reactivity, revealing α (4)β(7) +/CD4+ T cells as key targets in mucosal transmission. PLoS Pathog 2011;7:e1001301. doi:10.1371/journal.ppat.1001301.

287 Gosselin A, Monteiro P, Chomont N, Diaz-Griffero F, Said EA, Fonseca S *et al*. Peripheral blood CCR4+CCR6+ and CXCR3+CCR6+CD4+ T cells are highly permissive to HIV-1 infection. J Immunol 2010;184:1604-1616.

288 Monteiro P, Gosselin A, Wacleche VS, El-Far M, Said EA, Kared H *et al.* Memory CCR6+CD4+ T cells are preferentialtargets for productive HIV type 1 infection regardless of their expression of integrin β7. J Immunol 2011;186:4618-4630.

289 Sugimoto C, Nakamura S, Hagen SI, Tsunetsugu-Yokota Y, Villinger F, Ansari AA *et al*. Glycosylation of SIV influences immune-tissue targeting during primary infection that leads to immunodeficiency or viral control. J Virol 2012;86: 9323-9336.

290 Fiebig EW, Wright DJ, Rawal BD, Garrett PE, Schumacher RT, Peddada L *et al.* Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. AIDS 2003;17:1871–1879.

291 Reinhart TA, Rogan MJ, Huddleston D, Rausch DM, Eiden LE, Haase AT. Simian immunodeficiency virus burden in tissues and cellular compartments during clinical latency and AIDS. J Infect Dis 1997;176:1198–1208.

292 Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. Proc Natl Acad Sci U S A 1998;95:8869-8873.

293 Kelly CG, Shattock RJ. Specific microbicides in the prevention of HIV infection (Symposium). J Intern Med 2011; 270: 509–519.

294 Anderson R M, May R M. Infectious Diseases of Humans-Dynamics and Control. Oxford, England: Oxford University Press; 1992.

295 Kurth AE, Celum C, Baeten JM, Vermund SH, Wasserheit JN. Combination HIV prevention: significance, challenges and opportunities. Curr HIV/AIDS Rep 2011; 8:62-72.

296 Morris M, Kretzschmar M. Concurrent sexual partnerships and the spread of HIV. AIDS 1997; 11:641-648.

297 Mah TL, Halperin DT. The evidence for the role of concurrent partnerships in Africa's HIV epidemics: a response to Lurie and Rosenthal. AIDS Behav 2010;14:25-28.

298 Morris M. Barking up the wrong evidence tree. Comment on Lurie & Rosenthal, 'Concurrent partnerships as a driver of the HIV epidemic in sub-Saharan Africa? The evidence is limited'. AIDS Behav 2010;14:31-33.

299 Coates TJ, Richter L, Caceres C. Behavioural strategies to reduce HIV transmission: how to make them work better. Lancet 2008;372:669-684.

300 Gupta GR, Parkhurst JO, Ogden JA, Aggleton P, Mahal A. Structural approaches to HIV prevention. Lancet 2008;372:764-775.

301 Sperling RS, Shapiro DE, Coombes RW Todd JA, Herman SA, McSherry GD *et al*. Maternal viral load, zidovudine treatment and the risk of transmission of human immunodeficiency virus type 1. N Eng J Med 1996; 335:1621-1629.

302 Jourdain G, Mary JY, Coeur SL, Ngo-Giang-Huong N, Yuthavisuthi P, Limtrakul A *et al*. Perinatal HIV Prevention Trial Group, Thailand. [Risk factors for in utero or intrapartum mother-to-child transmission of human immunodeficiency virus type 1 in Thailand.]. J Infect Dis 2007;196:1629-1636.

303 Mnyani C, McIntyre J. Preventing mother-to-child transmission of HIV. BJOG 2009; 116 (suppl.1): 71-76.

304 Sturt AS, Dokubo EK, Sint TT. Antiretroviral therapy (ART) for treating HIV infection in ART-eligible pregnant women. Cochrane Database of Systematic Reviews 2010;3:CD008440. doi: 10.1002/14651858.CD008440.

305 Attia S, Egger M, Muller M, Zwahlen M, Low N. Sexual transmission of HIV according to viral load and antiretroviral therapy: systematic review and meta-analysis. AIDS 2009; 23: 1397–1404.

306 Sulivan P, Kayitenkore K, Chomba E,Karita E, Mwananyanda L *et al.* Reduction of HIV Transmission Risk and High Risk Sex while Prescribed ART:Results from Discordant Couples in Rwanda and Zambia. 16th Conference on Retroviruses and Opportunistic Infections, Montreal, Canada; Feb 8-11, 2009. Abstract 52b, Late Breaker.

307 Reynolds S, Makumbi F, Nakigozi G, Kagaayi J, Gray R, Wawer M, Quinn T, Serwadda D. HIV-1 transmission among HIV-1 discordant couples before and after the introduction of antiretroviral therapy. AIDS 2011;25:473-477.

308 Anglemyer A, Rutherford GW, Baggaley RC, Egger M, Siegfried N. Antiretroviral therapy for prevention of HIV transmission in HIV-discordant couples. Cochrane Database of Systematic Reviews 2011; 8: CD009153. doi: 10.1002/14651858.CD009153.pub2.

309 Donnell D, Baeten JM, Kiarie J, Thomas KK, Stevens W, Cohen CR *et al.;* Partners in Prevention HSV/HIV Transmission Study Team. Heterosexual HIV-1 transmission after initiation of antiretroviral therapy: a prospective cohort analysis. Lancet. 2010;375:2092-2098.

310 Cohen MS, Chen YQ, McCauley M, Gamble T, Hosseinipour MC, Kumarasamy N *et al.* Prevention of HIV-1 infection with early antiretroviral therapy. NEng J Med 2011; 365: 493-505.

311 Dunkle KL, Stephenson R, Karita E, Chomba E, Kayitenkore K, Vwalika C *et al.* New heterosexually transmitted HIV infections in married or cohabiting couples in urban Zambia and Rwanda: an analysis of survey and clinical data. Lancet 2008; 371: 2183-2191.

312 Eyawo O, de Walque D, Ford N, Gakii G, Lester RT, Mills EJ. HIV status in discordant couples in sub-Saharan Africa: a systematic review and meta-analysis. Lancet Infect Dis. 2010;10:770-777.

313 Coburn BJ, Gerberry DJ, Blower S. Quantification of the role of discordant couples in driving incidence of HIV in sub-Saharan Africa. Lancet Infect Dis 2011; 11: 263-264.

314 Vernazza P, Hirschel B, Bernasconi E, Flepp M. Les personnes séropositives ne souffrant d'aucune autre MST et suivant un traitement antirétroviral efficace ne transmettent pas le VIH par voie sexuelle. Bulletin des médecins suisses 2008;89:165-169.

315 Kalichman S, Di Berto G, Eaton L. Human immunodeficiency virus viral load in blood plasma and semen: review and implications of empirical findings. Sex Trans Dis 2008; 35:55–60.

316 Marcelin AG, Tubiana R, Lambert-Niclot S, Lefebvre G, Dominguez S, Bonmarchand M, Vauthier-Brouzes D, Marguet F, Mousset-Simeon N, Peytavin G, Poirot C.Detection of HIV-1 RNA in seminal plasma samples from treated patients with undetectable HIV-1 RNA in blood plasma. AIDS 2008 ;22:1677-1679.

317 Sheth PM, Kovacs C, Kemal KS, Jones RB, Raboud JM, Pilon R et al; Toronto Mucosal Immunology Group. Persistent HIV RNA shedding in semen despite effective antiretroviral therapy. AIDS 2009;23:2050-2054.

318 Pasquier C, Sauné K, Raymond S, Moinard N, Daudin M, Bujan L, Izopet J.Determining seminal plasma human immunodeficiency virus type 1 load in the context of efficient highly active antiretroviral therapy. J Clin Microbiol 2009; 47:2883-2887.

319 Anderson BL, Cu-Uvin S. Determinants of HIV shedding in the lower genital tract of women. Current Infectious Disease Reports 2008; 10:505–511.

320 Taylor S and Davies S. Antiretroviral drug concentrations in the male and female genital tract: implications for the sexual transmission of HIV. Curr Opin HIV AIDS 2010;5:335-343.

321 Ghosn J, Viard JP, Katlama C, de Almeida M, Tubiana R, Letourneur F *et al*. Evidence of genotypic resistance diversity of archived and circulating viral strains in blood and semen of pre-treated HIV-infected men. AIDS 2004;18:447-457.

322 Diem K, Nickle DC, Motoshige A, Fox A, Ross S, Mullins JI, Corey L, Coombs RW, Krieger JN. Male genital tract compartmentalization of human immunodeficiency virus type 1 (HIV). AIDS Res Hum Retroviruses 2008;24:561-571.

323 HIV Trialists' Collaborative Group. Zidovudine, didanosine, and zalcitabine in the treatment of HIV infection: meta analyses of the randomised evidence. Lancet 1999;353:2014-2025.

324 Palella FJ, Jr., Deloria-Knoll M, Chmiel JS, Moorman AC, Wood KC, Greenberg AE, Holmberg SD; HIV Outpatient Study Investigators. Survival benefit of initiating antiretroviral therapy in HIV-infected persons in different CD4+ cell strata. Ann Intern Med 2003;138:620-626.

325 When To Start Consortium, Sterne JA, May M, Costagliola D, de Wolf F, Phillips AN, Harris R, et al. Timing of initiation of antiretroviral therapy in AIDS-free HIV-1-infected patients: a collaborative analysis of 18 HIV cohort studies. Lancet 2009;373:1352-1363.

326 Severe P, Juste MA, Ambroise A, Eliacin L, Marchand C, Apollon S *et al.* Early versus standard antiretroviral therapy for HIV-infected adults in Haiti. N Engl J Med 2010;363:257-265.

327 HIV-CAUSAL Collaboration, Cain LE, Logan R, Robins JM, Sterne JA, Sabin C, Bansi L *et al.* When to initiate combined antiretroviral therapy to reduce mortality and AIDS defining illness in HIV-infected persons in developed countries: an observational study. Ann Intern Med 2011;154:509-515.

328 Kitahata MM, Gange SJ, Abraham AG, Merriman B, Saag MS, Justice AC *et al*. Effect of early versus deferred antiretroviral therapy for HIV on survival. Na-Accord Investigators. N Engl J Med 2009;360:1815-1826.

329 Writing Committee of the CASCADE Collaboration. Timing of HAART initiation and clinical outcomes in human immunodeficiency virus type 1 seroconverters. Arch Intern Med 2011;171:1560-1569.

330 Strategies for Management of Antiretroviral Therapy (SMART) Study Group, Emery S, Neuhaus JA, Phillips AN, Babiker A, Cohen CJ, Gatell JM *et al*. Major clinical outcomes in antiretroviral therapy (ART)-naive participants and in those not receiving ART at baseline in the SMART study. J Infect Dis 2008;197:1133-1144.

331 The START Study Homepage. Available at: http://thestartstudy.org/index.html. Accessed July 13 2012.

332 Granich RM, Gilks CF, Dye C, De Cock KM, Williams BG. Universal voluntary HIV testing with immediate antiretroviral therapy as a strategy for elimination of HIV transmission: a mathematical model. Lancet 2009;373:48-57.

333 Granich R, Kahn JG, Bennett R, Holmes CB, Garg N, Serenata C *et al*. Expanding ART for treatment and prevention of HIV in South Africa: estimated cost and cost-effectiveness 2011-2050. PLoS One. 2012;7:e30216. doi:10.1371/journal.pone.0030216.

334 PopART-Reducing HIV transmission at a population level. Available at: http://www1.imperial.ac.uk/medicine/research/researchthemes/infection/infectious_diseases/hiv_trials/hiv_prevention_technologies/popart/. Accessed July 11, 2012.

335 HIV, viral hepatitis and sexually transmissible infections in Australia: annual surveillance report 2011. Sydney, Australia: The Kirby Institute, University of New South Wales; 2011.

336 Wilson DP. HIV Treatment as Prevention: Natural Experiments Highlight Limits of Antiretroviral Treatment as HIV Prevention. PLoS Med 2012; 9: e1001231. doi:10.1371/journal.pmed.1001231.

337 Das M, Chu PL, Santos GM, Scheer S, Vittinghoff E *et al*. Decreases in community viral load are accompanied by reductions in new HIV infections in San Francisco. PLoS ONE 2010; 5: e11068. doi:10.1371/journal.pone.0011068.

338 Law MG, Woolley I, Templeton DJ, Roth N, Chuah J, Mulhall B *et al*.; Australian HIV Observational Database (AHOD). Trends in detectable viral load by calendar year in the Australian HIV observational database. J Int AIDS Soc 2011;14:10. doi:10.1186/1758-2652-14-10.

339 Gardner EM, McLees MP, Steiner JF, Del Rio C, Burman WJ. The spectrum of engagement in HIV care and its relevance to test-and-treat strategies for prevention of HIV infection. Clin Infect Dis 2011;52:793-800.

340 Lange JMA.Editorial Commentary: “Test and Treat”: Is It Enough? Clin Infect Dis 2011; 52: 801-802.

341 Flynn NM, Forthal DN, Harro CD, Judson FN, Mayer KH, Para MF; rgp120 HIV Vaccine Study Group. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J Infect Dis 2005; 191: 654–665.

342 Pitisuttithum P, Gilbert P, Gurwith M, Heyward W, Martin M, van Griensven F *et al.* Randomized, double-blind placebo controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. J Infect Dis 2006; 194: 1661-1671.

343 Kim JH, Rerks-Ngarm S, Excler J, Michael NL. HIV Vaccines: lessons learned and the way forward. Curr Opin HIV AIDS 2010; 5: 428-434.

344 Mascola JR, Montefiori DC. The role of antibodies in HIV vaccines. Annu Rev Immunol 2010; 28:413–444.

345 Mc Elrath MJ, Haynes BF. Induction of immunity to human immunodeficiency virus type 1 by vaccination. Immunity 2010; 33: 542-544.

346 Jones NG, DeCamp A, Gilbert P, Peterson ML, Gurwith M, Cao H. AIDSVAX immunisation induces HIV specific CD8+ T cell responses in High-risk, HIV-negative volunteers who subsequently acquire HIV infection. Vaccine 2009; 27: 1136-1140.

347 Buchbinder SP, Mehrota DV, Duerr A, Fitzgerald DW, Mogg R, Li D, Gilbert PB *et al*. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomized, placebo-controlled, test of concept trial. Lancet 2008; 372: 1881-1893.

348 McElrath MJ, De Rosa SC, Moodie Z, Dubey S, Kierstead L, Janes H *et al.* HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. Lancet 2008;372:1894–1905.

349 Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S *et al.* Vaccination with ALVAC and AIDSVAX to prevent HIV -1 infection in Thailand. N Engl J Med 2009; 361: 2209-2220.

350 Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM *et al*. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N Engl J Med 2012; 366:1275-1286.

351 Edlefsen P, Rolland M, Bonsignori M, Liao H-X, Haynes B, Gilbert *et al*. RV144 Sequencing Study Group: Sieve Analysis and Epitope Mapping Support Anti-V2 Antibody-mediated Immune Pressure of ALVAC-AIDSVAX Vaccination in the RV144 Trial. 19th Conference on Retroviruses and Opportunistic Infections, Seattle, USA; March 5-8, 2012. Abstract 421.

352 Virgin HW, Walker BD. Immunology and the elusive AIDS vaccine. Nature 2010; 464: 224-231.

353 Auvert B, Talijaard D, Lagarde E, Sobngwi-Tambekou J, Sitta R, Puren A *et al.* Randomised, controlled intervention trial of male circumcision for reduction of HIV infection risk: the ANRS 1265 Trial. PLoS Med 2005;2:e298.
doi/ 10.1371/journal.pmed.0020298.

354 Gray RH, Kigozi G, Serwadda D, Makumbi F, Watya S, Nalugoda F *et al.* Male circumcision for HIV prevention in men in Rakai, Uganda: a randomized trial. Lancet 2007; 369: 657-666.

355 Bailey RC, Moses S, Parker CB, Agot K, Maclean I, Krieger JN *et al*. Male circumcision for HIV prevention in young men in Kisumu, Kenya: a randomized controlled trial. Lancet 2007; 369: 643-656.

356 Hargreave T. Male circumcision: towards a World Health Organisation normative practice in resource limited settings. Asian J Androl 2010;12:628-638.

