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Localised cyclical variations in immunoproteins in the female genital tract and the implications on the design and assessment of mucosal infection and therapies.

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Running head:

Effects of cyclical mucosal immunological changes

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Abstract

Problem

Fluctuating hormones regulate reproductive processes in the female genital tract. Consequent changes in the local immunological environment are likely to affect cellular interaction with infectious agents and the assessment of therapies that target mucosal infections.

Method of study

We compared Softcup and Weck-Cel sampling protocols and assessed the changes in the concentrations of 39 soluble proteins with menstrual cycle progression in the mucosal and peripheral compartments.

Results

We demonstrate that the mucosal immunological profile is distinct from serum with inflammatory and migratory signatures that are localised throughout the cycle. The analytes highlighted in the mucosal compartment were generally highest at the follicular phase with a tendency to fall as the cycle progressed through ovulation to the luteal phase.

Conclusion

Our results underscore the need to consider these localised cyclical differences in studies aimed at assessing the outcome of disease and the efficacy of mucosal vaccines and other therapies.

Introduction

The female genital mucosa is home to cellular mediators of immunity such as Langerhans cells, CD4^{+} and CD8^{+} T cells, macrophages, dendritic cells, neutrophils, NK cells and B cells[1]. These cells mediate the innate and adaptive components of mucosal immunity often via the production of a plethora of chemokines, cytokines and other immunoproteins. CD4+ T cells expressing receptors like CD4 and CCR5 also constitute targets for infectious viral particles that traverse the epithelial barrier[2]. Layers of intact stratified squamous cells line the vagina and ectocervix whilst the single columnar epithelium maintains the integrity of the endometrium and endocervix[2]. Together these cells in the most exposed regions of the female genital tract provide a reasonable physical barrier to the entry of infectious pathogens like HIV and an additional secretory mucosal component containing amongst other things innate antimicrobial proteins like secretory leukocyte protease inhibitor (SLPI) [3] and defensins[4].

Varying levels of endogenous female hormones like oestradiol and progesterone regulate the onset of ovulation, implantation, menstruation and other reproductive processes in the female genital tract[5]. These hormones are also known to induce changes in the appearance and function of mucosal tissue. For instance, over the course of the menstrual cycle hormonal fluctuations are believed to induce changes in the viscosity of the secretory component of the mucosal environment [6]. The epithelial barrier is also reportedly affected by these changes as evidenced in the progesterone induced thinning observed in macaques [7]. These changes have been shown to increase tissue susceptibility to Simian Immunodeficiency Virus (SIV) and are likely to affect HIV infection[8], as well as the uptake and response to vaccines and topical microbicides. Although absolute numbers of the

immune cells are believed to fluctuate across the menstrual cycle [9], phenotypic changes and

consequent variations in the levels of secreted chemokines, cytokines and immunoproteins

in the periphery and in mucosal tissue have not been fully elucidated. There is some

evidence to show that the concentrations of immunoproteins in the mucosal compartment

vary with the menstrual cycle [10]. A recent study of cervicovaginal secretions obtained from

Tanzanian women considered to be at high risk of HIV demonstrated increases in a number

of mucosal analytes from pre- to post-ovulation phases of the menstrual cycle [11]. Such

significant variations in the levels of secreted immunoproteins over the menstrual cycle

particularly if they are unique to the mucosal compartment are likely to affect the outcome

and monitoring of therapies that target the mucosal compartment, thus making a case for

the consideration of these differences in the evaluation of mucosal infections in general and

in clinical studies aimed at designing or assessing interventions against HIV infection.

Identifying the best sampling approach for evaluating mucosal responses to infection and

therapy has been a priority, not only for HIV vaccine development, but also for the

evaluation of microbicides [12, 13]. Sample quality, ease of collection, processing and

downstream analyses are common factors considered in selecting the appropriate

approach. In this study we assessed the levels of 39 different soluble immunoproteins in

secreted samples collected from the cervicovaginal compartment and compared them with

those in the endocervix collected at a single time point corresponding to the follicular

phase. In doing so we provide a direct comparison of two important mucosal sampling

techniques. We also collected secretions from the cervicovaginal compartment at three

distinct points that correspond with the follicular (proliferative), ovulatory and luteal

(secretory) phases of the menstrual cycle and compared them with levels seen in the

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peripheral compartment (serum) at the corresponding time points. Analytes assessed

include cytokines, growth factors, chemokines, antimicrobial (including antiviral) proteins

and immunoglobulins. We produced profiles of the immunological landscape across the

menstrual cycle highlighting critical points that need to be considered in designing and

assessing mucosal interventions against pathogens like HIV.

Results

Characteristics of participants

Although samples were collected from 31 women during the course of the study, 19

participants were excluded on account of the participants not being available for sampling

across all the time points (Figure 1), or not providing matching samples from all sampled

compartments for comparison. Of the analysed participants, 4 were on combined oral

contraception, one was on combined transdermal contraception whilst the rest of the

analysed participants used barrier contraception (condoms). Participants were between the

ages of 18 and 45 years with a median age of 27 years.

Endocervical and cervicovaginal compartments display distinctive immunological analyte

signatures in the follicular phase of the menstrual cycle

Samples were collected from two compartments at visit 1 corresponding to days 5-10 of the

menstrual cycle from study participants. We measured the concentrations of 39 analytes in

the samples collected from the cervical os representing endocervical secretions or the upper

vaginal region using Weck-Cel® spears or an Instead ® Softcup respectively. We found the

analyte signatures to be similar overall (Figure 2A) with strong correlations (r = 0.96; p =

0.0001) (Figure 2B). However, there were significant differences between the two

compartments with regards to a few analytes. Although the average analyte concentrations

were generally higher in the vaginal samples we observed significantly higher

concentrations in 6/39 analytes (IL-6, p=0.0137; IFN-β, p=0.0005; SLPI, p=0.0005; MIP-3α,

p=0.0010; P-Selectin, =0.0005; and G-CSF, p=0.0244) in the samples collected by direct

adsorption from the cervical os with Weck-Cel® spears (Supplementary Table 1). Notably

there were no significant differences in the concentrations of mucosal antibodies (IgG1-4,

IgA or IgM) recorded using both sampling techniques. Due to the observed correlation

between the analyte signatures obtained using both techniques we opted to use Softcups in

subsequent analysis as it was generally considered to be more acceptable and less invasive

for study participants.

Serum and cervicovaginal compartments display distinctive immunological analyte

signatures across the menstrual cycle

We compared analyte concentrations in the samples obtained from the upper vaginal

region with those seen in the serum compartment and found the signature of each

compartment to be distinct at visit 1 (Figure 3 and Table 1) . The differences in analyte

concentrations in both compartments were significant for 29/39 analytes at visit 1 (Table 1).