357 Weiss HA, Hankins CA, Dickson K. Male circumcision and risk of HIV infection in women: a systematic review and meta-analysis. Lancet Infect Dis 2009; 9: 669-677.

358 Wawer MJ, Makumbi F, Kigozi G, Serwadda D, Watya S, Nalugoda F *et al.* Circumcision in HIV-infected men and its effect on transmission to female partners in Rakai, Uganda: a randomized controlled trial. Lancet 2009;374: 229-237.

359 Grosskurth H, Mosha F, Todd J, Mwijarubi E, Klokke A, Senkoro K *et al*. Impact of improved treatment of sexually transmitted diseases on HIV infection in rural Tanzania: randomised controlled trial. Lancet 1995;346:530-536.

360 Wawer MJ, Sewankambo NK, Serwadda D, Quinn TC, Paxton LA, Kiwanuka N *et al*. Control of sexually transmitted diseases for AIDS prevention in Uganda: a randomised community trial. Rakai Project Study Group. Lancet 1999;353:525-535.

361 Kamali A, Quigley M, Nakiyingi J, Kinsman J, Kengeya-Kayondo J, Gopal R *et al*. Syndromic management of sexually-transmitted infections and behaviour change interventions on transmission of HIV-1 in rural Uganda: a community randomised trial. Lancet 2003;361:645-652.

362 Kaul R, Kimani J, Nagelkerke NJ, Fonck K, Ngugi EN, Keli F *et al*; Kibera HIV Study Group. Monthly antibiotic chemoprophylaxis and incidence of sexually transmitted infections and HIV-1 infection in Kenyan sex workers: a randomized controlled trial. JAMA 2004;291:2555-2562.

363 Celum C, Wald A, Hughes J, Sanchez J, Reid S, Delany-Moretlwe S *et al*. HPTN 039 protocol team. Effect of aciclovir on HIV 1 acquisition in herpes simplex virus type 2 seropositive women and men who have sex with men: a randomized double-blind, placebo-controlled trial. Lancet 2008; 371:2109-2119.

364 Watson-Jones D, Weiss HA, Rusizoka M, Changalucha J, Baisley K, Mugeye K *et al.* Effect of herpes simplex suppression on incidence of HIV among women in Tanzania. N Eng J Med 2008; 358:1560-1571.

365 Celum C, Wald A, Lingappa JR, Magaret AS, Wang RS, Mugo N *et al*. Partners in Prevention HSV/HIV Study Team. Acyclovir and transmission of HIV-1 from persons infected with HIV-1 and HSV-2. N Engl J Med 2010; 362: 427-439.

366 Vanpouille C, Lisco A, Margolis L. Acyclovir: a new use for an old drug. Curr Opin Infect Dis 2009;23:479-483.

367 Johnson KE, Sherman ME, Ssempiija V, Tobian AA, Zenilman JM, Duggan MA *et al*. Foreskin inflammation is associated with HIV and herpes simplex virus type-2 infections in Rakai, Uganda. AIDS 2009;23:1807-1815.

368 Johnson KE, Redd AD, Quinn TC, Collinson-Streng AN, Cornish T, Kong X *et al.* Effects of HIV-1 and herpes simplex virus type 2 infection on lymphocyte and dendritic cell density in adult foreskins from Rakai, Uganda. J Infect Dis 2011;203:602-609.

369 Holmes KK, Levine R, Weaver M. Effectiveness of condoms in preventing sexually transmitted infections. Bull World Health Organ 2004;82:454-461.

370 Mindel A, Sawleshwarkar S. Condoms for sexually transmissible infection prevention: politics versus science. Sex Health 2008; 5:1-8.

371 Crepaz N, Lyles CM, Wolitski RJ, Passin WF, Rama SM, Herbst JH *et al*; HIV/AIDS Prevention Research Synthesis (PRS) Team. Do prevention interventions reduce HIV risk behaviours among people living with HIV? A meta-analytic review of controlled trials. AIDS 2006;20:143-157.

372 Johnson WD, Diaz RM, Flanders WD, Goodman M, Hill AN, Holtgrave D *et al.* Behavioral interventions to reduce risk for sexual transmission of HIV among men who have sex with men. Cochrane Database Syst Rev 2008;3:CD001230.

373 McCoy SI, Kangwende RA, Padian NS. Behavior change interventions to prevent HIV infection among women living in low and middle income countries: a systematic review. AIDS Behav 2010 ;14:469-482.

374 Wariki WM, Ota E, Mori R, Koyanagi A, Hori N, Shibuya K. Behavioral interventions to reduce the transmission of HIV infection among sex workers and their clients in low- and middle-income countries. Cochrane Database Syst Rev 2012;2:CD005272.

375 Krishnan S, Dunbar MS, Minnis AM, Medlin CA, Gerdts CE, Padian NS. Poverty, gender inequities, and women's risk of human immunodeficiency virus/AIDS. Ann N Y Acad Sci. 2008;1136:101-110.

376 Vijayakumar G, Mabude Z, Smit J, Beksinska M, Lurie M. A review of female-condom effectiveness: patterns of use and impact on protected sex acts and STI incidence Int J STD AIDS 2006 17: 652-659.

377 Hoffman S, Mantell J, Exner T, Stein Z. The Future of the Female Condom. Perspectives on Sexual and Reproductive Health 2004; 36: 120–126.

378 Matthews J, Harrison T. An update on female-controlled methods for HIV prevention: Female condom, microbicides and cervical barriers. Southern African Journal of HIV Medicine 2006;7:7-11.

379 Dowdy D, Sweat M, Holtgrave D. Country-wide distribution of the nitrile female condom(FC 2) in Brazil and South Africa: a cost-effectiveness analysis. AIDS 2006;20:2091-2098.

380 Free C, Roberts IG, Abramsky T, Fitzgerald M, Wensley F. A systematic review of randomised controlled trials of interventions promoting effective condom use. J Epidemiol Community Health 2011; 65: 100 – 110.

381 Stein ZA. HIV prevention: the need for methods women can use. Am J Public Health. 1990; 80:460–462.

382 Stone A. Microbicides: A new approach to preventing HIV and other sexually transmitted infections. Nat Rev Drug Discov 2002;1: 977–985.

383 McGowan I. Rectal microbicides: a new focus for HIV prevention. Sex Transm Infect 2008; 84: 413-417.

384 Boily MC, Dimitrov D, Abdool Karim SS, Mâsse B. The future role of rectal and vaginal microbicides to prevent HIV infection in heterosexual populations: implications for product development and prevention. Sex Transm Infect 2011;87:646-653.

385 Fuchs EJ, Lee LA, Torbenson MS, Parsons TL, Bakshi RP *et al*. Hyperosmolar sexual lubricant causes epithelial damage in the distal colon: Potential implication for HIV transmission. J Infect Dis 2007; 195: 703–710.

386 Anton PA, Saunders T, Elliott J, Khanukhova E, Dennis R, *et al*. First phase 1 double-blind, placebo-controlled, randomized rectal microbicide trial using UC781 gel with a novel index of ex vivo efficacy. PLoS ONE 2011; 6: e23243. doi:10.1371/journal.pone.0023243

387 McGowan I. Rectal microbicides: can we make them and will people use them? AIDS Behav 2011;15, Suppl 1:S66-71.

388 Klasse PJ, Shattock RJ, Moore JP. Which topical microbicides for blocking HIV-1 transmission will work in the real world? PLoS Med 2006;3:e351. doi:10.1371/journal.pmed.0030351

389 Rohan LC, Hillier SL, Dezzuti CS. Preventing the sexual transmission of HIV with topical microbicides: another piece of the equation. J Infect Dis 2007;196: 1285-1287.

390 Lard-Whiteford SL, Matecka D, O'Rear JJ, Yuen IS, Litterst C, Reichelderfer P. Recommendations for the nonclinical development of topical microbicides for prevention of HIV transmission: an update. J Acquir Immune Defic Syndr 2004;36:541–552.

391 Buckheit RW Jr, Watson KM, Morrow KM, Ham AS. Development of topical microbicides to prevent the sexual transmission of HIV. Antiviral Res 2010;85:142-158.

392 Doncel GF, Clark MR. Preclinical evaluation of anti-HIV microbicide products: New models and biomarkers. Antiviral Res 2010;88:S10-18.

393 Draize JH, Woodard G, Calvery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J Pharmacol Exp Ther 1944; 82:377–390.

394 Eckstein P, Jackson MC, Millman N, Sobrero AJ. Comparison of vaginal tolerance tests of spermicidal preparations in rabbits and monkeys. J Reprod Fertil 1969;20:85–93.

395 Ramjee G, Kamali A and McCormack S.The last decade of microbicide clinical trials in Africa: from hypothesis to facts. AIDS 2010; 24:S40-S49.

396 Kreiss J, Ngugi E, Holmes K, Ndinya-Achola J, Waiyaki P, Roberts PL *et al.* Efficacy of nonoxynol 9 contraceptive sponge use in preventing heterosexual acquisition of HIV in Nairobi prostitutes. JAMA 1992;268:477-482.

397 Roddy RE, Zekeng L, Ryan KA, Tamoufé U, Weir SS, Wong EL. A controlled trial of nonoxynol 9 film to reduce male-to-female transmission of sexually transmitted diseases. N Engl J Med 1998;339:504-510.

398 Van Damme L, Ramjee G, Alary M, Vuylsteke B, Chandeying V, Rees H *et al*. Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomized controlled trial. Lancet 2002; 360:971-977.

399 Feldblum PJ, Adeiga A, Bakare R, Wevill S, Lendvay A, Obadaki F *et.al.* Savvy vaginal gel (C31G) for prevention of HIV infection: a randomized controlled trial in Nigeria. PLoS One 2008; 3:e1474. doi:10.1371/journal.pone.0001474.

400 Peterson L, Nanda K, Opoku BF, Ampofo WK, Owusu-Amoako M, Boakye AY *et al*. SAVVY® (C31G) Gel for Prevention of HIV infection in Women: A Phase 3, Double-Blind, Randomized, Placebo-Controlled Trial in Ghana**.** PLoS One 2007; 2: e1312. doi:10.1371/journal.pone.0001312.

401 Hillier SL, Moench T, Shattock R, Black R, Reichelderfer P, Veronese F. In vitro and in vivo: the story of nonoxynol-9. J Acquir Immune Defic Syndr 2005; 39: 1-8.

402 Beer BE, Doncel GF, Krebs FC, Shattock RJ, Fletcher PS, Buckheit RW Jr *et al.* In vitro preclinical testing of nonoxynol-9 as potential anti-human immunodeficiency virus microbicide: a retrospective analysis of results from five laboratories. Antimicrob Agents Chemother 2006;50:713-723.

403 Mbopi-Keou FX, Trottier S, Omar RF, Nkele NN, Fokoua S, Mbu ER *et al.* A randomized, double-blind, placebo-controlled Phase II extended safety study of two Invisible Condom formulations in Cameroonian women. Contraception 2010;81:79-85.

404 Lacey CJ, Woodhall S, Qi Z, Sawant S, Cowen M, McCormack S, Jiang S. Unacceptable side-effects associated with a hyperosmolar vaginal microbicide in a phase 1 trial. Int J STD AIDS 2010;21:714-717.

405 Cohen CR, Brown J, Moscicki AB, Bukusi EA, Paull JR, Price CF, Shiboski S. A phase I randomized placebo controlled trial of the safety of 3% SPL7013 Gel (VivaGel®) in healthy young women administered twice daily for 14 days. PLoS One 2011;6:e16258. doi:10.1371/journal.pone.0016258.

406 McGowan I, Gomez K, Bruder K, Febo I, Chen BA, Richardson BA *et al*; MTN-004 Protocol Team. Phase 1 randomized trial of the vaginal safety and acceptability of

SPL7013 gel (VivaGel) in sexually active young women (MTN-004). AIDS 2011;25:1057-1064.

407 Williams DL, Newman DR, Ballagh SA, Creinin MD, Barnhart K, Weiner DH *et al.* Phase I safety trial of two vaginal microbicide gels (Acidform or BufferGel) used with a diaphragm compared to KY jelly used with a diaphragm. Sex Transm Dis 2007;34:977-984.

408 von Mollendorf CE, Van Damme L, Moyes JA, Rees VH, Callahan MM, Mauck CK *et al*. Results of a safety and feasibility study of the diaphragm used with ACIDFORM Gel or K-Y Jelly. Contraception 2010;81:232- 239.

409 Scordi-Bello IA, Mosoian A, He C, Chen Y, Cheng Y, Jarvis GA *et al*. Candidate sulfonated and sulfated topical microbicides: comparison of anti-human immunodeficiency virus activities and mechanisms of action.Antimicrob Agents Chemother 2005;49:3607-3615.

410 Skoler-Karpoff S, Ramjee G, Ahmed K *et al*. Efficacy of Carraguard for prevention of HIV infection in women in South Africa: a randomised, double-blind, placebo-controlled trial. Lancet 2008; 372:1977-1987.

411 Van Damme L, Govinden R, Mirembe FM, Guédou F, Solomon S, Becker ML *et al.* Lack of effectiveness of cellulose sulfate gel for prevention of HIV transmission. N Eng J Med 2008; 359:463-472.

412 Halpern V, Ogunsola F, Obunge O, Wang CH, Onyejepu N, Oduyebo O *et al.* Effectiveness of cellulose sulfate vaginal gel for the prevention of HIV infection: results of a Phase III trial in Nigeria. PLoS One 2008;3:e3784. doi:10.1371/journal.pone.0003784.

413 Mesquita P M M, Cheshenko N, Wilson S, Mhatre M, Guzman E, Fakioglu E *et al*. Disruption of tight junctions by cellulose sulphate facilitates HIV infection: Model of microbicide safety. J Infect Dis 2009; 200: 599-608.

414 Trifonova RT, Doncel GF , Fichorova RN **.** Polyanionic Microbicides Modify Toll-Like Receptor-Mediated Cervicovaginal Immune Responses. Antimicrob Agents Chemother 2009; 53;1490- 1500.

415 Abdool Karim SS, Richardson BA, Ramjee G, Hoffman IF, Chirenje ZM, Taha T *et al* ; HIV Prevention Trials Network (HPTN) 035 Study Team Safety and effectiveness of BufferGel and 0.5% PRO2000 gel for the prevention of HIV infection in women. AIDS 2011;25:957-966.

416 McCormack S, Ramjee G, Kamali A, Rees H, Crook A, Gafos M. PRO2000 vaginal gel for prevention of HIV-1 infection (Microbicides Development Programme 301): a phase 3, randomised, double-blind, parallel-group trial**.** Lancet 2010;376: 1329-1337.

417 Grant RM, Hamer D, Hope T, Johnston R, Lange J, Lederman MM *et al.* Whither or wither microbicides? Science 2008;321:532-534.

418 Poynten M, Millwood IY, Falster MO Law MG, Andresen DN, Van Damme L, Kaldor JM. The safety of candidate vaginal microbicides since nonoxynol-9: a systematic review of published studies. AIDS 2009; 23:1245-1254.

419 Barber TJ, Benn PD. Post exposure prophylaxis for HIV following sexual exposure. Curr Opin HIV AIDS 2010; 5: 322-326.

420 Abdool Karim Q, Abdool Karim SS, Frohlich JA, Grobler AC, Baxter C, Mansoor LE *et al.*; CAPRISA 004 Trial Group. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. Science 2010;329:1168-1174. Erratum in: Science 2011;333:524.

421 Kashuba ADM, Abdool Karim SS, Kraft E, *et al.* Do systemic and genital tract tenofovir concentrations predict HIV seroconversion in the CAPRISA 004 tenofovir gel trial? XVIII International AIDS Conference; Vienna, Austria; July 18–23, 2010. Abstract TUSS0503.

422 Karim SS, Kashuba AD, Werner L, Karim QA. Drug concentrations after topical and oral antiretroviral pre-exposure prophylaxis: implications for HIV prevention in women. Lancet 2011;378:279-281.

423 Fischer W, Hunt G, Sibeko S, Naranbhai V, Abdool Karim Q, Abdool Karim S *et al*. Tenofovir resistance mutation frequencies assessed by deep pyrosequencing of plasma virus from breakthrough HIV infections: CAPRISA 004 Microbicide Trial. 19th Conference on Retroviruses and Opportunistic Infections, Seattle, USA; March 5-8, 2012. Abstract 1063.

424 Wei X, Morris L, Naranbhai V, Sibeko S, Abdool Karim Q, Kashuba A, Passmore J-A *et al.* Sensitive tenofovir resistance screening of HIV-1 from the genital tract of women with breakthrough Infections: CAPRISA 004 Tenofovir Gel Trial. 19th Conference on Retroviruses and Opportunistic Infections, Seattle, USA; March 5-8, 2012. Abstract 33.

425 Li JF, Lipscomb JT, Wei X, Martinson NA, Morris L, Heneine W, Johnson JA. Detection of low-level K65R variants in nucleoside reverse transcriptase inhibitor-naive chronic and acute HIV-1 subtype C infections. J Infect Dis 2011;203:798-802.

426 Andrei G, Lisco A, Vanpouille C, Introini A, Balestra E, van den Oord J. Topical tenofovir, a microbicide effective against HIV, inhibits herpes simplex virus-2 replication. Cell Host Microbe 2011;10:379-389.

427 Grant RM, Lama JR, Anderson PL, McMahan V,Liu AY, Vargas L *et al*.; iPrEx Study Team. Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. N Engl J Med 2010;363:2587–2599.

428 R Grant, V McMahan, A Liu *et al*. Completed observation of the randomized placebo-controlled phase of iPrEx: daily oral FTC/TDF pre-exposure HIV prophylaxis among men and trans women who have sex with men. 6th International AIDS Society Conference on HIV Pathogenesis, Treatment and Prevention (IAS 2011). Rome, Italy; July 17-20, 2011. Abstract WELBC04.

429 Thigpen MC, Kebaabetswe PM, Paxton LA, Smith DK, Rose CE, Segolodi TM *et al*; the TDF2 Study Group . Antiretroviral Preexposure Prophylaxis for Heterosexual HIV Transmission in Botswana. N Engl J Med 2012 Jul 11 [Epub ahead of print]. doi. 10.1056/NEJMoa1110711 Available at: http://www.nejm.org/doi/pdf/10.1056/NEJMoa1110711 . Accessed July 13 2012.

430 Baeten JM, Donnell D, Ndase P, Mugo NR, Campbell JD, Wangisi J *et al*.;the Partners PrEP Study Team. Antiretroviral Prophylaxis for HIV Prevention in Heterosexual Men and Women. N Engl J Med. 2012 Jul 11[Epub ahead of print]. doi. 10.1056/NEJMoa1108524 Available at: http://www.nejm.org/doi/pdf/10.1056/NEJMoa1108524. Accessed July 13 2012.

431 Liegler T, Abdel-Mohsen M, Atchison R, Mehotra M, Schmidt T, Eden V, D *et al.;* iPrEx Study Team. Drug Resistance and Minor Drug Resistant Variants in iPrEx .18th Conference on Retroviruses and Opportunistic Infections, Boston, Massachucetts, USA; Feb 27–March 2, 2011. Abstract 97LB.

432 Mulligan K, Glidden D, Gonzales P, Ramirez-Cardich M-E, LiuA, Namwongprom S, *et al.* Effects of FTC/TDF on bone mineral density in seronegative men from 4 continents: DEXA results of the global iPrEx study. 18th Conference on Retroviruses and Opportunistic Infections, Boston, Massachucetts, USA Feb 27–March 2, 2011. Abstract 94LB.

433 Roehr B. HIV prevention trial in women is abandoned after drugs show no impact on infection rates. BMJ 2011;342:d2613.

434 Van Damme L, Corneli A, Ahmed K, Agot K, Lombaard J, Kapiga S *et al*.; the FEM-PrEP Study Group. Preexposure Prophylaxis for HIV Infection among African Women. N Engl J Med. 2012 Jul 11. [Epub ahead of print] doi 10.1056/NEJMoa1202614. Available at : http://www.nejm.org/doi/pdf/10.1056/NEJMoa1202614. Accessed July 13 2012.

435 The VOICE Study: Vaginal and Oral Interventions to Control the Epidemic: Backgrounder. Pittsburgh, Pennsylvania , USA: Microbicides Trial Network, February 8, 2012. Available at: http://www.mtnstopshiv.org/news/studies/mtn003/backgrounder. Accessed July 11 2012.

436 MTN statement on decision to discontinue use of oral tenofovir tablets in VOICE, a major HIV prevention study in women. Pittsburgh, Pennsylvania, USA: Microbicides Trial Network, September 28, 2011. Available at: http://www.mtnstopshiv.org/node/3619. Accessed July 11 2012.

437 FDA approves first drug for reducing the risk of sexually acquired HIV infection. Bethesda, Maryland, USA; United States Food and Drug Administration, United States Department of Health and Human Services. Available at: http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm312210.htm. Accessed July 17 2012.

438 Next steps for tenofovir gel: Conrad and TIA sign license agreement. Arlington, Virginia, USA: Conrad News; June 14, 2011. Available at: http://www.conrad.org/news-pressreleases-66.html. Accessed July 17 2012.

439 Facts 001 Study. Available at:http://www.facts-consortium.co.za/?page_id=83. Hillbrow, South Africa: Facts Consortium; 2012. Accessed July 17 2012.

440 Malcolm RK, Edwards KL, Kiser P, Romano J, Smith TJ. Advances in microbicide vaginal rings. Antiviral Res 2010;88 (Suppl. 1):S30-S39.

441 Nuttall JP, Thake DC, Lewis MG, Ferkany JW, Romano JW, Mitchnick MA. Concentrations of dapivirine in the rhesus macaque and rabbit following once daily intravaginal administration of a gel formulation of [14C]dapivirine for 7 days. Antimicrob Agents Chemother 2008;52:909-914.

442 Nel AM, Coplan P, van de Wijgert JH, Kapiga SH, von Mollendorf C, Geubbels E *et al.* Safety, tolerability, and systemic absorption of dapivirine vaginal microbicide gel in healthy, HIV-negative women. AIDS 2009;23:1531-1538.

443 Nel AM, Coplan P, Smythe SC, McCord K, Mitchnick M, Kaptur PE, Romano J. Pharmacokinetic assessment of dapivirine vaginal microbicide gel in healthy, HIV-negative women. AIDS Res Hum Retroviruses 2010;26:1181-1190.

444 Nel AM, Smythe SC, Habibi S, Kaptur PE, Romano JW. Pharmacokinetics of 2 dapivirine vaginal microbicide gels and their safety vs. hydroxyethyl cellulose-based universal placebo gel. J Acquir Immune Defic Syndr 2010;55:161-169.

445 Romano J, Variano B, Coplan P, Van Roey J, Douville K, Rosenberg Z *et al.* Safety and availability of dapivirine (TMC120) delivered from an intravaginal ring. AIDS Res Hum Retroviruses 2009;25:483-488.

446 Nel A, Smythe S, Young K, Malcolm K, McCoy C, Rosenberg Z, Romano J. Safety and pharmacokinetics of dapivirine delivery from matrix and reservoir intravaginal rings to HIV-negative women. J Acquir Immune Defic Syndr. 2009;51:416-423.

447 The Ring Study. IPM website. Available at: http://www.ipmglobal.org/the-ring-study. Silver Spring, Maryland, USA: The International Partnership for Microbicides; 2012. Accessed July 17 2012.

448 Phase III trial of dapivirine ring begins in Africa: new HIV prevention approach for women. Pittsburg, Pennsylvania, USA: Microbicide Trials Network; Press Release, July 24 2012. Available at: http://www.eurekalert.org/pub_releases/2012-07/mtn-pit072412.php. Accessed August 17 2012.

449 Dumond JB, Patterson KB, Pecha AL, Werner RE, Andrews E, Damle B *et al.*Maraviroc concentrates in the cervicovaginal fluid and vaginal tissue of HIV-negative women. J Acquir Immune Defic Syndr 2009;51:546-553.

450 NIH News. NIH to test maraviroc-based drug regimens for HIV prevention. Bethesda, Maryland USA: National Institutes of Health, United States Department of Health and Human Services, USA; July 19 2012. Available at: http://www.nih.gov/news/health/jul2012/niaid-18.htm. Accessed July 19 2012.

451 Veazey RS, Ketas TJ, Dufour J, Moroney-Rasmussen T, Green LC, Klasse PJ, Moore JP. Protection of rhesus macaques from vaginal infection by vaginally delivered maraviroc, an inhibitor of HIV-1 entry via the CCR5 co-receptor. J Infect Dis 2010; 202: 739–744.

452 IPM product pipeline. Available at: http://www.ipmglobal.org/products-development. Silver Spring, Maryland, USA : The International Partnership for Microbicides; 2012. Accessed on July 17 2012.

453 CHAARM Project Website. Available at: http://chaarm.eu/. London, England: CHAARM; 2012. Accessed on July 17 2012.

454 Hamers RL, Wallis CL, Kityo C, Siwale M, Mandaliya K, Conradie F *et al*.; Pharm Access African Studies to Evaluate Resistance (PASER). HIV-1 drug resistance in antiretroviral-naive individuals in sub-Saharan Africa after rollout of antiretroviral therapy: a multicentre observational study. Lancet Infect Dis 2011;11:750-759.

455 Hamers RL, Sigaloff KC, Wensing AM, Wallis CL, Kityo C, Siwale M *et al*.; Pharm Access African Studies to Evaluate Resistance (PASER) Patterns of HIV-1 drug resistance after first-line antiretroviral therapy (ART) failure in 6 sub-Saharan African countries: implications for second-line ART strategies. Clin Infect Dis 2012;54:1660-1669.

456 Lederman MM, Veazey RS, Offord R, Mosier DE, Dufour J, Mefford M *et al.* Prevention of vaginal SHIV transmission in rhesus macaques through inhibition of CCR5. Science 2004; 306: 485-487

457 Gaertner G, Cerini F, Escola J-M , Kuenzi G, Melotti A, Offord R *et al*. Highly potent, fully recombinant anti-HIV chemokines: reengineering a low cost microbicide. Proc Natl Acad SciUSA 2008;105:17706–17711.

458 Alexandre KB, Gray ES, Mufhandu H, McMahon JB, Chakauya E, O'Keefe BR *et al.* The lectins griffithsin, cyanovirin-N and scytovirin inhibit HIV-1 binding to the DC-SIGN receptor and transfer to CD4(+) cells.Virology 2012 20;423:175-186.

459 Tsai CC, Emau P, Jiang Y, Agy MB, Shattock RJ, Schmidt A *et al.* Cyanovirin- N inhibits AIDS virus infections in vaginal transmission models. AIDS Res. Hum Retroviruses 2004; 20: 11-18.

460 Tsai CC, Emau P, Jiang Y , Tian B, Morton WR, Gustafson KR, Boyd MR *et al.* Cyanovirin- N gel as a topical microbicide prevents rectal transmission of SHIV89.6P in macaques. AIDS Res Hum. Retroviruses 2003; 19: 535-541.

461 Lagenaur LA, Sanders-Beer BE, Brichacek B, Pal R, Liu X, Liu Y *et al*. Prevention of vaginal SHIV transmission in macaques by a live recombinant Lactobacillus. Mucosal Immunol 2011;4:648-657.

462 Huskens D, Vermeire K, Vandemeulebroucke E, Balzarini J, Schols D. Safety Concerns for the potential use of cyanovirin-N as a microbicidal anti-HIV agent. Int J Biochem Cell Biol 2008; 40: 2802-2814.

463 O'Keefe BR, Vojdani F, Buffa V, Shattock RJ, Montefiori DC, Bakke J *et al*. Scaleable manufacture of HIV-1 entry inhibitor griffithsin and validation of its safety and efficacy as a topical microbicide component. Proc Natl Acad Sci 2009; 106: 6099-6104.

464 Robbins JB, Schneerson R, Szu SC. Perspective: Serum IgG antibody is sufficient to confer protection against infectious diseases by inactivating the inoculum. J Infect Dis 1995; 171:1387-1398.

465 Burton DR, Desrosiers RC, Doms RW, Koff WC, Kwong PD, Moore JP *et al.* HIV vaccine design and the neutralizing antibody problem. Nat Immunol 2004;5:233-236.

466 Amana IJ, Messaoudi, Slifka MK. Protective immunity following vaccination: how is it defined? Hum Vaccine 2008,4:316-319.

467 Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975; 256: 495–497.

468 Keizer RJ, Huitema ADR, Schellens JHM, Beijnen JH. Clinical pharmacokinetics of therapeutic monoclonal antibodies. Clin Pharmacokinet 2010; 49: 493-507.

469 Cone RA, Whaley KJ. Monoclonal antibodies for reproductive health: Part I. Preventing sexual transmission of disease and pregnancy with topically applied antibodies. Am J Reprod Immunol 1994;32:114-131.

470 Zeitlin L, Cone R A, Whaley KJ. Using monoclonal antibodies to prevent mucosal transmission of epidemic infectious diseases. Emerging Infectious Diseases 1999; 1; 54-64.

471 Burton DR, Pyati J, Koduri R, Sharp SJ, Thornton GB, Parren PW *et al*. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science 1994; 266:1024–1027.

472 Pantophlet R, Ollmann Saphire E, Poignard P, Parren PWHI, Wilson IA , Burton DR. Fine mapping of the interaction of neutralizing and nonneutralizing monoclonal antibodies with the CD4 binding site of human immunodeficiency virus type 1 gp120. J Virol 2003;77:642–658.

473 Trkola A, Purtscher M, Muster T, Ballaun C, Buchacher A, Sullivan N *et al*. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J Virol 1996; 70:1100–1108.

474 Sanders RW, Venturi M, Schiffner L, Kalyanaraman R, Katinger H, Lloyd KO *et al*. The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. J Virol 2002;76:7293-7305.

475 Platt EJ, Gomes MM, Kabat D. Kinetic mechanism for HIV-1 neutralization by antibody 2G12 entails reversible glycan binding that slows cell entry. Proc Natl Acad Sci U S A. 2012;109:7829-7834.

476 Muster T, Steindl F, Purtscher M, Trkola A, Klima A, Himmler G *et al.* A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J Virol 1993; 67:6642–6647.

477 Stiegler G, Kunert R, Purtscher M, Wolbank S, Voglauer R, Steindl F *et al*. A potent cross-clade neutralizinghuman monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. AIDS Res Hum Retrovir 2001; 17:1757–1765.

478 Zwick MB, Labrijn AF, Wang M, Spenlehauer C, Ollmann Saphire E, Binley JM *et al.* Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. J Virol 2001; 75:10892–10905.

479 Alam SM, Morelli M, Dennison SM, Liao HX, Zhang R, Xia SM *et al.* Role of HIV membrane in neutralization by two broadly neutralizing antibodies. Proc Natl Acad Sci U S A 2009;106:20234-20239.

480 Sun ZY, Oh KJ, Kim M, Yu J, Brusic V, Song L *et al.* HIV-1 broadly neutralizing antibody extracts its epitope from a kinked gp41 ectodomain region on the viral membrane. Immunity 2008;28:52-63.

481 Cardoso RM, Zwick MB, Stanfield RL, Kunert R, Binley JM, Katinger H *et al*. Broadly neutralizing anti-HIV antibody 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. Immunity 2005;22:163-173.

482 Frey G , Peng H, Rits-Volloch S, Morelli M , Cheng Y,Chen BC. A fusion-intermediate state of HIV-1 gp41 targeted by broadly neutralizing antibodies. Proc Natl Acad Sci USA 2008; 105:3739–3744. doi:10.1073/pnas.0800255105

483 Frey G, ChenJ, Rits-Volloch S, Freeman MM, Zolla-Pazner S, Chen B. Distinct conformational states of HIV-1 gp41 are recognized by neutralizing and non-neutralizing antibodies. Nat Struct Mol Biol 2010;17:1486–1491.

484 Binley JM, Wrin T, Korber B, Zwick MB, Wang M, Chappey C *et al*. Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. J Virol 2004: 78:13232–13252.

485 Walker LM, Phogat SK, Chan-Hui, PY Wagner D, Phung P, Goss JL *et al.* Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science 2009; 326:285-289.

486 Mehandru S, Wrin T, Galovich J Stiegler G, Vcelar B, Hurley A *et al*. Neutralization profiles of newly transmitted human immunodeficiency virus type 1 by monoclonal antibodies 2G12, 2F5, and 4E10. J Virol 2004; 78:14039–14042.