Of the 29 analytes that were found to be different 14 analytes (IL-6, IL-1α, IL-1β, IL-10, IL-2,

β-defensin 3, Elafin, Hnp 1-3, IL-8, MIP-3α, IP-10, P-Selectin, G-CSF, and TGF-β) were higher

in the mucosal compartment whilst 15 analytes (IL-12, IL-4, IL-15, IFN-β, SLPI, MCP-2,

RANTES, L-Selectin, GM-CSF, IgG1, IgG2, IgG3, IgG4, IgA and IgM) were higher in the serum

compartment. We observed similar differences in compartmental analyte signature was

seen at visits 2 and 3 (Supplementary Figure 1 A-C). We then sought to determine how the

unique signatures identified in the serum and vaginal compartments varied throughout the

menstrual cycle. Overall we recorded more significant fluctuations in the concentrations of

secreted analytes in the vaginal compartment across the menstrual cycle compared to the

serum compartment where analyte concentrations appeared to vary less.   Specifically we

recorded significant differences in 14/39 (IL-1β, IL-12, IL-16, IL-4, MCP-1, MIP-1β, RANTES,

SDF-1β, L-Selectin, IgG1, IgG2, IgG3, IgG4, and IgM) analytes sampled in the cervicovaginal

compartment (Figure 4 and Supplementary Figure 2) and found that the concentrations of

these analytes decreased across the menstrual cycle phases represented by the three time

points that we sampled (i.e. decreased with progression from visits 1 to 3). The functional

analyte groups highlighted by this trend included chemokines and antibody isotypes even

though changes were recorded for analytes belonging to at least three other functional

groups. Interestingly the concentrations of secreted analytes in the vagina appeared to be

stable between the ovulation and luteal phases.

We recorded significant differences in just 3/39 (SLPI, P-Selectin, and IL-7) analytes in the

serum compartment (Figure 4 and Supplementary Figure 2) across the menstrual cycle with

all but IL-7 increasing with cycle progression. Fluctuations in serum analyte concentrations

across the cycle were significant for analytes belonging to three functional groups

(Haemopoietic/growth factors, antimicrobial and cell homing). Immunoglobulin levels

remained largely stable in the serum compartment throughout the cycle. Notably there was

no overlap between the analytes that were seen to vary across the menstrual cycle in the

serum and vaginal compartments (Supplementary Figure 2).

Discussion

Our study demonstrates that significant differences exist between the genital immunological secretome and the periphery throughout the menstrual cycle by producing profiles for 39 analytes that are relevant to the investigation of mucosal infections and the evaluation of vaccine responses [14, 15] at three key points of the menstrual cycle. This knowledge is critical because the mucosal environment remains a key route of acquisition and transmission for pathogens like HIV [2]. The presence of strong local innate and adaptive mucosal immune responses is thought to be important for effective prevention and control of HIV infection [16, 17]. It is also crucial to establish a baseline profile for these responses against which any intervention that targets infectious pathogens can be gauged.

Our assessment of two mucosal sampling techniques show a direct correlation in the results obtained. Absorbent sampling materials like Weck-Cel® or ophthalmic sponges/swabs are purportedly advantageous over cervicovaginal lavage (CVL) sampling methods that require sample dilution at the point of collection [14]. However both methods are not necessarily optimal for volume standardisation and ease of sample collection. In studies comparing sampling of mucosal secretions using swabs or CVL, the latter resulted in higher concentrations of immunoproteins despite the extensive dilution of samples that occurred during CVL sample collection [13]. Another study looking at cell recoveries for mucosal sampling techniques noted that the CVL approach enabled the recovery of the lowest amount of viable mononuclear leukocytes in comparison with collection with cytobrushes or biopsies [18]. Softcups rank high in user acceptability [12] and have become increasingly popular due to the practical advantages of their use by study participants without the need for

clinical personnel, and possibility of obtaining cervicovaginal secretions as a concentrate

without dilution leading to better detection of analytes in comparison with CVL [14]. We

sought to determine whether differences exist in the secreted analyte signatures observed

using two sampling methodologies that have not been compared directly before. Whereas

previous studies have highlighted the need to consider differences that may arise as a result

of methodology or the location of sampling in the genital tract [13, 14, 19] we found strong

correlations between the secreted analyte signatures observed in samples collected and

eluted from   Weck-Cel® sponges and those collected using Softcups. However analyte

concentrations were distinctly higher in the samples collected using the Softcups. The latter

observation is in agreement with a previous study comparing Softcups with CVL sampling [14].

This combined with the higher acceptability of self-sampling provided the rationale for using

Softcups for the rest of this study. The notable exceptions to this observation (IL-6, IFN-β,

SLPI, MIP-3α, P-Selectin and G-CSF) may reflect some effect of the different sampling

methodology, or actual differences in the quantities of secreted analytes at various sites in

genital tract that need to be taken into account when either technique is being utilised.

Differences in administration of the sampling techniques used in this study mean that the

Weck-Cel® sponges would have absorbed secretions around the cervical os, whilst the

insertion of the Softcup in the vaginal would facilitate the collection of samples from several

regions of the cervicovaginal tract.

We demonstrate fluctuations in mucosal analytes (IL-1β, IL-12, IL-16, IL-4, MCP-1, MIP-1β,

RANTES, SDF-1β and L-Selectin) across the time points that were examined in this study.

Evaluation of the functional associations of the analytes that fluctuated significantly across

the menstrual cycle in the reproductive compartment revealed the prominence of cellular

chemoattractants and proteins associated with the migration of hematopoietic cells. These

factors have been shown to target a range of immune cell types including CD4^{+} T cells, NK

cells, B cells and monocytes [20-23]. Changes in mucosal immunoglobulins and in proteins

associated with the modulation of the inflammatory response (IL-1β) [24] and the release of

inflammatory cytokines (IL-12) [25, 26] were also demonstrated. The analytes highlighted by

this study in the mucosal compartment were generally highest at the follicular phase (visit 1)

with a tendency to fall as the cycle progressed through ovulation (visit 2) to the luteal phase

(visit 3). A study by Francis *et al* that examined the changes in analyte concentrations pre-

and post-ovulation also revealed a menstrual cycle phase signature in which the

concentrations of chemoattractants like MCP-1 and SDF-1β decreased as the cycle

progressed beyond ovulation whilst IL-18, IL-10, IL-17 and TGF-β increased. Consistent with

our data and those of others [27, 28], they also saw a fall in the concentration of mucosal

immunoglobulins [11]