487 Baba TW, Liska V, Hofmann-Lehmann R, Vlasak J, Xu W, Ayehunie S *et al.* Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian human immunodeficiency virus infection. Nat Med 2000; 6:200–206.

488 Xu W, Smith-Franklin BA, Li PL, Wood C, He J, Du Q *et al.* Potent neutralization of primary human immunodeficiency virus clade C isolates with a synergistic combination of human monoclonal antibodies raised against clade B. J Hum Virol 2001;4:55-61.

489 Zwick MB, Wang M, Poignard P, Stiegler G, Katinger H, Burton DR *et al.* Neutralization synergy of human immunodeficiency virus type 1 primary isolates by cocktails of broadly neutralizing antibodies. J Virol 2001 ;75:12198-208.

490 Shibata R, Ingarashi T, Haigwood N, Buckler- White A. Ogert R, Ross W *et al*. Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. Nat Med 1999; 5: 204-210.

491 Mascola JR, Lewis MG, Stiegler G, Harris D, VanCott T C, Hayes D *et al.* Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J Virol 1999; 73:4009–4018.

492 Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE *et al*. Protection of macaques against vaginal transmission of a pathogenic HIV_ 1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat Med 2000; 6:207–210.

493 Parren PW, Marx PA, Hessell AJ, Luckay A, Harouse J, Cheng-Mayer C *et al*. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. J Virol 2001; 75:8340–83407.

494 Hofmann-Lehmann R, Vlasak J, Rasmussen RA, Smith BA, Baba TW, Liska V *et al.* Postnatal passive immunization of neonatal macaques with a triple combination of human monoclonal antibodies against oral simian-human immunodeficiency virus challenge. J Virol 2001;75:7470-7480.

495 Hofmann-Lehmann R, Vlasak J, Rasmussen RA, Jiang S, Li PL, Baba TW *et al.* Postnatal pre- and postexposure passive immunization strategies: protection of neonatal macaques

against oral simian-human immunodeficiency virus challenge. J Med Primatol 2002 ;31:109-119.

496 Ferrantelli F, Hofmann-Lehmann R, Rasmussen RA, Wang T, Xu W, Li PL *et al*. Post-exposure prophylaxis with human monoclonal antibodies prevented SHIV89.6P infection or disease in neonatal macaques. AIDS 2003;17:301-309.

497 Ferrantelli F, Rasmussen RA, Buckley KA, Li PL, Wang T, Montefiori DC *et al*. Complete protection of neonatal rhesus macaques against oral exposure to pathogenic simian-human immunodeficiency virus by human anti-HIV monoclonal antibodies. J Infect Dis 2004;189:2167-2173.

498 Ferrantelli F, Buckley KA, Rasmussen RA, Chalmers A, Wang T, Li PL *et al.* Time dependence of protective post-exposure prophylaxis with human monoclonal antibodies against pathogenic SHIV challenge in newborn macaques. Virology 2007;358:69-78.

499 Hessell AJ, Rakasz E G, Tehrani D M, Huber M, Weisgrau K L, Landucci G *et al*. Broadly Neutralizing Monoclonal Antibodies 2F5 and 4E10 Directed against the Human Immunodeficiency Virus Type 1 gp41 Membrane-Proximal External Region Protect against Mucosal Challenge by Simian-Human Immunodeficiency Virus $SHIV_{Ba-L}$
J Virol; 2010 84: 1302-1313.

500 Veasey RS, Shattock RJ, Pope M, Kirijan JC, Jones J, Hu Q *et al*. Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat Med 2003;9:343-346.

501 Armbruster C, Stiegler GM, Vcelar BA, Jager W, Michael NL, Vetter N *et al*. A phase I trial with two human monoclonal antibodies (hmAb 2F5, 2G12) against HIV-1. AIDS 2002;16:227-233.

502 Stiegler G, Armbruster C, Vcelar B, Stoiber H, Kunert R, Michael N *et al.* Antiviral activity of the neutralizing antibodies 2F5 and 2G12 in asymptomatic HIV-1-infected humans: a phase I evaluation. AIDS 2002; 16:2019–2025

503 Armbruster C, Stiegler GM, Vcelar BA, Jäger W, Köller U, Jilch R *et al.* Passive immunization with the anti-HIV-1 human monoclonal antibody (hmAb) 4E10 and the hmAb combination 4E10/2F5/2G12. J Antimicrob Chemother 2004; 54:915-920.

504 Trkola A, Kuster H, Rusert P, Joos B, Fischer M, Leemann C *et al*. Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies. Nat Med 2005;11:615-22

505 Mehandru S, Vcelar B, Wrin T, Stiegler G, Joos B, Mohri H *et al.* Adjunctive Passive Immunotherapy in Human Immunodeficiency Virus Type 1-Infected Individuals Treated with Antiviral Therapy during Acute and Early Infection. J Virol 2007; 81:11016-11031.

506 Vcelar B, Stiegler G, Wolf HM, Muntean W, Leschnik B, Mehandru S *et al*. Reassessment of autoreactivity of the broadly neutralizing HIV antibodies 4E10 and 2F5 and retrospective analysis of clinical safety data. AIDS 2007;21:2161-21670.

507 Nakowitscha S, Quendlera H, Feketea H, Kunert R, Katinger H, Stiegler G. HIV-1 mutants escaping neutralization by the human antibodies 2F5, 2G12, and 4E10: in vitro experiments versus clinical studies. AIDS 2005; 19*:*1957–1966.

508 Buchacher A, Predl R, Strutzenberger K, Steinfellner W, Trkola A, Purtscher M *et al.* Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. AIDS Res Hum Retroviruses 1994;10:359-369.

509 Kunert R, Steinfellner W, Purtscher M, Assadian A, Katinger H. Stable recombinant expression of the anti HIV-1 monoclonal antibody 2F5 after IgG3/IgG1 subclass switch in CHO cells. Biotechnol Bioeng 2000;67:97-103.

510 Sherwood JK, Zeitlin L, Chen X, Whaley KJ, Cone RA, Saltzman WM. Residence half-life of IgG administered topically to the mouse vagina. Biology of Reproduction 1996; 54:264-269.

511 Moench T, Blumenthal P, Cone R, Whaley K. Antibodies may provide prolonged microbicidal activity due to their long residence time in the vagina. Microbicides Conference 2000; Washington DC, USA; March 13-16, 2000. Abstract published in: AIDS 2001; 15: PS42.

512 Tien D, Schnaare R L, Kang F, Cohl G, Mc Cormick T J, Moench T *et al. In vitro* and *in vivo* characterization of a potential universal placebo designed for use in vaginal microbicide clinical trials. AIDS Res Hum Retrovir 2005;21: 845-853.

513 Schwartz JL, Ballagh SA, Kwok C, Mauck CK, Weiner DH, Rencher WF, Callahan M M. Fourteen-day safety and acceptability study of the universal placebo gel. Contraception 2007; 75: 136-141.

514 Dhillon A K, Donners H, Pantophlet R, Johnson WE, Decker JM, Shaw GM *et al.* Dissecting the neutralizing antibody specificities of broadly neutralizing sera from human immunodeficiency virus type 1-infected donors. J Virol 2007; 81:6548–6562.

515 Shen X, Parks RJ, Montefiori DC, Kirchherr JL, Keele BF, Decker JM *et al*. In vivo gp41 antibodies targeting the 2F5 monoclonal antibody epitope mediate human immunodeficiency virus type 1 neutralization breadth. J Virol 2009; 83: 3617–3625.

516 Haynes BF, Moody MA, Verkoczy L, Kelsoe G, Alam SM. Antibody polyspecificity and neutralization of HIV-1: a hypothesis. Hum Antibodies 2005; 14:59–67.

517 Haynes BF, Fleming J, St Clair WE, Katinger H, Stiegler G, Kunert R *et al.* Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. Science 2005;308: 1906-1908.

518 Scherer EM, Zwick MB, Teyton L, Burton DR. Difficulties in eliciting broadly neutralizing anti-HIV antibodies are not explained by cardiolipin autoreactivity AIDS 2007;21:2131-2139.

519 Weiss L, You JF, Giral P, Alhenc-Gelas M, Senger D, Kazatchkine MD *et al.* Anticardiolipin antibodies are associated with antiendothelial cell antibodies but not with antibeta 2 glycoprotein I antibodies in HIV infection. Clin Immunol Immunopathol 1995; 77:69-74.

520 Abuaf N, Laperche S, Rajoely B, Carsique R, Deschamps A, Rouquette AM *et al.* Autoantibodies to phospholipids and to the coagulation proteins in AIDS. Thromb Haemost 1997; 77: 856-861.

521 Petrovas C, Vlachoyiannopoulos PG, Kordossis T , Moutsopoulos HM. Antiphospholipid antibodies in HIV infection and SLE with or without antiphospholipid syndrome: comparisons of phospholipid specificity, avidity and reactivity with beta2-GPI. J Autoimmun 1999: 13:347-355.

522 Loizou S, Singh S, Wypkema E, Asherson RA. Anticardiolipin, antibeta(2)-glycoprotein I and antiprothrombin antibodies in black South African patients with infectious disease. Ann Rheum Dis 2003; 62:1106-1111.

523 Jespers V, Francis SC, Van der Wijgert J, Crucitti T. Methodological Issues in Sampling the Local Immune System of the Female Genital Tract in the Context of HIV Prevention Trials. Am J Reprod Immunol 2011;65:368-376.

524 Jespers V, Harandi AM, Hinkula J, Medaglini D, Le Grand R, Stahl-Hennig C *et al.* Assessment of mucosal immunity to HIV-1. Expert Rev Vaccines 2010; 9:381-394

525 Crowley-Nowick PA, Bell MC, Brockwell R, Edwards RP, Chen S, Partridge EE, Mestecky J. Rectal immunization for induction of specific antibody in the genital tract of women. J Clin Immunol 1997; 17: 370–379.

526 Hildesheim A, McShane LM, Schiffman M, Bratti MC, Rodriguez AC , Herrero R *et al.* Cytokine and immunoglobulin concentrations in cervical secretions: reproducibility of the Weck-cel collection instrument and correlates of immune measures. J Immunol Methods 1999;225:131–143.

527 Kozlowski PA, Lynch RM, Patterson RR, Cu-Uvin S, Flanigan TP, Neutra MR. Modified wick method using Weck-Cel sponges for collection of human rectal secretions and analysis of mucosal HIV antibody. J Acquir Immune Defic Syndr 2000;24: 297–309.

528 Belec L, Meillet D, Levy M, Georges A, Tevi-Benissan C, Pilot J. Dilution assessment of cervicovaginal secretions obtained by vaginal washing for immunological assays. Clin Diagn Lab Immunol 1995; 2: 57-61.

529 Snowhite IV, Jones WE, Dumestre J, Dunlap K, Braly PS, Hagensee ME. Comparative analysis of methods for collection and measurement of cytokines and immunoglobulins in cervical and vaginal secretions of HIV and HPV infected women. J Immunol Methods 2002: 263: 85-95.

530 Van der Wijgert J, Altini L, Jones H, de Kock A, Young T, Williamson AL *et al*. Two methods of self-sampling compared to clinician sampling to detect reproductive tract infections in Gugulethu, South Africa. Sex Transm Dis 2006; 33:516-523

531 Gok M, Heideman DA, van Kemenade FJ, Berkhof J, Rozendaal L, Spruyt JW *et al*. HPV testing on self collected cervicovaginal lavage specimens as screening method for women who do not attend cervical screening cohort study. BMJ 2010; 340:1040.

532 Brink AA, Meijer CJ, Wiegerinck MA, Nieboer TE, Kruitwagen RF,van Kemenade F *et al*. High concordance of results of testing for humanpapillomavirus in cervicovaginal samples collected by two methods,with comparison of a novel self-sampling device to a conventional endocervical brush. J Clin Microbiol 2006; 44:2518–2523.

533 Jones HE, Wiegerinck MA, Nieboer TE, Mol BW, Westhoff CL. Women in the Netherlands prefer self-sampling with a novel lavaging device to clinician collection of specimens for cervical cancer screening. Sex Transm Dis 2008; 35:916–991.

534 Chernesky M A, Hook E W. III, Martin D H, Lane J, Johnson R, Jordan J A, *et al.* Women find it easy and prefer to collect their own vaginal swabs to diagnose Chlamydia trachomatis or Neisseria gonorrhoeae infections. Sex Trans Dis 2005; 32:729-733.

535 Schachter J, Chernesky MA, Willis D E, Fine P M, Martin D H, Fuller D *et al.* Vaginal swabs are the specimens of choice when screening for Chlamydia trachomatis and Neisseria gonorrhoeae: results from a multicenter evaluation of the APTIMA assays for both infections. Sex Transm Dis 2005;32:725-728.

536 Boskey ER, Moench TR, Hees PS, Cone RA: A self-sampling method to obtain large volumes of undiluted cervicovaginal secretions. Sex Transm Dis 2003; 30:107–109.

537 Usala SJ, Schumacher GF. Volumetric self-sampling of cervicovaginal fluid: a new approach to ovulation timing. Fertil Steril 1983; 39: 304-309.

538 Min SS, Corbett AH, Rezk N, Cu-Uvin S, Fiscus SA, Petch L *et al.* Protease inhibitor and non nucleoside reverse transcriptase inhibitor concentrations in the genital tract of HIV-1 infected women. J AIDS 2004;37:1577–1580.

539 Dumond JB, Yeh RF, Patterson KB, Corbett AH, Jung BH, Rezk NL *et al*. Antiretroviral drug exposure in the female genital tract: implications for oral pre- and post-exposure prophylaxis. AIDS 2007; 21: 1899–1907.

540 Giorgi A, Torriani S, Dellaglio F, Bo G, Stola E, Bernuzzi L. Identification of vaginal lactobacilli from asymptomatic women. Microbiologica 1987;10:377–384.

541 Antonio MAD, Hawes SE, Hillier SL. The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species**.** J InfectDis 1999; 180**:**1950-1956.

542 De Backer E, Verhelst R, Verstraelen H, Alqumber MA, Burton JP, Tagg J R *et al.* Quantitative determination by real-time PCR of four vaginal *Lactobacillus species*, *Gardnerella vaginalis* and *Atopobium vaginae* indicates an inverse relationship between *L. gasseri* and *L. iners.* BMC Microbiol 2007; 7: 115.

543 Tamrakar R, Yamada T, Furuta I, Cho K, Morikawa M, Yamada H *et al.* Association between *Lactobacillus* species and bacterial vaginosis-related bacteria, and bacterial vaginosis scores in pregnant Japanese women. BMC Infect Dis 2007;7:128. doi:10.1186/1471-2334-7-128.

544 Easmon CSF, PE Hay, C Ison. Bacterial Vaginosis: a diagnostic approach. Genitourin Med 1992;68:134-138.

545 Kalra A, Palcu CT, Sobel JD, Akins RA. Bacterial vaginosis: culture- and PCR-based characterizations of a complex polymicrobial disease's pathobiology. Curr Infect Dis Rep 2007;9: 485–500.

546 Mirmonsef P, Gilbert D, Zariffard MR, Hamaker BR, Kaur A, Landay AL, Spear GT. The effects of commensal bacteria on innate immune responses in the female genital tract. Am J Reprod Immunol 2011; 65: 190–195.

547 Hillier SL: The vaginal microbial ecosystem and resistance to HIV. AIDS Res Hum Retroviruses 1998; 14:S17–S21.

548 Mattsby-Baltzer I, Platz-Christensen JJ, Hosseini N, Rosen P. IL- 1beta, IL-6, TNF alpha, fetal fibronectin, and endotoxin in the lower genital tract of pregnant women with bacterial vaginosis. Acta Obstet Gynecol Scand 1998; 77:701–706.

549 Watts DH, Krohn MA, Hillier SL, Eschenbach DA. Bacterial vaginosis as a risk factor for post-cesarean endometritis. Obstet Gynecol 1990;75:52-58.

550 McGregor JA, French JI, Jones W, Milligan K, McKinney PJ, Patterson E *et al*. Bacterial vaginosis is associated with prematurity and vaginal fluid mucinase and sialidase: results of a controlled trial of topical clindamycin cream. Am J Obstet Gynecol 1994;170:1048-59.

551 Larsson PG, Platz-Christensen JJ, Thejls H, Forsum U, Pahlson C. Incidence of pelvic inflammatory disease after first-trimester legal abortion in women with bacterial vaginosis after treatment with metronidazole: a double-blind, randomized study. Am J Obstet Gynecol 1992;166:100-103.

552 Ness RB, Hillier SL, Kip KE, Soper DE, Stamm CA, McGregor JA et al. Bacterial Vaginosis and Risk of Pelvic Inflammatory Disease. Obstet Gynecol Surv 2005;60:99-100.

553 Mauck C, Rosenberg Z,Van Damme L; for the International Working Group on Microbicides. Recommendations for the Clinical development of topical microbicides. AIDS 2001; 15:857-868.

554 Watts DH, Rabe L, Krohn MA, Aura J, Hillier SL. The effects of three nonoxynol-9 preparations on vaginal flora and epithelium. J Infect Dis 1999; 180:426-437.

555 Clarke J G, Peipert JF, Hillier SL, Heber W, Boardman L, Moench T R, Mayer K. Microflora changes with the use of a vaginal microbicide. Sex Transm Dis 2002; 29:288-293.

556 Hugenholtz P, Goebel B M, Pace NR. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 1998; 180:4765-4774.

557 Van der Wijgert J, Shattock R. Vaginal Microbicides: moving ahead after an unexpected set-back. AIDS 2007; 21: 2368-2376.

558 Sha BE, Chen HY, Wang Q J, Zariffard MR, Cohen MH, Spear GT. Utility of Amsel criteria, Nugent score, and quantitative PCR for Gardnerella vaginalis, Mycoplasma hominis, and Lactobacillus spp. for diagnosis of bacterial vaginosis in human immunodeficiency virus-infected women. J Clin Microbiol 2005; 43:4607-4612.

559 Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Delanghe J, Van Simaey L *et al.* Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between Atopobium vaginae, Gardnerella vaginalis and bacterial vaginosis. BMC Microbiol 2002; 4:16. doi:10.1186/1471-2180-4-16.

560 Zariffard MR, Saifuddin M, Sha BE, Spear GT. Detection of bacterial vaginosis-related organisms by real-time PCR for Lactobacilli, Gardnerella vaginalis and Mycoplasma hominis. FEMS Immunol Med Microbiol 2002; 34:277-281.

561 Nugent R, Krohn M, Hillier S. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. J Clin Microbiol 1991;29:297-301.

562 Donadoni C, Bisighini C, Scotti L, Diomede L, Ngyen M, Nouhin J *et al.* Setting of Methods for Analysis of Mucosal Antibodies in Seminal and Vaginal Fluids of HIV

Seropositive Subjects from Cambodian and Italian Cohorts. PLoS ONE 2010; 5: e9920. doi:10.1371/journal.pone.0009920.

563 Lindner JG, Plantema FH, Hoogkamp K. Quantitative studies of the vaginal flora of healthy women and of obstetric and gynaecological patients. J Med Microbiol 1978; 11:233-241.

564 Odds FC. Candida and Candidosis; A review and bibliography. 2nd edition. London, England: Bailliere Tindall; 1988.

565 Hopwood V, Crowley T, Horrocks CT, Milne JD, Taylor PK, Warnock DW. Vaginal candidosis: relation between yeast counts and symptoms and clinical signs in non-pregnant women. Genitourin Med 1988; 64:331-334.

566 Odds FC, Webster CE, Mayuranathan P, Simmons PD. Candida concentrations in the vagina and their association with signs and symptoms of vaginal candidosis. Journal of Medical & Veterinary Mycology 1988; 26:277-283.

567 El-Din SS, Reynolds MT, Ashbee HR, Barton RC, Evans EG. An investigation into the pathogenesis of vulvo-vaginal candidosis. Sex Transm Infect 2001; 77:179-183.

568 Moraes PS. Recurrent vaginal candidiasis and allergic rhinitis: a common association. Annals of Allergy, Asthma, & Immunology 1998; 81:165-169.

569 Dunlop DC, Ulrich A, Appelmelk BJ , Burton DR, Dwek RA, Zitzmann N and Scanlan C N. Antigenic mimicry of the HIV envelope by AIDS-associated pathogens. AIDS 2008; 22 : 2214-2217.

570 Procedures for the collection of diagnostic blood specimens by venipuncture. Approved Standard, 6th Edition. Document H3-A6. Wayne, Pennsylvania, USA: Clinical and Laboratory Standards Institute; 2007. Available at: www.clsi.org. Accessed May 1 2011.

571 The Medicines for Human Use (Clinical Trials) Regulations 2004. United Kingdom: National Legislative Archives. Available at: http://www.legislation.gov.uk/uksi/2004/1031/contents/made. Accessed May 1 2011.

572 An international multi-centre, randomised, double-blind, placebo-controlled trial to evaluate theefficacy and safety of 0.5% and 2% PRO 2000/5 gels for the prevention of vaginally acquired HIV infection. Microbicides Development Programme (MDP) 301 Trial Protocol (version 1.3, June 2006). Available at. http://www.mdp.mrc.ac.uk/publications_documents/MDP301_1.4_291107.pdf. Accessed September 22 2011.

573 The Division of AIDS Table for Grading the Severity of Adult and Pediatric Adverse Events, Version 1.0, December 2004. Bethesda, Maryland, USA: United States National

Institutes of Health Division of AIDS, United States Department for Health and Human Services. Available at: http://www.ucdmc.ucdavis.edu/clinicaltrials/documents/DAIDS_AE_GradingTable_FinalDec2004.pdf. Accessed September 22 2011.

574 CONRAD/WHO Manual for the Standardization of Colposcopy for the Evaluation of Vaginal Products-Update 2004. Geneva, Switzerland: World Health Organization, Department of Reproductive Health and Research; 2004. Available at: http://www.conrad.org/publications. Accessed May 1 2011.

575 Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol 1990;28:495-503.

576 Byun R, Nadkarni MA, Chhour KL, Martin FE, Jacques NA, Hunter N. Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. J Clin Microbiol. 2004;42:3128–3136.

577 Freund RJ, Wilson WJ. Statistical Methods. 2nd Edition. San Diego, California, USA: Academic Press; 2002.

578 Bland M. An Introduction to Medical statistics. 3rd Edition. Oxford, England:Oxford University Press; 2000.

579 Greenblatt DJ. Elimination half-life of drugs: value and limitations. Ann Rev Med 1985; 36: 421-427.

580 Schulz KF, Altman DG, Moher D; for the CONSORT Group. CONSORT 2010 Statement: updated guidelines for reporting parallel group randomized trials. Open Med 2010;4:60-68.

581 Nardelli-Haefliger D, Wirthner D, Schiller JT, Lowy DR, Hildesheim A, Ponci F *et al.* Specific antibody levels at the cervix during the menstrual cycle of women vaccinated with human papillomavirus 16 virus-like particles. J Natl Cancer Inst 2003;95:1128–1137.

582 Bland JM, Altman DG. Statistics Notes. Measurement error proportional to the mean. BMJ 1996; 313: 106.

583 Oxford Concise Medical Dictionary. 5th Edition. Oxford, England: Oxford University Press; 1995.

584 Lobo ED, Hansen RJ, Balthasar JP. Antibody pharmacokinetics and pharmacodynamics. Journal of Pharmaceutical Sciences 2004; 93: 2645-2668.

585 Wang W, Wang EQ, Balthasar JP. Monoclonal antibody pharmacokinetics and pharmacodynamics. Clinical Pharmacology and Therapeutics 2008; 84:545-558.

586 Linke R, Klein A and Seimetz D. Catumoxamab: Clinical development and future directions. mAbs 2010;2: 129-136.

587 The CATT Research Group. Ranibizumab and Bevacizumab for neovascular age-related macular degeneration. N Engl J Med 2011; 364:1897-1908.

588 Maillet A, Congy-Jolivet N, Le Guellec S, Vecellio L, Hamard S, Courty Y *et al.* Aerodynamical, immunological and pharmacological properties of the anticancer antibody cetuximab following nebulization. Pharm Res 2008;25:1318-1326.

589 Zeitlin L, Whaley KJ, Sanna PP, Moench TR, Bastidas R, De Logu A, Williamson RA, Burton DR, Cone RA. Topically applied human recombinant monoclonal IgG1 antibody and its Fab and F(ab')2 fragments protect mice from vaginal transmission of HSV-2. Virology 1996; 225:213-215.

590 Chen J, Dave SK, Simmons A. Prevention of genital herpes in a guinea pig model using a glycoprotein D-specific single chain antibody as a microbicide. Virol J 2004; 1:11.doi:10.1186/1743-422X-1-11.

591 Barnhart KT, Pretorius ES, Stolpen A, Malamud D. Distribution of topical medication in the human vagina as imaged by MRI. Fertil Steril 2001;76:189–195.

592 Barnhart K, Kulp JL, Rosen M, Shera DM. A randomized trial to determine the distribution of four topical gel formulations in the human vagina. Contraception 2009; 79 : 297–303.

593 Barnhart KT, Pretorius ES, Shaunik A, Timbers K, Nasution M, Mauck C. Vaginal distribution of two volumes of the novel microbicide gel cellulose sulfate (2.5 and 3.5 mL). Contraception 2005;72:65–70.

594 Barnhart KT, Pretorius ES, Timbers K, Shera D, Shabbout M, Malamud D. In vivo distribution of a vaginal gel: MRI evaluation of the effects of gel volume, time, and simulated intercourse. Contraception 2004;70:498–505.

595 Tolley EE, Harrison PF, Goetghebeur E, Morrow K, Pool R, Taylor D *et al.* Adherence and its measurement in phase 2/3 microbicide trials. AIDS Behav 2010;14:1124-1136.

596 Dumond JB, Patterson KB, Pecha AL, Werner RE, Andrews E, Damle B *et al*. Maraviroc Concentrates in the Cervicovaginal Fluid and Vaginal Tissue of HIV-Negative Women. J Acquir Immune Defic Syndr 2009; 51: 546–553.

597 Yeh RF, Rezk NL, Kashuba AD, Dumond JB, Tappouni HL, Tien HC *et al*.Genital Tract, Cord Blood, and Amniotic Fluid Exposures of Seven Antiretroviral Drugs During and After Pregnancy in HIV-1 Infected Women. Antimicrob Agents Chemother 2009;53:2367-2374.

598 Liu H, Gaza-Bulseco G, Faldu D, Chumsae C, Sun J. Heterogeneity of monoclonal antibodies. J Pharm Sci 2008; 97:2426-2447.

599 Tuck MK, Chan DW, Chia D, Godwin AK, Grizzle WE, Krueger KE *et al.* Standard operating procedures for serum and plasma collection: Early Detection Research Network Consensus Statement Standard Operating Procedure Integration Working Group. Journal of Proteome Research 2009; 8: 113–117.

600 Pierce Protein Biology Products. Overview of ELISA. Rockford Ilinois, USA: Thermo Fisher Scientific; 2012. Available at: http://www.piercenet.com/browse.cfm?fldID=F88ADEC9-1B43-4585-922E-836FE09D8403 . Accessed May 15 2012.

601 Mestecky J, Moldoveanu Z , Russell MW . Immunologic uniqueness of the genital tract: challenge for vaccine development. Am J Reprod Immunol 2005; 53: 208-214.

602 Das Neves J, Bahia MF. Gels as vaginal drug delivery systems. International Journal of Pharmaceutics 2006;318: 1–14.

603 Owen DH, Peters JJ, Katz DF. Comparison of the rheological properties of Advantage-S and Replens. Contraception 2001; 64: 393–396.

604 Roge AB, Firke SN, Gaikwad SB, Gunjkar VN, Vadvalker SM. Bioadhesive polymer:Review. Journal of Pharmaceutical and Biomedical Sciences 2011; 5: 25.

605 Magnusson K E, Stjernstrom I. Mucosal barrier mechanisms.Interplay between secretory IgA, IgG and mucins on the surface properties and association of salmonellae with intestine and granulocytes. Immunology 1982; 45:239-248.

606 McSweegan E, Burr DH,Walker RI. Intestinal mucus gel and secretory antibody are barriers to Campylobacterjejuni adherence to INT 407 cells. Infect Immun 1987; 55:1431-1435.

607 Olmsted SS, PadgettJ L, Yudin A I, Whaley KJ, Moench TR, Cone RA. Diffusion of macromolecules and virus-like particles in human cervical mucus, Biophys J 2001;81:1930–1937.

608 Saltzman WM, Radomsky ML, Whaley KJ, Cone RA , Antibody diffusion in human cervical-mucus, Biophys J 1994;66: 508–515.

609 SK Lai, Wang Y, Hida K, Cone R, Hanes J. Nanoparticles reveal that human cervicovaginal mucus is riddled with pores larger than viruses. Proc Natl Acad Sci U S A 2010;107: 598-603.

610 Lai SK, Wang YY, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Adv Drug Deliv Rev 2009;61:158-171.

611 Kieweg SL, Geonnotti AR, Katz DF. Gravity-induced coating flows of vaginal gel formulations: in vitro experimental analysis. J Pharm Sci 2004;93:2941-2952.

612 Kieweg SL, Katz DF. Squeezing flows of vaginal gel formulations relevant to microbicide drug delivery. J Biomech Eng 2006; 128:540–553.

613 Barnhart KT, Stolpen A, Pretorius ES, Malamud D. Distribution of a spermicide containing Nonoxynol-9 in the vaginal canal and the upper female reproductive tract. Hum Reprod 2001;16:1151-1154.

614 Mauck CK, Katz D, Sandefer EP, Nasution MD, Henderson M, Digenis GA *et al*. Vaginal distribution of Replens and K-Y Jelly using three imaging techniques. Contraception. 2008;77:195-204.

615 Das Neves J, Amaral MH, Bahia MF. Vaginal Drug Delivery. In: Pharmaceutical Sciences Encyclopedia: Drug Discovery, Development, and Manufacturing. Hoboken, New Jersey, USA: Wiley; 2010. pp 1-70.

616 Chodirker WB, Tomasi TB Jr. Gamma-globulins: Quantitative relationships in human serum and non-vascular fluids.Science 1963;142:1080-1081.

617 Brandtzaeg P. Mucosal immunity in the female genital tract. J Reprod Immunol 1997; 36: 23–50.

618 Bouvet J P, Bélec L, Pirès R, Pillot J. Immunoglobulin G antibodies in human vaginal secretions after parenteral vaccination. Infect Immun 1994; 62: 3957–3961.

619 Lowe RS, Brown DR, Bryan JT, Cook JC, George HA, Hofmann K J *et al*. Human papillomavirus type 11 (HPV-11) neutralizing antibodies in the serum and genital mucosal secretions of African green monkeys immunized with HPV-11 virus- like particles expressed in yeast. J Infec Dis 1997;176:1141-1145.

620 Kozlowski PA, Cu-Uvin S, Neutra MR, Flanigan TP. Comparison of the oral, rectal, and vaginal immunization routes for induction of antibodies in rectal and genital tract secretions of women. Infect Immun 1997;65:1387-1394.

621 Belec L,Dupré T, Prazuck T, Tévi-Bénissan C, Kanga J, Pathey O *et al*.Cervicovaginal overproduction of specific IgG to human immunodeficiency virus (HIV) contrasts with normal or impaired IgA local response in HIV infection. J Infec Dis 1995;172:691-697.

622 Hocini H, Barra A, Belec L, Iscaki S, Preud'homme JL, Pillot J *et al*. Systemic and secretory humoral immunity in the normal human vaginal tract. Scand J Immunol 1995;42:269-274.

623 Crowley-Nowick PA, Bell M, Edwards RP, McCallister D, Gore H, Kanbour-Shakir A *et al*. Normal uterine cervix: characterization of isolated lymphocyte phenotypes and immunoglobulin secretion. Am J Reprod Immunol 1995; 34: 241-247.

624 Jilanti R, Isliker M. Immunoglobulin in human cervico-vaginal secretions. Int Arch Allergy Appl Immunol 1977;53:402-408.

625 Kaushic C, Wira CR. IgA and reproductive tract immunity. In: Kaetzel C, editor. Mucosal Immune Defense: Immunoglobulin A. New York, USA: Kluwer Academic; 2008. pp. 291–320.