Endometrial function and the menstrual cycle is tightly regulated by hormones such as

oestrogen and progesterone to ensure the sustenance of progeny. Progesterone increases

soon after ovulation resulting in the beginning of what is generally known as the secretory

phase of the menstrual cycle where it prepares for the reception of a fertilised egg. This

release peaks around day 22 after which progesterone withdrawal occurs as a result of the

disintegration of the corpus luteum[5]. Mouse experiments and *in vitro* studies using human

cells show that the withdrawal of the anti-inflammatory effect of progesterone in the

follicular phase results in an increase in inflammatory analytes under the direct control of

NF-κB [29 30, 31] and a steady rise in the population of mucosal leucocyte [32] and antigen

presenting cells [33]. In addition to higher levels of inflammatory markers we found evidence

of high levels of analytes that direct the influx of immune cells in mucosal samples obtained

in the follicular phase (visit 1).   This very specific signature appeared to be localised and

conspicuously absent in the peripheral compartment where we recorded significant changes

for just three analytes (IL-7, P-Selectin and SLPI). Although distinct from the mucosal

compartment, the changes seen in the periphery could be significant in so much as they

indicate the initiation of immune cell homeostasis, migration to mucosal sites and priming

for any infection as a result of rising levels of inflammation. [34] [35, 36]. The inflammatory and

migratory signature seen in the mucosal compartment was significantly reduced by visit 2

which coincides with ovulation and rising levels of progesterone in the mucosal

compartment.

These findings can inform our understanding of mucosal infections and the approach to

evaluating interventions. For example, a localised surge in mucosal inflammation and

migratory signals in the follicular phase may create an enabling environment for HIV entry

into susceptible cells that have migrated into the mucosal environment [2, 37] and found their

way into its secretions [18]. A number of the analytes that were highlighted in our results have

been implicated in the provision of migratory stimulus to antigen presenting cells [38],

including lymphocytes which are capable of viral uptake and the presentation of HIV

antigens [2]. An influx of antigen presenting cells (APCs) in response to these migratory and

inflammatory or danger signals in the late luteal and follicular[33] phase may result in the

uptake of virus upon exposure and the subsequent migration of APCs to the draining lymph

nodes where further dissemination can occur [2]. Targeting of mucosal vaccines to the late

luteal or follicular phase for instance may enhance their uptake or efficacy.

In this study we have measured 39 analytes to provide a picture of how they vary in the

mucosal versus peripheral compartment and across the menstrual cycle. In doing so we also

provide a comparison of key mucosal sampling methods to inform therapeutic research.

The limitations of this study includes the small sample size and the absence of a direct

quantification of hormone levels in study participants. However we reasoned that samples

collected at visits 1 and 3 in normal cycling women would enable us to compare the

immunological profiles at the peak of oestrogen and progesterone release, respectively. In

addition we have not adjusted our findings to reflect any difference that may arise from

reported behaviours described in the methods section such as the use of oral contraception,

or unreported/undiagnosed infections in volunteers. It is also worth pointing out that four

of the participants in this study were on oral combined contraception whilst one was on

combined transdermal contraception even though they all had regular withdrawal bleeding.

Nevertheless our data highlight the need to standardise the timing of mucosal sampling in

clinical studies. One approach could be to ensure that sampling in all study participants

occurs within a similar period of the menstrual cycle to ensure that the differences in local

cyclical effects are minimised. A similar advantage would be applicable if the administration

and assessment of therapies are designed to fall within similar periods within the menstrual

cycle. As an example, the period defined by visits 2-3 of our study showed the least amount

variation in analyte concentrations (Figure 4), presenting a relatively long window to

accommodate the challenges of scheduling of volunteer visits for clinical studies.   Where

practically possible, studies could also be designed to target this period, or other periods of

the cycle.

There is currently a need to design and evaluate vaccines that are capable of eliciting

protective mucosal immunological responses that can prevent HIV acquisition and

transmission[39-41], particularly in women[2] who have been reported to be more susceptible to

infection compared to age-matched males [42]. Indeed a number of studies have evaluated

aspects of this approach such as the linkage between the nasal and genital mucosal

compartments[43], and the quality of the responses that are elicited by various antigens and

delivery methods[44, 45]. None of these studies have taken into account the role that the

unique and dynamic cervicovaginal immunological landscape might have on the outcome of

vaccine regimens. Our data demonstrates the need to fully consider this in the design and

execution of future vaccine studies.

Methods

Study design and ethical approval

The CASHIR (Characterisation of the activity and stability of anti-HIV-1 agents in the presence of female genital secretions and establishing methods to measure immune response) study was initiated to help in the development of novel agents against mucosal infections such as HIV. Its main aim was to provide some clarity on reliable methods used to measure immune responses in the female genital tract. As such it was designed as a longitudinal prospective cohort study to enable the standardization and comparison of methods and sampling approaches. We set out to explore the measurement of cytokine, chemokine and antibody responses in cervical/vaginal secretions and serum, taking into cognisance the effect of the menstrual cycle on these factors. Samples were collected at The Vaccine Institute, St George's University of London. All volunteers gave full informed and written consent. All relevant approvals were in place and the study was conducted in accordance with the relevant Clinical Regulations and guidelines on Good Clinical Practice.

Subjects and sample collection protocol

Screening was focused on healthy female volunteers aged 18-45 years who were HIV negative, recruited through the use of staff and student mail shots and through the placement of posters within the campuses of St George's University of London and St George's Foundation Trust Hospital notice boards. Volunteers were questioned by a doctor or trained research nurse for any history of sexually transmitted infections like HIV and administered with a pregnancy test. Exclusion criteria were set out as a) Inability to consent b) women who were actively menstruating c) women who have had unprotected (condoms) sexual intercourse 24 hours prior to sample collection, d) women who had intrauterine

contraceptive device in situ e) ability to communicate effectively with clinical team. In

addition, participants who developed bacterial vaginosis, thrush or went on antibiotics were

made to wait until the next cycle for collection with repeats to first sample collection

initiated where necessary. Screened women were eligible to participate if they had regular

menstrual cycles of 28-day duration on average. Samples were collected during three

scheduled visits coinciding with days 5-10 (end of menstruation; follicular phase), days 14-

16 (ovulation), and days 19-24 (luteal phase) of the menstrual cycle. Day 1 coincided with

the first day of their menses.

Serum was collected for antibody and analyte screening at all three visits. Briefly, 10 ml of

peripheral blood was centrifuged in sterile collection tubes for ten minutes at 400 $x\,g$ to

obtain serum. Serum samples were immediately stored at ≤ -15 ^{o}C in 500 µl aliquots.

Cervicovaginal secretions were collected by study participants at all three visits using

INSTEAD Softcup ® (Evofem Inc). The Softcup was self-inserted into the upper vaginal region

and left in situ for one hour before being placed in a sterile 50 ml collection container

(Beckton Dickinson, UK) for immediate storage at -80 ^{o}C prior to analysis. For analysis,

volumes of samples were measured using a positive displacement pipette and diluted in

extraction buffer [250 mM NaCl, 1× protease inhibitor cocktail set 1 (Calbiochem) in

phosphate buffered saline (1XDPBS, Gibco)] prior measuring analyte concentrations by

Luminex. Additional endocervical secretion samples were collected from volunteers at visit

1 (corresponding to days 5-10 of the menstrual cycle and at the same time that

corresponding CVS was collected) under speculum examination using a Weck-Cel®

ophthalmic sponge. Two sponges were placed in the cervical os for 1 minute each to

passively absorb cervical secretions. After removal the Weck-Cels® were immediately

transferred into Costar® Spin-X® polypropylene microcentrifuge filter tubes (Corning Inc)

containing extraction buffer and stored at -80 until analysis.

Processing of samples for Immunological assays

Prior to immunological analysis serum sample aliquots were heat inactivated at 56 $^\circ$C with

slow mixing for 35 minutes. Softcups containing secretions in 50 ml collection tubes were

removed from -80 $^\circ$C, allowed to thaw on ice, and centrifuged at 400 x g for 15 minutes at 4

$^\circ$C. An equal volume of extraction buffer was then added to the secretion samples (after

removal of the cup) with mixing. Processed aliquots were then used in immunological assays

or frozen at -80 $^\circ$C. Similarly Spin–X tubes containing Weck-Cel® spears (sponges) were

thawed on ice, centrifuged at 13,000 rpm in a micro-centrifuge at 4 $^\circ$C. 300µl extraction

buffer was added to the top chamber of the Spin–X tube containing the spears centrifuged

again for an additional 15 minutes at 13,000rpm and 4 $^\circ$C to further extract any residual

secretions. Sponges were removed and eluates used in assays or aliquoted for storage at -

80°C.

Measurement of Immunological proteins

Thirty-three soluble immune proteins were quantified in all processed samples using an in-

house Luminex assay comprising of four panels. The proteins analysed were as follows;

Panel 1- IL-7, IL-6, G-CSF, MIP-3α, IL-8, MCP-1. **Panel 2**- GM-CSF, IP-10, IL-12, MIG, IL-16, IL-

2, RANTES, MCP-2, IL-4, MIP-1β, IL-1α, SDF-1, IFN-γ, IFN-β, TGFβ, IL-1β, IL-15, TNFα. **Panel 3**-

β3-Defensin, IL-17, IL-10, β4-Defensin, L-Selectin. **Panel** 4- SLPI, Elafin, Hnp 1-3 and P-

Selectin. Additionally, six immunoglobulin (Ig) isotypes (IgG1, IgG2, IgG3, IgG4, IgA, IgM)

were measured using a Bio-Plex Pro Human Isotyping 6-plex panel kit from Bio-rad

Laboratories, In all cases, a set of standard curves were run on each plate. UK according the

manufacturer's instructions. Analytes were quantified using Bio-Plex® 100 Luminex analyzer

(Bio-rad Laboratories). Data was collected for three replicates per sample using Bio-Plex

Manager Software version 6. A five parameter logistic regression formula was used to derive

sample concentrations from the standard curves. Analytes below the lower limit of

detection were assigned a concentration of half the lower limit of quantification (LLOQ).

Statistical analysis

Data was characterised using a range of statistical methods. We did not assume a normal

distribution for analyte concentrations in our population. The comparison of analyte

concentrations in paired samples collected from various compartments was performed

using the non-parametric Wilcoxon matched-pairs signed ranked test. The same method

was applied to the evaluation of analyte fluctuations in each compartment over the

menstrual cycle. Spearman correlation was used to calculate the relationships between

analyte sampling techniques and compartmental secretions. p-values were generally

considered not significant at ≥0.05. All statistical analyses were performed and graphs

drawn using Graphpad Prism 6 Software.

Author contributions: JE-M, CJ and SS ran all experiments and sample analysis. AB, SB and CAC provided clinical direction for sample collection and processing. RJS provided scientific direction and support throughout the study.

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Table 1: Median analyte concentrations in the peripheral (serum) and mucosal (cervicovaginal) compartments in the follicular phase of the menstrual cycle

Functional classification	Analyte	Unit	Cervicovaginal region (Softcup) median (range)	Serum median (range)	*p* value
Pro-inflammatory	IL-6	pg/ml	351.7 (2.0-2300)	17.3 (2.0-36.7)	**0.0098**
	IL-1α	pg/ml	1023.3 (556.7-11943.3)	4.0 (1.0-10.3)	**0.0010**
	IL-1ß	pg/ml	323.3 (123.3-3300)	30.0 (4.0-43.3)	**0.0010**
	IL-12	pg/ml	4.7 (2.0-20)	36.7 (19.3-60.0)	**0.0010**
	IL-16	pg/ml	56.7 (17-10683.3)	205.0 (205.0-231.0)	0.3496
	IL-17	pg/ml	35.0 (35.0-196.7)	164.0 (17.0-164.0)	0.5449
	TNF-α	pg/ml	2.0 (2.0-73.3)	17.3 (2.0-26.7)	0.0996
Anti-inflammatory	IL-10	pg/ml	133.0 (6.0-244.0)	66.0 (22.0-98.0)	**0.0371**
Adaptive	IL-2	pg/ml	146.7 (20.0-356.7)	22.0 (22.0-151.33)	**0.0049**
	IL-4	pg/ml	44.0 (20.0-280.0)	515.7 (57.0-913.3)	**0.0010**
	IL-15	pg/ml	1.0 (1.0-1.0)	4.0 (1.0-10.7)	**0.0313**
	IFN-γ	pg/ml	19.0 (17.0-110.0)	18.7 (3.0-86.7)	0.3096
Antimicrobial	IFN-ß	pg/ml	1.0 (0.3-10.0)	100.7 (16.0-233.3)	**0.0010**
	ß-defensin 3	ng/ml	18.8 (8.0-48.8)	3.8 (1.0-5.3)	**0.0020**
	ß-defensin 4	pg/ml	2.0 (2.0-603.3)	3.0 (2.0-110.0)	0.6250
	SLPI	ng/ml	10.6 (4.5-18.7)	33.9 (23.7-48.7)	**0.0010**
	Elafin	ng/ml	4473.5 (27.5-4906.2)	6.5 (2.1-10.5)	**0.0010**
	Hnp-1-3	µg/ml	21.4 (8.8-250.0)	0.2 (0.007-1.3)	**0.0010**
Chemokine	IL-8	pg/ml	30120.0 (10846.6-76113.3)	2.5 (2.0-2.5)	**0.0020**
	MCP-1	pg/ml	170.0 (4.7-2656.7)	260.0 (156.7-430.0)	0.7646
	MCP-2	pg/ml	2.0 (2.0-36.7)	110.0 (73.3-120.0)	**0.0010**
	MIP-1ß	pg/ml	20.0 (2.0-706.7)	53.3 (43.3-93.3)	0.4502
	MIP-3α	pg/ml	900.0 (126.7-7626.7)	36.5 (36.5-39.5)	**0.0010**
	RANTES	pg/ml	10.0 (1.0-116.7)	32826.7 (11133.3-47480.0)	**0.0010**
	IP10	pg/ml	606.7 (290.0-4700.0)	113.3 (60.0-226.7)	**0.0010**
	SDF-1ß	pg/ml	43.3 (33.3-980.0)	78.7 (13.0-250.0)	0.7646
	MIG	pg/ml	120.0 (50.7-826.7)	166.7 (123.3-193.3)	0.8948
Cell homing	L-Selectin	ng/ml	0.6 (0.1-11.2)	103.8 (73.4-136.0)	**0.0020**
	P-Selectin	ng/ml	189.8 (124.1-436.9)	111.5 (50.3-268.4)	**0.0010**
Hematopoietic/ Growth factor	IL-7	pg/ml	9.0 (7.0-93.3)	13.5 (4.0-55.7)	0.7480
	GCSF	pg/ml	2008.3 (506.7-7650.0)	24.0 (1.5-107.2)	**0.0020**
	GM-CSF	pg/ml	7.3 (2.0-26.7)	86.7 (48.7-103.3)	**0.0010**