626 Simister NE, Mostov KE. An Fc receptor structurally related to MHC class I antigens. Nature 1989; 337:184–187.

627 Burmeister W P, Gastinel LN, Simister NE, Blum ML, Bjorkman P J. Crystal structure at 2.2 Å resolution of the MHC-related neonatal Fc receptor. Nature 1994; 372:336–343.

628 Brambell FW. The transmission of immunity from mother to young and the catabolism of immunoglobulins. Lancet 1966; 2: 1087–1093 .

629 Jones E A, Waldmann TA. The mechanism of intestinal uptake and transcellular transport of IgG in the neonatal rat. J Clin Invest 1972; 51:2916–2927.

630 Leach JL, Sedmak DD, Osborne JM, Rahill B, Lairmore MD, Anderson CL. Isolation from human placenta of the IgG transporter, FcRn, and localization to the syncytiotrophoblast: implications for maternal–fetal antibody transport. J Immunol 1996; 157: 3317–3322.

631 Simister NE, Story C M, Chen HL. Hunt J S. An IgG-transporting Fc receptor expressed in the syncytiotrophoblast of human placenta. EurJ Immunol. 1996; 26, 1527–1531.

632 Raghavan M, Bonagura V R, Morrison, SL, Bjorkman PJ. Analysis of the pH dependence of the neonatal Fc receptor/immunoglobulin G interaction using antibody and receptor variants. Biochemistry 1995;34: 14649–14657.

633 Kim JK, Tsen MF, Ghetie V, Ward ES. Localization of the site of the murine IgG1 molecule that is involved in binding to the murine intestinal Fc receptor. Eur J Immunol 1994; 24: 2429–2434.

634 Simister NE, Rees AR. Isolation andcharacterization of an Fc receptor from neonatal rat small intestine. Eur. J. Immunol. 1985; 15, 733–738.

635 Jones E A, Waldmann T A. The mechanism of intestinal uptake and transcellular transport of IgG in the neonatal rat. J Clin Invest. 1972; 51: 2916–2927.

636 Ober R J, Martinez C, Vaccaro C , Zhou J, Ward ES. Visualizing the site and dynamics of IgG salvage by the MHC class I-related receptor, FcRn. J Immunol 2004; 172: 2021–2029.

637 Ober R J, Martinez C, Lai X, Zhou J, Ward ES. Exocytosis of IgG as mediated by the receptor, FcRn: an analysis at the single-molecule level. Proc Natl Acad Sci USA 2004;101: 11076–11081.

638 Israel EJ, Taylor S, Wu Z, Mizoguchi E, Blumberg RS, Bhan A, Simister NE. Expression of the neonatal Fc receptor, FcRn, on human intestinal epithelial cells. Immunology 1997; 92: 69-74.

639 Dickinson BL, Badizadegan K, Wu Z, Ahouse JC, Zhu X, Simister NE *et al.* Bidirectional FcRn-dependent IgG transport in a polarized human intestinal epithelial cell line. J Clin Invest 1999; 104: 903–911.

640 Spiekermann G M, Finn PW, Ward ES, Dumont J, Dickinson BL, Blumberg RS, Lencer WI. Receptor-mediated immunoglobulin G transport across mucosal barriers in adult life: functional expression of FcRn in the mammalian lung. J Exp Med 2002; 196:303–310.

641 Haymann, JP, Levraud JP, Bouet S, Kappes V, Hagège J, Nguyen G *et al.* Characterization and localization of the neonatal Fc receptor in adult human kidney. J Am Soc Nephrol 2000; 11: 632–639.

642 Kobayashi N, Suzuki Y, Tsuge T, Okumura K, Ra C, Tomino Y. FcRn-mediated transcytosis of immunoglobulin G in human renal proximal tubular epithelial cells. Am J Physiol Renal Physiol 2002;282: F358–F365.

643 Telleman P, Junghans R P. The role of the Brambell receptor (FcRB) in liver: protection of endocytosed immunoglobulin G (IgG) from catabolism in hepatocytes rather than transport of IgG to bile. Immunology 2000; 100: 245 – 251.

644 Antohe F, Radulesc L, Gafencu A, Ghetie V, Simionescu M. Expression of functionally active FcRn and the differentiated bidirectional transport of IgG in human placental endothelial cells. Hum Immunol 2001; 62: 93–105

645 Roopenian D C, Akilesh S. FcRn: the neonatal Fc receptor comes of age. Nature Reviews Immunol 2007; 7:215-725.

646 Zhu X, Meng G, Dickinson BL, Li X, Mizoguchi E, Miao L *et al.* MHC class I‑related neonatal Fc receptor for IgG is functionally expressed in monocytes, intestinal macrophages, and dendritic cells. J Immunol 2001; 166: 3266–3276.

647 Akilesh S, Christianson GJ, Roopenian DC, Shaw AS. Neonatal FcR expression in bone marrow-derived cells functions to protect serum IgG from catabolism. J Immunol; 2007; 179: 4580-4588.

648 Yoshida M, Claypool SM, Wagner JS, Mizoguchi E, Mizoguchi A, Roopenian DC *et al.* Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. Immunity 2004; 20: 769–783.

649 Bitonti AJ, Dumont JA, Low SC, Peters RT, Kropp KE, Palombella VJ *et al.* Pulmonary delivery of an erythropoietin Fc fusion protein in non-human primates through an immunoglobulin transport pathway. Proc Natl Acad Sci USA 2004;101:9763-9768.

650 Dumont JA, Bitonti AJ, Clark D, Evans S, Pickford M, Newman SP. Delivery of an erythropoietin-Fc fusion protein by inhalation in humans through an immunoglobulin transport pathway. J Aerosol Med 2005 ;18:294-303.

651 Lu L, Palaniyandi S, Zeng R, Bai Y, Liu X, Wang Y *et al.* A neonatal Fc receptor-targeted mucosal vaccine strategy effectively induces HIV-1 antigen-specific immunity to genital infection. J Virol 2011; 85: 10542-10553.

652 Li Z, Palaniyandi S, Zeng R, Tuo W, Roopenian D C, Zhu X. Transfer of IgG in the female genital tract by MHC class 1- related neonatal Fc receptor (FcRn) confers protective immunity to vaginal infection. Proc Natl Acad Sci USA 2011;108; 4388-4393.

653 Tiwari B, Junghans R P. Functional analysis of the mouse Fcgrt 5' proximal promoter. Biochim Biophys Acta 2005; 1681: 88–98.

654 Mikulska JE, Simister NE. Analysis of the promoter region of the human FcRn gene. Biochim Biophys Acta 2000; 1492: 180–184 .

655 Yoshida M, Masuda A, Kuo TT, Kobayashi K, Claypool SM, Takagawa T *et al*. IgG transport across mucosal barriers by neonatal Fc receptor for IgG and mucosal immunity. Springer Semin Immunopathol 2006; 28: 397–403.

656 Gill RK, Mahmood S, Sodhi CP, Nagpaul JP, Mahmood A. IgG binding and expression of its receptor in rat intestine during postnatal development. Indian J Biochem Biophys 1999; 36; 252–257 .

657 Mayer B, Kis Z, Kaján G, Frenyó LV, Hammarström L, Kacskovics I *et al*. The neonatal Fc receptor (FcRn) is expressed in the bovine lung. Vet Immunol Immunopathol 2004; 98: 85–89.

658 Sakagami M, Omidi Y, Campbell L, Kandalaft LE, Morris CJ, Barar J, Gumbleton M. Expression and transport functionality of FcRn within rat alveolar epithelium: a study in primary cell culture and in the isolated perfused lung. Pharm Res 2006;23: 270–279.

659 Thompson IOC, van der Bijl P, van Wyk CW, van Eyk AD. A comparative light-microscopic, electron-microscopic and chemical study of human vaginal and buccal epithelium. Archives of Oral Biology 2001; 46: 1091-1098.

660 Cauza K, Hinterhuber G, Dingelmaier-Hovorka R, Brugger K, Klosner G, Horvat R *et al*. Expression of FcRn, the MHC class I-related receptor for IgG, in human keratinocytes. J Invest Dermatol 2005;124:132-139.

661 Lencer WI, Blumberg RS. A passionate kiss, then run: exocytosis and recycling of IgG by FcRn. Trends in Cell Biology 2005; 15:5-9.

662 Connell GE, Painter RH. Fragmentation of immunoglobulin during storage. Can J Biochem 1966; 44:371–379.

663 Turner MW, Bennich HH, Natvig JB. Pepsin digestion of human G-myeloma proteins of different subclasses. II. Immunochemical investigations of the products of peptic digestion. Clin Exp Immunol 1970 ;7:627-640.

664 Gearing AJ, Thorpe SJ, Miller K, Mangan M, Varley PG, Dudgeon T *et al.* Selective cleavage of human IgG by the matrix metalloproteinases, matrilysin and stromelysin. Immunol Lett 2002; 81:41–48.

665 Berasain P, Carmona C, Frangione B, Dalton JP, Goni F. Fasciola hepatica: Parasite-secretedproteinases degrade all human IgG subclasses: Determination of the specific cleavage sites andidentification of the immunoglobulin fragments produced. Exp Parasitol 2000; 99–110.

666 Jefferis R, Weston PD, Stanworth DR, Clamp JR. Relationship between the papain sensitivity of human gammaG immunoglobulins and their heavy chain subclass. Nature 1968;219:646-649.

667 Taylor A. Aminopeptidases:structure and function. FASEBJ 1993;7: 290-298.

668 Provenzano D, Alderete J F. Analysis of human immunoglobulin-degrading cysteine proteinases of Trichomonas vaginalis. Infect Immun 1995; 63: 3388-3395.

669 Kilian M, Mestecky J, Russell MW. Defence mechanisms involving Fc dependent functions of immunoglobulin A and their subversion by bacterial immunoglobulin A proteases. Microbiol Rev 1988;52:296–303.

670 Vanhoof G, Goossens F, De Meester I, Hendriks D, Scharpe S. Proline motifs in peptides and their biological processing. FASEB J 1995; 9:736-744.

671 Loomes LM, Kerr MA, Senior BW. The cleavage of immunoglobulin G in vitro and in vivo by a proteinase secreted by the urinary tract pathogen Proteus mirabilis. J Med Microbiol 1993; 39: 225-232.

672 Pham CTN. Neutrophil serine proteases: specific regulators of inflammation. Nature Rev Immunol 2006; 6: 541-550.

673 Ward ES, Zhou J, Ghetie V, Ober RJ. Evidence to support the cellular mechanism involved in serum IgG homeostasis in humans. Int Immunol 2003;15: 187–195 .

674 Brambell HW, Morris IG. A Theoretical Model of gamma-Globulin Catabolism. Nature 1964; 203: 1352-1355.

675 Junghans RP. Finally! The Brambell receptor (FcRB). Mediator of transmission of immunity and protection from catabolism for IgG. Immunol Res 1997; 16: 29–57.

676 Joos B, Trkola A, Kuster H, Aceto L, Fischer M, Stiegler G *et al*. Long-term multiple-dose pharmacokinetics of human monoclonal antibodies (mAbs) against human

immunodeficiency virus type 1 envelope gp120 (mAb 2G12) and gp41 (mAbs 4E10 and 2F5). Antimicrob Agents Chemother 2006;50:1773-1779.

677 Waldmann TA, Strober W. Metabolism of immunoglobulins. Prog Allergy 1969;13 :1–110.

678 Martin WL, West AP Jr, Gan L, Bjorkman PJ. Crystal structure at 2.8 A of an FcRn/heterodimeric Fc complex: mechanism of pH-dependent binding. Mol Cell 2001;7:867-877.

679 Ghetie V, Ward ES. Multiple roles for the major histocompatibility complex class I-related receptor FcRn. Annu Rev Immunol 2000;18:739-766.

680 Kim JK, Tsen MF, Ghetie V, Ward ES. Identifying amino acid residues that influence plasma clearance of murine IgG1 fragments by site-directed mutagenesis. Eur J Immunol 1994; 24:542–548.

681 Ghetie V, Popov S, Borvak J, Radu C, Matesoi D, Medesan C *et al.* Increasing the serum persistence of an IgG fragment by random mutagenesis. Nat Biotechnol 1997; 15:637–640.

682 Hinton PR, Johlfs MG, Xiong JM, Hanestad K, Ong KC, Bullock C *et al*. Engineered human IgG antibodies with longer serum half-lives in primates. J Biol Chem 2004; 279:6213–6216.

683 Hinton PR, Xiong JM, Johlfs MJ, Tang MT, Keller S, Tsurushita N. An engineered human IgG1 antibody with longer serum half-life. J Immunol 2006; 176:346–356.

684 Yeung YA, Leabman MK, Marvin JS, Qiu J, Adams CW, Lien S *et al.* Engineering human IgG1 affinity to human neonatal Fc receptor: impact of affinity improvement on pharmacokinetics in primates. J Immunol 2009;182:7663-7671.

685 Dall'Acqua WF, Woods RM, Ward ES, Palaszynski SR, Patel NK, Brewah YA *et al*. Increasing the affinity of a human IgG1 for the neonatal Fc receptor: biological consequences. J Immunol 2002; 169:5171–5180.

686 Vaccaro C, Zhou J, Ober RJ, Ward ES. Engineering the Fc region of immunoglobulin G to modulate in vivo antibody levels. Nat Biotechnol 2005; 23:1283–1288.

687 Datta-Mannan A, Witcher DR, Tang Y, Watkins J, Jiang W, Wroblewski VJ. Humanized IgG1 variants with differential binding properties to the neonatal Fc receptor: relationship to pharmacokinetics in mice and primates. Drug Metab. Dispos 2007;35: 86–94.

688 Wang W, Lu P, Fang Y, Hamuro L, Pittman T, Carr B, Hochman J, Prueksaritanont T. Monoclonal antibodies with identical Fc sequences can bind to FcRn differentially with pharmacokinetic consequences . Drug Metab Dispos 2011;39:1469-1477.

689 Suzuki T, Ishii-Watabe A, Tada M, Kobayashi T, Kanayasu-Toyoda T, Kawanishi T, Yamaguchi T. Importance of neonatal FcR in regulating the serum half-life of therapeutic proteins containing the Fc domain of human IgG1: a comparative study of the affinity of monoclonal antibodies and Fc-fusion proteins to human neonatal FcR. J Immunol 2010 ; 15;184:1968-1976.

690 Rudd PM, Dwek RA. Glycosylation: heterogeneity and the 3D structure of proteins. Crit Rev Biochem Mol Biol. 1997;32 :1-100.

691 Bonomelli C, Doores KJ, Dunlop DC, Thaney V, Dwek RA, Burton DR *et al*. The glycan shield of HIV Is predominantly oligomannose independently of production system or viral clade. PLoS ONE 2011; 6: e23521. doi:10.1371/journal.pone.0023521.

692 Calarese DA, Scanlan CN, Zwick MB, Deechongkit S, Mimura Y, Kunert R *et al.* Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. Science 2003; 300:2065-2071.

693 Ofek G, Tang M, Sambor A, Katinger H, Mascola JR, Wyatt R, Kwong PD. Structure and mechanistic analysis of the anti-human immunodeficiency virus type 1 antibody 2F5 in complex with its gp41 epitope. J Virol 2004;78:10724 –10737.

694 Matyas GR, Beck Z, Karasavvas N, Alving CR. Lipid binding properties of 4E10, 2F5, and WR304 monoclonal antibodies that neutralize HIV-1. Biochimica et Biophysica Acta 2009; 1788:660–665.

695 Nieva JL, Apellaniz B, Huarte N, Lorizate M. A new paradigm in molecular recognition? Specific antibody binding to membrane-inserted HIV-1 epitopes. J Mol Recognit 2011;24:642-646.

696 Maeso R, Huarte N, Julien JP, Kunert R, Pai EF, Nieva JL. Interaction of anti-HIV type 1 antibody 2F5 with phospholipid bilayers and its relevance for the mechanism of virus neutralization. AIDS Res Hum Retroviruses 2011; 27:863-876.

697 Ofek G, McKee K, Yang Y, Yang Z, Skinner J, Guenaga FJ *et al*. Relationship between antibody 2F5 neutralization of HIV-1 and hydrophobicity of Its heavy chain third complementarity-determining region. J Virol 2010; 84: 2955-2962.

698 Franquelim HG, Chiantia S, Veiga AS, Santos NC, Schwille P, Castanho MA. Anti-HIV-1 antibodies 2F5 and 4E10 interact differently with lipids to bind their epitopes. AIDS 2011;25:419-428.

699 Nabel GJ. Close to the Edge: Neutralizing the HIV-1 Envelope. Science 2005; 308: 1878-1879.

700 Boswell CA, Tesar DB, Mukhyala K, Theil FP, Fielder PJ, Khawli LA. Effects of charge on antibody tissue distribution and pharmacokinetics. Bioconjugate Chem 2010; 21:2153-2163.

701 Belting M. Heparan sulphate proteoglycan as a plasma membrane carrier. Trends Biochem Sci 2003; 28: 145-151.

702 Kreuger J, Spillmann D, Li JP, Lindahl U. Interactions between heparin sulphate and proteins: the concept of specificity J Cell Biol 2006; 174; 323-327.

703 Devenny JJ, Wagner RC. Transport of immunoglobulin G by endothelial vesicles in isolated capillaries. Microcirc Endothelium Lymphatics 1985;2:15-26.

704 Hong G, Chappey O, Niel E, Scherrmann JM.Enhanced cellular uptake and transport of polyclonal immunoglobulin G and fab after their cationization J Drug Target 2000;8:67-77.

705 Igawa T, Tsunoda H, Tachibana T, Maeda A, Mimoto F, Moriyama C, Nanami M, Sekimori Y, Nabuchi Y, Aso Y, Hattori K. Reduced elimination of IgG antibodies by engineering the variable region. Protein Eng Des Sel 2010;23:385-392.

706 Clamp JR. The relationship between secretory immunoglobulin A and mucus. Biochem Soc Trans 1997; 5: 1579-1581.

707 Crowther R, Lichtman S, Forstner J, Forstner G. Failure to show secretory IgA binding by rat intestinal mucin. Fed Proc 1985; 44: 691.

708 Iontcheva I, Oppenheim FG, Troxler RF. Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin and histatins. J Dent Res 1997; 76: 734-743.

709 Kobayashi K, Ogata H, Morikawa M, Iijima S, Harada N, Yoshida T *et al*. Distribution and partial characterisation of IgG Fc binding protein in various mucin producing cells and body fluids. Gut 2002;51:169-176.

710 Roux KH, Zhu P, Seavy M, Katinger H, Kunert R, Seamon V. Electron microscopic and immunochemical analysis of the broadly neutralizing HIV-1-specific,anti-carbohydrate antibody, 2G12. Mol Immunol 2004;41:1001-1011.

711 Patton DL, Sweeney YC, Rabe LK, Hillier SL. The vaginal microflora of pig-tailed macaques and the effects of chlorhexidine and benzalkonium on this ecosystem. Sex Transm Dis 1996;23:489-93. Erratum in: Sex Transm Dis 1997;24:180.

712 Patton DL, Sweeney YC, Tsai CC, Hillier SL. Macaca fascicularis vs. Macaca nemestrina as a model for topical microbicide safety studies. J Med Primatol 2004;33:105-108.

713 De Los Santos GE, Zuniga S. Comparison of pH measurement devices for

determining vaginal pH in cynomolgus macaques. Contemp Top Lab Anim Sci 2004 ;43:39-40.

714 Krohn M A, Hillier S L, Eschenbach D A. Comparison of methods for diagnosing bacterial vaginosis among pregnant women. J Clin Microbiol 1989;27:1266–1271.

715 Green D. Interpreting coagulation assays. Blood, Coagulation & Fibrinolysis 2010; 21: S3-S6.

716 Poller L. International Normalized Ratios (INR): the first 20 years. J Thromb Haemost 2004; 2: 849–860.

717 Requirements for thromboplastins and plasma used to control oral anticoagulant therapy (Requirements for Biological Substances no. 30, revised 1982). In: WHO Expert Committee on Biological Standardization. Thirty-Third report. Annex 3, WHO Technical Report Series, no. 687. Geneva, Switzerland: World Health Organization;1983.

718 Horsti J, Uppa H, Vilpo JA. Poor agreement among prothrombin time international normalized ratio methods: comparison of seven commercial reagents. Clin Chem 2005; 51: 553–560.

719 Jackson CM, Esnouf MP. Has the time arrived to replace the quick prothrombin time test for monitoring oral anticoagulant therapy? . Clin Chem 2005; 51 : 483–485.

720 El-Sadr WM, Mayer KH, Maslankowski L, Hoesley C, Justman J, Gai F *et al*. Safety and acceptability of cellulose sulfate as a vaginal microbicide in HIV-infected women. AIDS. 2006;20:1109-1116.

721 Kamali A, Byomire H, Muwonge C, Bakobaki J, Rutterford C, Okong P *et al*. A randomised placebo-controlled safety and acceptability trial of PRO 2000 vaginal microbicide gel in sexually active women in Uganda. Sex Transm Infect 2010; 86:222-226.

722 Joshi S, Bell B, Profy A, Kuruc J, Fang G, Maslankowski L *et al.* Documenting intermenstrual bleeding in a vaginal microbicide study: case reports and lessons learned. Aids Res Hum Retroviruses 2006; 22:294-296.

723 Roter DL, Hall JA. Physician gender and patient-centered communication: a critical review of empirical research. Annu Rev Public Health 2004;25:497-519.

724 Mauck CK, Weiner DH, Lai JJ, Schwartz JL. Colposcopy: still useful in microbicide safety trials? Sex Transm Dis 2012;39:465-469.

725 Mauck C, Doncel G, Archer DF, Creinin MD, Jou-Lai J, Weiner DH *et al*. Assessing markers of inflammation after vaginal product use: nonoxynol-9, cellulose sulfate, and HEC placebo comparative double-blind phase I trial. Microbicides Conference 2010, Pittsburgh, Pennsylvania, USA; May 22-25, 2010. Oral abstract.

726 Zhou X, Brown CJ, Abdo Z, Davis CC Hansmann M A, Joyce P *et al.* Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. ISME J*;* 2007;1:121–133.

727 Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL *et al*. Microbes and Health Sackler Colloquium: vaginal microbiome of reproductive-age women. Proc Natl Acad Sci, USA 2011; 108 (Suppl.1) 4680-4687.

728 Amsel R, Totten P A, Spiegel C A, Chen K C S, Eschenbach D A, Holmes K K. Nonspecific vaginitis: diagnostic criteria and epidemiologic associations. Am J Med 1983; 74**:**14–22.

729 Gutman RE, Peipert JF, Weitzen S, Blume J. Evaluation of clinical methods for diagnosing bacterial vaginosis. Am J Obstet Gynecol 2005; 105: 551-556.

730 Holst E, Wathne B, Hovelius B, Mårdh P-A. Bacterial vaginosis: microbiological and clinical findings.Eur J Clin Microbiol 1987; 6: 536-541.

731 Hellberg D, Nilsson, Mardh P-A. The diagnosis of bacterial vaginosis and vaginal flora changes. Arch Gynecol Obstet 2001; 265 :11-15.

732 Wagner G, Ottesen B. Vaginal physiology during menstruation. Ann Intern Med 1982;96:921-923.

733 Caillouette J C, Sharp CF, Zimmerman G J, Roy S. Vaginal pH as a marker for bacterial pathogens and menopausal status. Am J Obstet Gynecol 1997;176:1270-1275.

734 Brabin L, Roberts SA, Fairbrother E, Mandal D, Higgins SP, Chandiok S *et al.* Factors affecting vaginal pH levels among female adolescents attending genitourinary medicine clinics.Sex Transm Infect 2005;81**:**483-487.

735 Hunter CA Jr, Long KR. Vaginal and cervical pH in normal women and in patients with vaginitis. Am J Obstet Gynecol 1958;75:872-874.

736 Schwebke JR, Hillier SL, Sobel JD, McGregor JA, Sweet RL. Validity of the vaginal gram stain for the diagnosis of bacterial vaginosis. Obstet Gynecol 1996;88:573-576.

737 Tam M T, Yungbluth M, Myles T. Gram stain method shows better sensitivity than clinical criteria for detection of bacterial vaginosis in surveillance of pregnant low-income women in a clinical setting. Infect Dis Obstet Gynecol 1998; 6:273–275.

738 Gratacos E, Figueras F, Barranco M, Ros R, Andreu A, Alonso P L, Cararach V. Prevalence of bacterial vaginosis and correlation of clinical to Gram stain diagnostic criteria in low risk pregnant women. Eur J Epidemiol 1999; 15:913–916.

739 Joesoef MR, Hillier SL, Josodiwondo S, Linnan M. Reproducibility of a scoring system for gram stain diagnosis of bacterial vaginosis. J Clin Microbiol 1991;29:1730–1731.

740 Hillier S L, Krohn M L, Rabe LK, Klebanoff S J, Eschenbach DA. The normal vaginal flora, H2O2-producing lactobacilli, and bacterial vaginosis in pregnant women. Clin Infect Dis 1993;16(Suppl. 4):S273–S281.

741 Delaney M L, Onderdonk A B, for the Microbiology and Prematurity Study Group. Nugent score related to vaginal culture in pregnant women. Obstet Gynecol 2001; 98:79–84.

742 Eschenbach DA, Davick PR, Williams BL, Klebanoff SJ, Young-Smith K, Critchlow CM, Holmes KK. Prevalence of hydrogen Peroxide-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. J Clin Microbiol 1989;27:251-256.

743 Falsen E, Pascual C, Sjoden B, Ohlen M, Collins MD. Phenotypic and phylogenetic characterization of a novel *Lactobacillus* species from human sources: description of *Lactobacillus iners* sp.nov. Intl J Syst Bacteriol 1999; 49: 217-221.

744 Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Van Simaey L, De Ganck C, De Backer E, Temmerman M, Vaneechoutte M: Comparison between Gram stain and culture for the characterization of vaginal microflora: definition of a distinct grade that resembles grade I microflora and revised categorization of grade I microflora. BMC Microbiol 2005, 5:61. doi:10.1186/1471-2180-5-61.

745 Ison CA, Hay PE: Validation of a simplified grading of Gram stained vaginal smears for use in genitourinary medicine clinics. Sex Transm Infect 2002, 78:413-415.

746 Verstraelen H, Verhelst R, Roelens K, Claeys G, Weyers S, De Backer E, Vaneechoutte M, Temmerman M: Modified classification of Gram-stained vaginal smears to predict spontaneous preterm birth: a prospective cohort study. Am J Obstet Gynecol 2007; 196:528 e1-6.

747 Zhou X, Hansmann MA, Davis CC, Suzuki H, Brown CJ, Schütte U *et al.* The vaginal bacterial communities of Japanese women resemble those of women in other racial groups. FEMS Immunology and Med Microbiol. 2010;58:169–181.

748 Jakobsson T and Forsum U. *Lactobacillus iners*: a Marker of Changes in the Vaginal Flora? J Clin Microbiol 2007; 45: 3145.

749 Srinivasan S, Liu C, Mitchell CM, Fiedler TL, Thomas KK, Agnew JK *et al*. Temporal Variability of Human Vaginal Bacteria and Relationship with Bacterial Vaginosis. PLoS ONE 2010; 5: e10197 doi:10.1371/journal.pone.0010197.

750 Keane F E A, Ison C A, Taylor-Robinson D. A longitudinal study of the vaginal flora over a menstrual cycle. Int J STD AIDS 1997;8:489-494.

751 Brotman RM, Ravel J, Cone R A, Zenilman JM. Rapid fluctuation of the vaginal microbiota measured by Gram stain analysis. Sex Transm Infect 2010;86:297-302.

752 Rosenthal S L, Holmes W, L Maher. Australian men's experiences during a microbicide male tolerance study. AIDS Care 2009; 21: 125-130.

753 Ritchie J, Spencer L. Qualitative data analysis for applied policy research. In: A Bryman, RG Burgess, editors. Analysing Qualitative Data. London, England: Routledge 1994;pp 173-194.

754 Tolley EE, Eng E, Kohli R, Bently M E, Mehendale S, Bunce A *et al.* Examining the context of microbicide acceptability among married men and women in India. Culture, Health and Sexuality 2006; 8: 351-369.

755 Bentley ME, Morrow KM, Fullem A, Chesney MA, Horton SD, Rosenberg Z, Mayer KH. Acceptability of a novel vaginal microbicide during a safety trial among low-risk women. Fam Plann Perspect 2000;32:184-188.

756 Lopez Gonzalez L. Global Aids Funding: will the world pay up to end HIV? NAM Aidsmap HIV Treatment Update 2012;212; 4-9. Available at: http://www.aidsmap.com/v634770091449600000/file/1052486/HTU_212_Web.pdf. Accessed 13 July 2012.

757 Vincent KL, Bell BA, Rosenthal SL *et al*. Application of optical coherence tomography for monitoring changes in cervicovaginal epithelial morphology in macaques: potential for assessment of microbicide safety. Sex Transm Dis 2008; 35: 269-275.

758 Vincent KL, Bourne N, Bell BA *et al.* High resolution imaging of epithelial injury in the sheep cervicovaginal tract: a promising model for testing safety of candidate microbicides. SexTransm Dis 2009; 36: 312-318.

759 Vincent KL, Stanberry LR, Moench TR, Bretkopf CR, Loza ML, Wei J *et al.* Optical coherence tomography compared with colposcopy for assessment of vaginal epithelial damage: a randomised controlled trial. Obstet and Gynaecol 2011; 118:1354-1361.

760 Cummins JE Jr, Doncel, GF. Biomarkers of cervicovaginal inflammation for the assessment of microbicide safety. Sex Transm Dis 2009; 36 (Suppl.): S84-91.

761 Low-Beer N. The role of biopsy in vaginal microbicide development. J Acquir Immune Defic Syndr 2004;37(Suppl.3):S181-183.

762 Su HI, Schreiber CA, Fay C, Parry S, Elovitz MA, Zhang *et al.* Mucosal integrity and inflammatory markers in the female lower genital tract as potential screening tools for vaginal microbicides. Contraception 2011;84:525-532.

763 Dezzutti CS, Hendrix CW, Marrazzo JM, Pan Z, Wang L *et al.* Performance of swabs, lavage, and diluents to quantify biomarkers of female genital tract soluble mucosal mediators. PLoS ONE 2011;6: e23136. doi:10.1371/journal.pone.0023136.

764 Jespers V, Buvé A, Van Damme L. Safety trial of the vaginal microbicide cellulose sulfate gel in HIV-positive men. Sex Transm Dis 2007;34:519-522.

765 Morrow KM, Hendrix C. Clinical evaluation of microbicide formulations. Antiviral Res 2010; 88 (Suppl 1.): S40-S46.

766 Corti D, Langedijk JPM, Hinz A, Seaman MS, Vanzetta F *et al.* Analysis of Memory B Cell Responses and Isolation of Novel Monoclonal Antibodies with Neutralizing Breadth from HIV-1-Infected Individuals. PLoS ONE 2010;5: e8805. doi:10.1371/journal.pone.0008805

767 Davis D, Koornstra W, Mortier D, Fagrouch Z, Verschoor EJ, Heeney JL *et al.* Protection in macaques immunized with HIV-1 candidate vaccines can be predicted using the kinetics of their neutralizing antibodies. PLoS ONE 2011;6: e28974. doi:10.1371/journal.pone.0028974

768 Zwick MB and Burton DR. HIV-1 neutralization: mechanisms and relevance to vaccine design. Curr HIV Res 2007; 5: 608-624.

769 Wu X, Zhou T, O'Dell S, Wyatt RT, Kwong PD, Mascola JR. Mechanism of human immunodeficiency virus type 1 resistance to monoclonal antibody b12 that effectively targets the site of CD4 attachment. J Virol 2009; 83:10892–10907.

770 Chakrabarti BK, Walker LM, Guenaga JF, Ghobbeh A, Poignard P, Burton DR, Wyatt RT. Direct antibody access to the HIV-1 membrane-proximal external region positively correlates with neutralization sensitivity. J Virol 2011;85:8217-8226.

771 Ruprecht CR, Krarup A, Reynell L, Mann AM, Brandenberg OF, Berlinger L, Abela IA, Regoes RR, Günthard HF, Rusert P, Trkola A. MPER-specific antibodies induce gp120 shedding and irreversibly neutralize HIV-1. J Exp Med 2011;208:439-454.

772 Bomsel M, Heyman M, Hocini H, Lagaye S, Belec L, Dupont C, Desgranges C. Intracellular neutralization of HIV transcytosis across tight epithelial barriers by anti-HIV envelope protein dIgA or IgM. Immunity 1998; 9:277–287.