Page **1** of **2**

	TGF-ß	pg/ml	313.3 (33.3-6130.0)	126.7 (32.7-183.3)	**0.0146**
	IgG1	µg/ml	46.8 (14.1-119)	10138.0 (5491.9-14248.4)	**0.0039**
	IgG2	µg/ml	80.1 (38.9-214.4)	11272 (6433.5-16170.0)	**0.0039**
Antibody	IgG3	µg/ml	1.7 (0.2-10.9)	646.4 (266.6-1399.5)	**0.0039**
	IgG4	µg/ml	1.2 (0.0-2.9)	332.6 (6-855.2)	**0.0078**
	IgA	µg/ml	29.6 (8.2-113.3)	1427.7 (628.4-2674.6)	**0.0039**
	IgM	µg/ml	1.7 (0.5-91.6)	2108.2 (1743.4-2960.6)	**0.0039**

Wilcoxon matched-pairs signed rank Test applied to values recorded for study subjects at visit 1 with p values <0.05 considered to be significant
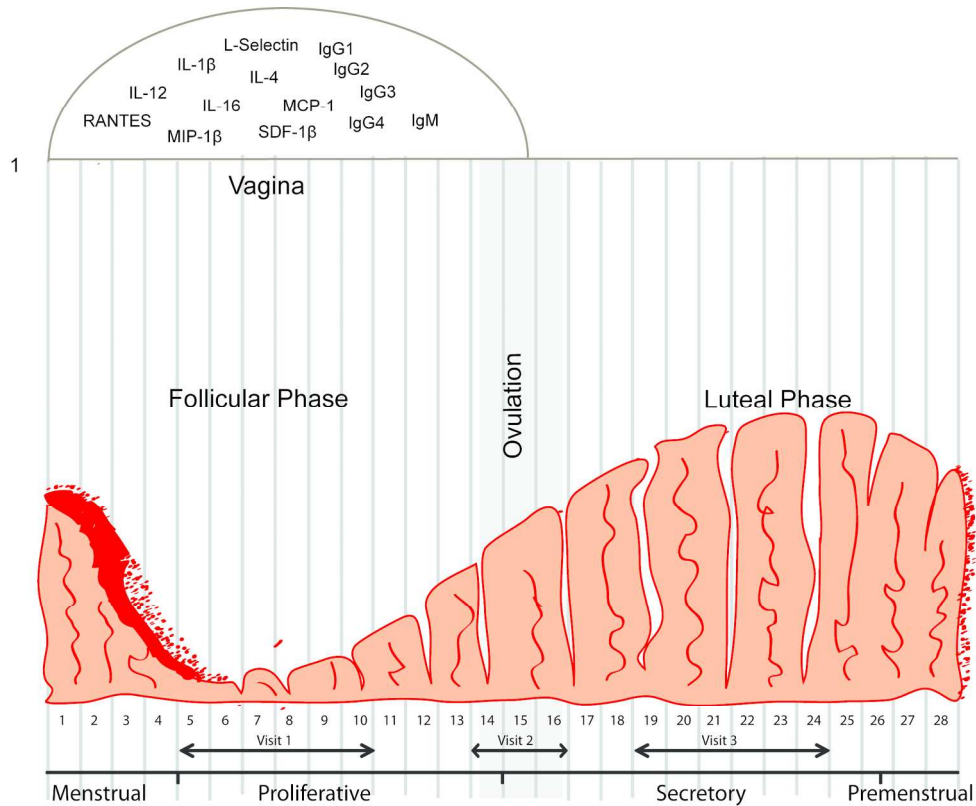
Figure 1: Illustration of the timeline for study visits with a list of the localised analyte changes occurring in the mucosal compartment. Phases of the menstrual cycle were specified for volunteers as the number of days since their last menstrual period where day 1 coincided with the first day of their menses. Samples were collected during three scheduled visits coinciding with days 5-10 (end of menstruation; follicular phase), days 14-16 (ovulation), and days 19-24 (luteal phase). More analyte fluctuations were recorded in study subjects over the time points that were investigated in the mucosal compartment compared to the serum compartment.