773 Devito C, Broliden K, Kaul R, Svensson L, Johansen K, Kiama P *et al*. Mucosal and plasma IgA from HIV-1-exposed uninfected individuals inhibit HIV-1 transcytosis across human epithelial cells. J Immunol 2000;165: 5170–5176.

774.Bomsel M, Pastori C, Tudor D, Alberti C, Garcia S, Ferrari D *et al.* Natural mucosal antibodies reactive with first extracellular loop of CCR5 inhibit HIV-1 transport across human epithelial cells. AIDS 2007;21: 13–22.

775 Tudor D, Derrien M., Diomede L, Drillet A.S, Houimel M, Moog C *et al*. HIV-1 gp41-specific monoclonal mucosal IgAs derived from highly exposed but IgG-seronegative individuals block HIV-1 epithelial transcytosis and neutralize CD4(+) cell infection: An IgA gene and functional analysis. Mucosal Immunol 2009; 2: 412–426.

776 De Parseval A, Bobardt MD, Chatterji A, Chatterji U, Elder JH *et al*. A highly conserved arginine in gp120 governs HIV-1 binding to both syndecans and CCR5 via sulphated motifs. J Biol Chem 2005;280: 39493-39504.

777 Mantis NJ, Palaia J, Hessell AJ, Mehta S, Zhu Z *et al*. Inhibition of HIV-1 infectivity and epithelial cell transfer by human monoclonal IgG and IgA antibodies carrying the b12 V region. J Immunol 2007; 179:3144-3152.

778 Hessell AJ, Hangartner L, Hunter M, Hangartner L, Landucci G, Forthal DN *et al*. Fc receptor but not complement binding is important in antibody protection against HIV. Nature 2007;449:101–104.

779 Hessell AJ, Poignard P, Hunter M, Hangartner L, Tehrani DM, Bleeker WK *et al.* Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. Nat Med 2009;15:951-955.

780 Hessell AJ, Rakasz EG, Poignard P, Hangartner L, Landucci G, Forthal DN *et al.* Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. PLoS Pathog 2009; 5: e10000433.doi:10.1371/journal.ppat.1000433.

781 Tudor D, Bomsel M. The broadly neutralizing HIV-1 IgG 2F5 elicits gp41-specific antibody-dependent cell cytotoxicity in a FcγRI-dependent manner. AIDS 2011; 25: 751-759.

782 Perez LG, Costa MR, Todd CA, Haynes BF, Montefiori DC. Utilization of immunoglobulin G Fc receptors by human immunodeficiency virus type 1: a specific role for antibodies against the membrane-proximal external region of gp41. J Virol 2009;83:7397-7410.

783 Klein JS, Webster A, Gnanapragadsam NP, Galimidi RP, Bjorkman PJ. A dimeric form of the HIV-1 antibody 2G12 elicits potent antibody-dependent cellular cytotoxicity. AIDS 2010; 24: 1633-1640.

784 Moldt B, Schultz N, Dunlop DC, Alpert MD, Harvey JD, Evans DT *et al*. A Panel of IgG1 b12 Variants with Selectively Diminished or Enhanced Affinity for Fcγ Receptors To Define the Role of Effector Functions in Protection against HIV. J Virol 2011;85:10572-10581.

785 Florese R H, Demberg T, Xiao P, Kuller L, Larsen K, Summers LE, Venzon D *et al.* Contribution of nonneutralizing vaccine-elicited antibody activities to improved protective efficacy in rhesus macaques immunized with Tat/Env compared with multigenic vaccines. J Immunol 2009;182:3718-3727.

786 Gomez-Roman V R, Patterson LJ, Venzon D, Liewehr D, Aldrich K, Florese R, Robert-Guroff M. Vaccine-elicited antibodies mediate antibody-dependent cellular cytotoxicity correlated with significantly reduced acute viremia in rhesus macaques challenged with SIVmac251. J Immunol 2005;174:2185-2189.

787 Forthal D N, Gilbert PB, Landucci G, Phan T. Recombinant gp120 vaccine-induced antibodies inhibit clinical strains of HIV-1 in the presence of Fc receptor-bearing effector cells and correlate inversely with HIV infection rate. J Immunol 2007;178:6596-6603.

788 Lambotte O, Ferrari G, Moog C, Yates NL, Liao XL, Parks RL *et al.* Heterogeneous neutralizing antibody and antibodydependent cell cytotoxicity responses in HIV-1 elite controllers. AIDS 2009;568 23:897-906.

789 Forthal DN, Landucci G, Bream J, Jacobson LP, Phan TB, Montoya B. FcgammaRIIa genotype predicts progression of HIV infection. J. Immunol 2007; 179: 7916–7923.

790 Kijak G, Li S, Paris R, Ngauy V, Michael N, Rerks-Ngarm S *et al.* for the MOPH–TAVEG Investigators. Modulation of vaccine effect by FcG receptor 3a genetic polymorphism in RV144. 19th Conference on Retroviruses and Opportunistic Infections, Seattle, USA; March 5-8, 2012. Abstract 420.

791 Moldt B, Shibata-Koyama M, Rakasz EG, Schultz N, Kanda Y, Dunlop DC *et al.* A non-fucosylated variant of the anti-HIV-1 MAb b12 has enhanced FcγRIIIa-mediated antiviral activity in vitro but not improved protection against mucosal SHIV challenge in macaques. JVirol 2012; 86:6189-6196.

792 Burton DR, Hessell AJ, Keele BF, Klasse PJ, Ketas TA, Moldt B *et al.* Limited or no protection by weakly or nonneutralizing antibodies against vaginal SHIV challenge of macaques compared with a strongly neutralizing antibody. Proc Natl Acad Sci USA. 2011;108:11181-11186.

793 Bomsel M, Tudor D, Drillet A-S, Alfsen A, Ganor Y, Roger M-G *et al*. Immunization with HIV-1 gp41 Subunit Virosomes Induces Mucosal Antibodies Protecting Nonhuman Primates against Vaginal SHIV Challenges Immunity 2011; 34 :269–280.

794 González N, Bermejo M, Calonge E, Jolly C, Arenzana-Seisdedos F, Pablos JL *et al*. SDF-1/CXCL12 Production by Mature Dendritic Cells Inhibits the Propagation of X4-Tropic HIV-1 Isolates at the Dendritic Cell-T-Cell Infectious Synapse. J Virol 2010; 84: 4341–4351.

795 Nishimura Y, Igarashi T, Haigwood N, Sadjadpour R, Plishka RJ, Buckler-White A *et al.* Determination of a statistically valid neutralization titer in plasma that confers protection against simian-human immunodeficiency virus challenge following passive transfer of high-titered neutralizing antibodies. J Virol 2002; 76: 2123–2130.

796 Salle B, Brouchard P, Bourry O, Abdelkrim M, Andrieu T, Prevot S *et al.* Infection of macaques after vaginal exposure to cell-associated simian immunodeficiency virus. J Infect Dis 2010;202: 337-344.

797 Chusainow J, Yang Y S, Yeo JH, Toh P C, Asvadi P, Wong N S, Yap MG. A study of monoclonal antibody-producing CHO cell lines: What makes a stable high producer?. Biotechnol Bioeng 2009;102: 1182–1196.

798 Paul M, Ma JK-C. Plant-made pharmaceuticals: Leading products and production platforms. Biotechnol Appl Biochem 2011;58:58–67.

799 Xu J, Dolan MC, Medrano G, Cramer CL, Weathers PJ. Green factory: Plants as bioproduction platforms for recombinant proteins. Biotechnol Adv 2012;30:1171-1184.

800 Giritch A, Marillonnet S, Engler C, van Eldik G, Botterman J, Klimyuk V, Gleba Y. Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. Proc Natl Acad Sci USA 2006; 103:14701–14706.

801 Huang Z, Phoolcharoen W, Lai H, Piensook K, Cardineau G, Zeitlin L *et al*. High-level rapid production of fullsize monoclonal antibodies in plants by a single-vector DNA replicon system.Biotechnol Bioeng 2010; 106: 9–17.

802 Whaley KJ, Hanes J, Shattock R, Cone RA, Friend DR. Novel approaches to vaginal delivery and safety of microbicides: biopharmaceuticals, nanoparticles, and vaccines. Antiviral Res 2010 ; 88 (Suppl 1): S55-66.

803 Ma JKC, Barros E, Bock R, Christou P, Dale PJ, Dix PJ *et al.* Molecular farming for new drugs and vaccines. Current perspectives on the production of pharmaceuticals in transgenic plants. EMBO 2005;Rep 6:593–599.

804 Sack M, Paetz A, Kunert R, Bomble M, Hesse F, Stiegler G *et al.* Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. FASEB J 2007; 21:1655–1664.

805 Strasser R, Castilho A, Stadlmann J, Kunert R, Quendler H, Gattinger P *et al*. Improved virus neutralization by plant-produced anti-HIV antibodies with a homogeneous beta1,4-galactosylated N-glycan profile. The Journal of Biological Chemistry 2009; 284: 20479-20485.

806 Rademacher T, Sack M, Arcalis E, Stadlmann J, Balzer S, Altmann F *et al*. Recombinant antibody 2G12 produced in maize endosperm efficiently neutralize HIV-1 and contains predominantly single-GlcNAc N-glycans. Plant Biotechnol J 2008;6:189–201.

807 Ramessar K, Rademacher T, Sack M, Stadlmann J, Platis D, Stiegler G *et al.* Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. Proc Natl Acad Sci USA 2008;105:3727–3732.

808 Forthal DN, Gach JS, Landucci G, Jez J, Strasser R, Kunert R, Steinkellner H. Fc-glycosylation influences Fcγ receptor binding and cell-mediated anti-HIV activity of monoclonal antibody 2G12. J Immunol 2010;185;6876-6882.

809 Deisenhofer J. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9- and 2.8-Å resolution. Biochemistry 1981; 20,2361–2370.

810 Jefferis R. Glycosylation as a strategy to improve antibody-based therapeutics. Nat Rev Drug Discov 2009;8:226-234.

811 Gomord V, Fitchette A C, Menu-Bouaouiche L, Saint-Jore-Dupas C, Michaud D, Faye L. Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol J 2010; 8: 564–587.

812 Garcia-Casado G, Sanchez-Monge R, Chrispeels M J, Armentia A, Salcedo G, Gomez, L. Role of complex asparagine-linked glycans in the allergenicity of plant glycoproteins.Glycobiology 1996; 6: 471–477.

813 Fox JL. HIV drugs made in tobacco. Nature Biotechnology 2011; 29: 852.

814 Morrow KM, Fava JL, Rosen RK, Christensen AL, Vargas S, Barroso C. Willingness to use microbicides varies by race/ethnicity, experience with prevention products, and partner type. Health Psychol 2007; 26; 777–786.

815 Montgomery CM, Lees S, Stadler J, Morar NS, Ssali A, Mwanza B *et al*. The role of partnership dynamics in determining the acceptability of condoms and microbicides. AIDS Care 2008; 20: 733–740.

816 Montgomery CM, Gafos M, Lees S, Morar NS, Mweemba O, Ssali A *et al.* ; MDP Team. Re-framing microbicide acceptability: findings from the MDP301 trial. Cult Health Sex 2010;12:649-662.

817 Bentley ME, Morrow K, Fullem A, Chesney MA, Horton SD, Rosenberg Z, Mayer KH. Acceptability of a novel vaginal microbicide during a safety trial among low-risk women. Fam Plann Perspect 2000; 32: 184–188.

818 Schwartz JL, Mauck C, Lai JJ, Creinin MD, Brache V, Ballagh SA *et al.* Fourteen-day safety and acceptability study of 6% cellulose sulfate gel: a randomized double-blind Phase I safety study. Contraception 2006 ;74:133-140.

819 Malonza IM, Mirembe F, Nakabiito C, Odusoga LO, Osinupebi OA, Hazari K *et al*. Expanded Phase I safety and acceptability study of 6% cellulose sulfate vaginal gel. AIDS 2005;19:2157-2163.

820 Dhookie J. An assessment of the acceptability of PRO 2000/5 microbicide gel in the MDP 301 trial in Johannesburg. Microbicides Conference 2008, New Delhi, India; February 24–27, 2008. Abstract 391.

821 Braunstein S, van de Wijgert J. Preferences and practices related to vaginal lubrication: implications for microbicide acceptability and clinical testing. J Womens Health 2005;14:424-433.

822 Nel A, Kamupira M, Woodsong C, Montgomery E, Nuttall J. Safety, acceptability, and adherence of monthly Dapiravine vaginal microbicide rings for HIV prevention. 19th Conference on Retroviruses and Opportunistic Infections, Seattle, Washington, USA; March 5-8, 2012. Abstract 1089.

823 van der Straten A, Montgomery ET, Cheng H, Wegner L, Masenga G, von Mollendorf C *et al*. High Acceptability of a vaginal ring intended as a microbicide delivery method for HIV prevention in African women. AIDS Behav May 27 2012. [Epub ahead of print]. doi

10.1007/s10461-012-0215-0. Available at:
http://www.springerlink.com/content/8h6662u2p8218276. Accessed July 20 2012.

824 Morrow RJ, Woolfson AD, Donnelly L, Curran R, Andrews G, Katinger D, Malcolm RK. Sustained release of proteins from a modified vaginal ring device. Eur J Pharm Biopharm 2011;77:3-10.

825 Lewis AD, Chen R, Montefiori DC, Johnson PR, Clark KR. Generation of neutralizing activity against human immunodeficiency virus type 1 in serum by antibody gene transfer. J Virol 2002; 76, 8769–8775.

826 Johnson PR, Schnepp BC, Zhang J, Connell MJ, Greene SM, Yuste E *et al*. Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys. Nature Medicine 2009;15: 901 - 906 .

827 Abdel-Motal UM, Sarkis PT, Han T, Pudney J, Anderson DJ, Zhu Q, Marasco WA. Anti-gp120 minibody gene transfer to female genital epithelial cells protects against HIV-1 virus challenge in vitro. PLoS One 2011; 6: e26473. doi: 10.1371/journal.pone.0026473.

828 Yang ZY, Li Y, Hogerkorp CM, Schief WR, Seaman MS, Zhou T *et al.* Rational design of envelope identifies broad neutralizing human monoclonal antibodies to HIV-1. Science 2010;329: 856–861.

829 Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien JP *et al*. Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature 2011; 477:466-470.

830 Falkowska E, Ramos A, Feng Y, Zhou T, Moquin S, Walker LM *et al*. PG V04, an HIV-1 gp120 CD4 binding site antibody, is broad and potent in neutralization but does not induce conformational changes characteristic of CD4. J Virol 2012; 86 :4394-4403.

831 Doria-Rose NA, Louder MK, Yang Z ,O'Dell S, Nason M, Schmidt SD *et al*. HIV-1 neutralization coverage is improved by combining monoclonal antibodies that target independent epitopes. J Virol 2012; 86:3393-3397.

832 Pejchal R, Doores KJ, Walker LM, Khayat R, Huang PS, Wang SK *et al.* A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield. Science 2011;334:1097-1103.

833 Ferir G, Huskens D, Palmer KE, Boudreaux DM, Swanson MM, Markovitz DM *et al*. Combinations of griffithsin with other carbohydrate-binding agents (CBAs) demonstrate superior activity against HIV-1, HIV-2 and selected CBA-resistant HIV-1 strains. AIDS Res Hum Retroviruses June 25 2012 [Epub ahead of print]. Available at http://online.liebertpub.com/doi/pdf/10.1089/AID.2012.0026. Accessed July 25 2012.

834 Barouch D H, Klasse PJ, Dufour J, Veazey RS, Moore JP. Macaque studies of vaccine and microbicide combinations for preventing HIV-1 sexual transmission. Proc Natl Acad Sci USA 2012; 109: 8694-8698.

835 Jespers J, Morris G, Nel A, Crucitti T, Lacey C. Vaginal microbiota assessment by Real Time PCR in the Mabgel1 study and the IPM013 dapivirine ring trial. Microbicides Conference 2012; Sydney, Australia; April 15-18, 2012. Oral abstract.

836 Combination HIV prevention: Tailoring and co-ordinating biomedical, behavioural and structural strategies to reduce new HIV infections. Geneva, Switzerland: Joint United Nations Programme on HIV/AIDS (UNAIDS); 2010.