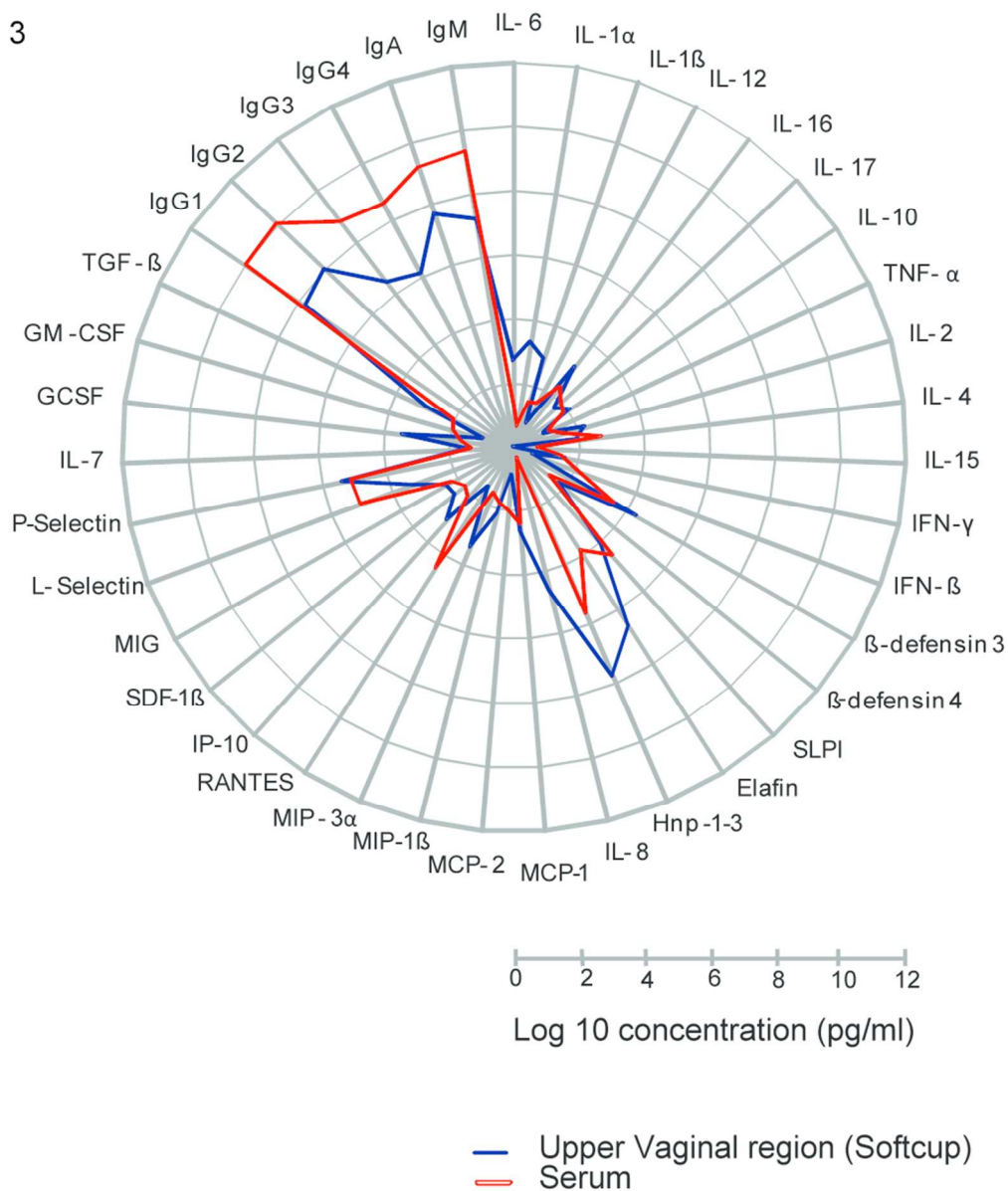
178x142mm (300 x 300 DPI)
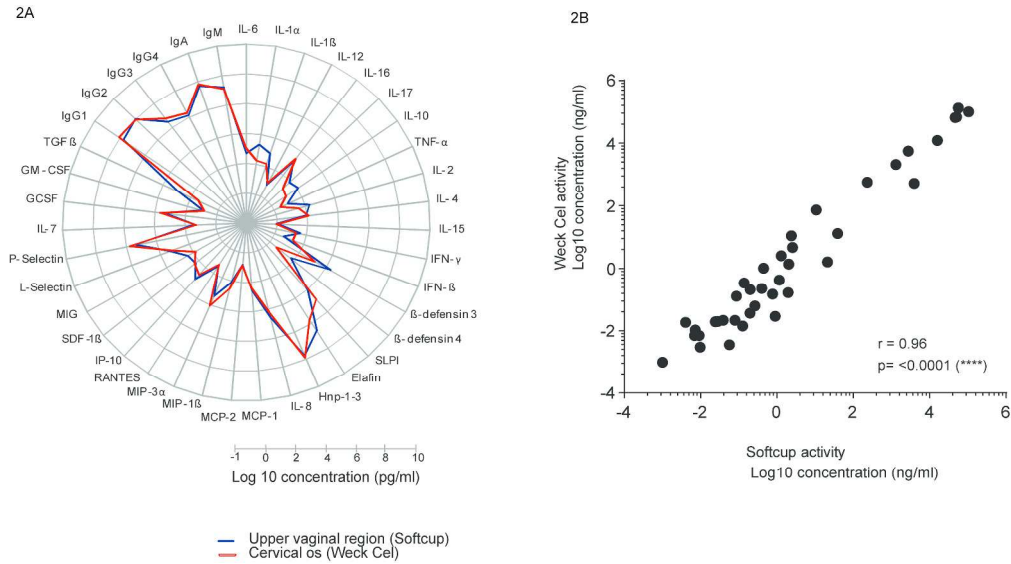
Figure 2A-B: Analysis of the secreted immunoproteome in the female genital tract. A) Sampling with Softcup and Weck Cel sampling techniques reveal similar analyte signatures at visit 1 (corresponding to days 5-10 of the menstrual cycle) in the vaginal and cervical os of 12 healthy women aged 18-45 years. Points represent mean analyte concentration (pg/ml) of three experimental replicates. B) Comparison of 39 analyte concentrations (activity; ng/ml) in matched vaginal and cervical os samples collected from 12 women by Spearman rank correlation. Concentrations shown as Log 10 values in ng/ml. p= <0.0001. 95% confidence interval = 0.9184-0.9781.

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Log 10 concentration (pg/ml)

— Upper Vaginal region (Softcup)
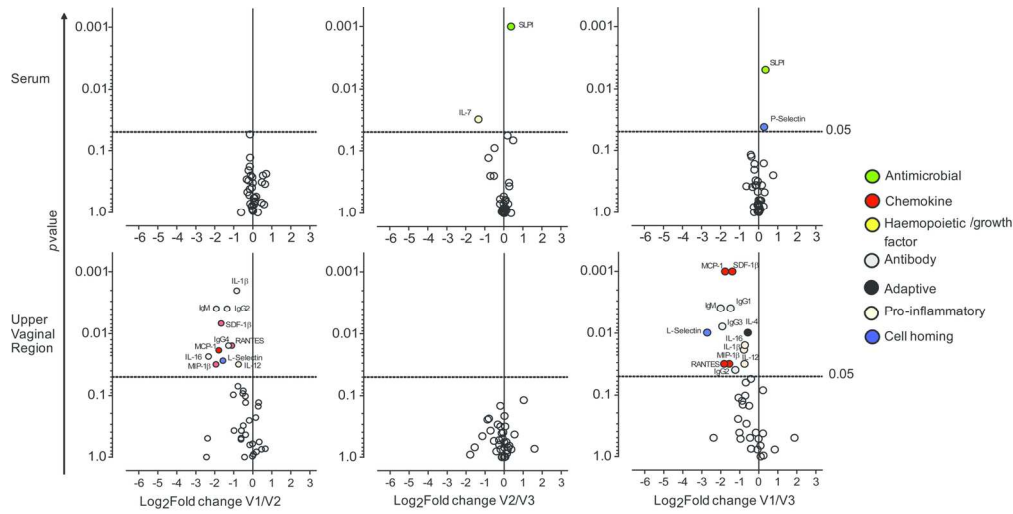— Serum

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Figure 4: More analyte fluctuations observed across the menstrual cycle in the vaginal compartment compared to the serum compartment. Vertical lines represent the points where no change in analyte concentration occurred. Horizontal lines indicate the points where significance is achieved. p-values < 0.05 were considered to be significant.

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Supplementary material

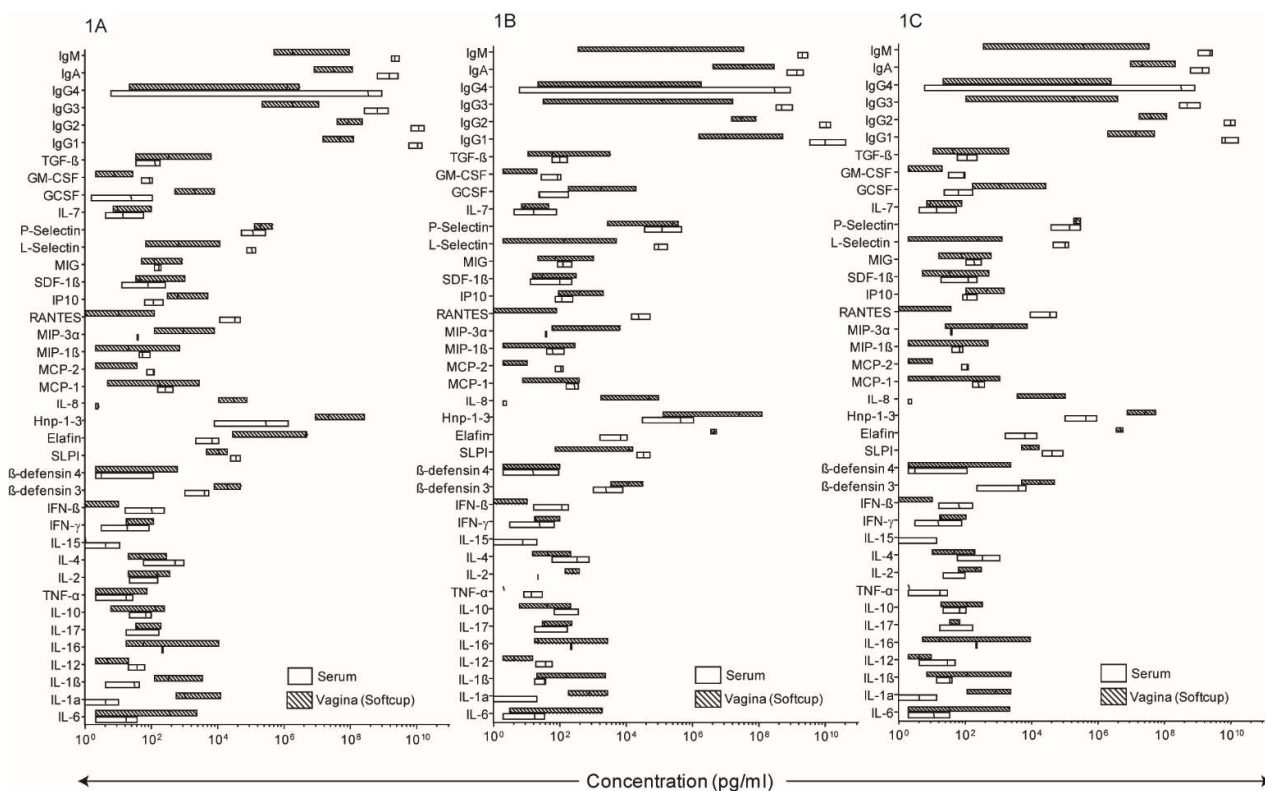Table 1: Median analyte concentrations in the mucosal sub-compartments in the follicular phase of the menstrual cycle

Functional classification	Analyte	Unit	Cervicovaginal region (Softcup) median (range)	Endocervical region (Weck Cel) median (range)	*p* value
Pro-inflammatory	IL-6	pg/ml	326.7 (2.0-2300)	713.3 (33.3-2490.0)	**0.0137**
	IL-1α	pg/ml	993.3 (556.7-11943.3)	98.3 (40.0-640.0)	**0.0005**
	IL-1ß	pg/ml	313.3 (40.0-3300.0)	83.3 (16.7-473.3)	**0.0049**
	IL-12	pg/ml	4.7 (2.0-20.0)	10.5 (9.0-12.0)	0.0586
	IL-16	pg/ml	48.3 (17.0-10683.3)	2050 (73.3-2050.0)	0.1514
	IL-17	pg/ml	35.0 (35.0-290.0)	25.0 (5.3-30.0)	**0.0010**
	TNF-α	pg/ml	2.0 (2.0-73.3)	2.0 (1.3-7.3)	0.6250
Anti-inflammatory	IL-10	pg/ml	138.7 (6.0-244.0)	19.0 (4.0-21.3)	**0.0049**
Adaptive	IL-2	pg/ml	180.0 (20.0-356.7)	36.7 (3.7-80.0)	**0.0010**
	IL-4	pg/ml	41.2 (10.0-280.0)	117.0 (80.7-186.7)	0.2734
	IL-15	pg/ml	1.0 (1.0-1.0)	1.0 (0.7-1.0)	>0.9999
	IFN-γ	pg/ml	19.0 (17.0-110.0)	19.0 (14.0-50.0)	0.4648
Antimicrobial	IFN-ß	pg/ml	2.2 (0.3-10.0)	19.0 (14.0-50.0)	**0.0005**
	ß-defensin 3	ng/ml	20.3 (8.0-48.8)	1.1 (0.3-8.6)	**0.0010**
	ß-defensin 4	pg/ml	2.0 (2.0-603.3)	2.0 (2.0-10.0)	>0.9999
	SLPI	ng/ml	10.7 (4.5-18.7)	68.0 (33.9-151.8)	**0.0005**
	Elafin	ng/ml	4424.9 (27.5-4906.2)	445.0 (146.2-1351.8)	**0.0010**
	Hnp-1-3	µg/ml	22.3 (8.8-250.0)	28.5 (40.72-285.6)	0.8501
Chemokine	IL-8	ng/ml	28.1 (10.8-76.1)	10.5 (2.0-35.1)	**0.0029**
	MCP-1	pg/ml	108.3 (4.6-2656.7)	178.3 (26.7-1053.3)	0.9697
	MCP-2	pg/ml	2.0 (2.0-36.7)	7.3 (4.7-10.0)	0.3145
	MIP-1ß	pg/ml	11.0 (2.0-706.7)	225 (10.0-2166.7)	0.1763
	MIP-3α	pg/ml	861.7 (126.7-7626.7)	9938.335 (97.0-20553.3)	**0.0010**
	RANTES	pg/ml	10.0 (1.0-116.7)	15 (0.0-73.3)	0.4004
	IP10	pg/ml	766.7 (290.0-4700.0)	321.7 (33.3-1453.3)	**0.0005**
	SDF-1ß	pg/ml	48.3 (33.3-980.0)	156.7 (10.7-920.0)	0.5195
	MIG	pg/ml	125.0 (50.7-826.7)	27 (10.7-920.0)	**0.0010**
Cell homing	L-Selectin	ng/ml	0.5 (0.1-11.2)	0.8 (0.1-7.7)	0.8984
	P-Selectin	ng/ml	200.7 (124.0-436.9)	543.9 (201.2-1112.6)	**0.0005**
Hematopoietic/ Growth factor	IL-7	pg/ml	9.0 (7.0-93.3)	20.0 (4.7-33.3)	0.8945
	GCSF	ng/ml	1.9 (0.5-7.7)	4.2 (0.1-14.1)	**0.0244**

	GM-CSF	pg/ml	7.3 (2.0-26.7)	6.0 (5.0-13.3)	>0.9999
	TGF-ß	pg/ml	331.7 (33.3-6130.0)	34 (2.0-56.7)	**0.0010**
Antibody	IgG1	µg/ml	46.8 (14.0-119.5)	63.9 (12.9-623.1)	0.0977
	IgG2	µg/ml	80.1 (38.9-214.4)	69.4 (47.8-274.6)	0.9102
	IgG3	µg/ml	1.8 (0.2-10.9)	1.6 (0.1-30.2)	0.2031
	IgG4	µg/ml	1.2 (0.0-2.8)	2.1 (0.0-3.5)	0.2031
	IgA	µg/ml	29.6 (8.1-113.3)	57.2 (5.1-137.3)	0.5703
	IgM	µg/ml	1.7 (0.5-91.6)	3.2 (0.3-78.3)	0.4961

Wilcoxon matched-pairs signed rank Test applied to values recorded for study subjects at visit 1 with
p values <0.05 considered to be significant

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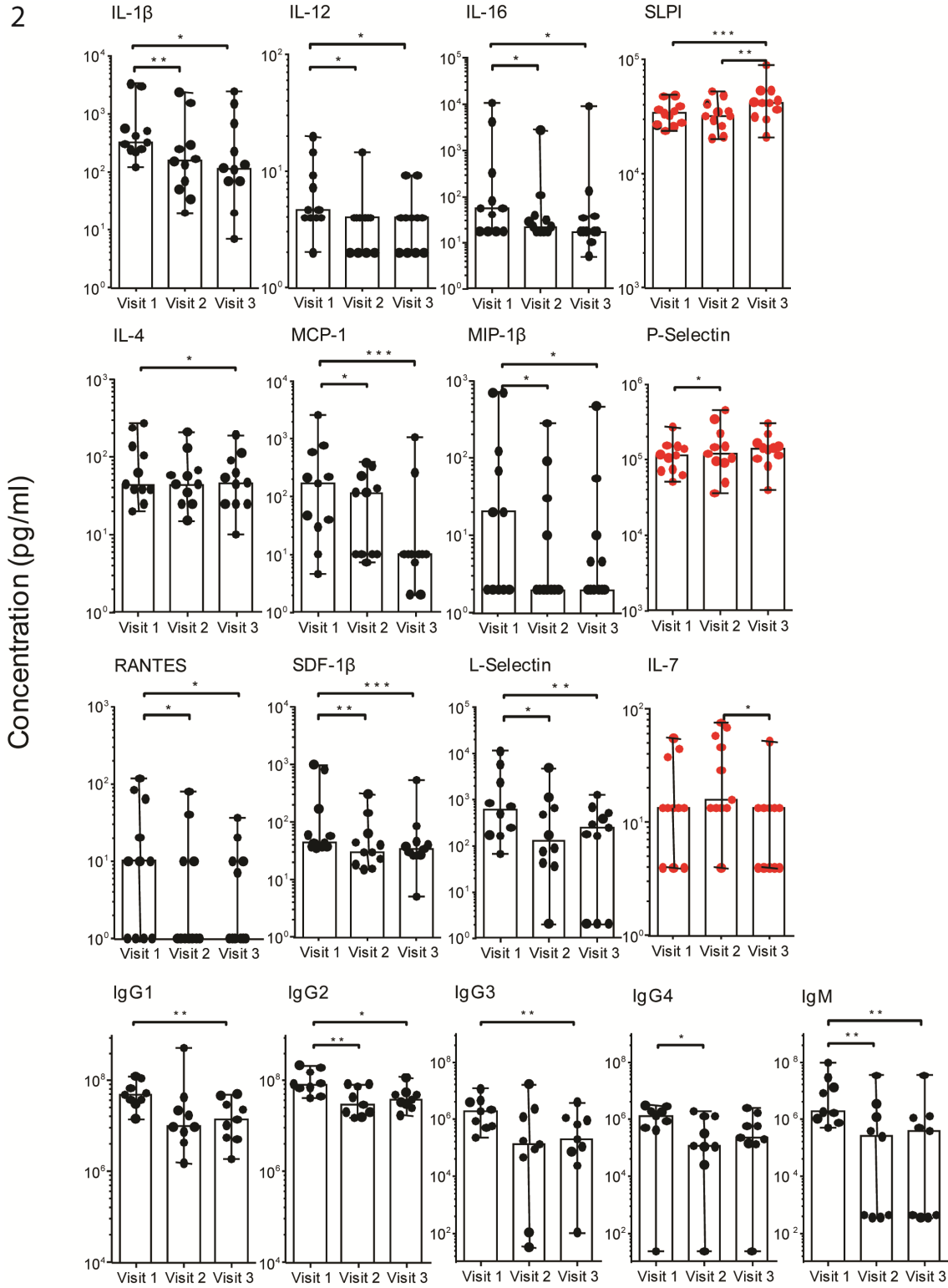
Supplementary Figure 1 A-C: Analyte signatures differ between peripheral (serum) and mucosal (Softcup) compartments across the menstrual cycle. Floating bars show maximum and minimum values recorded for 39 analytes in both compartments with a line at the median for samples collected from study participants at A) visit 1; days 5-10, B) visit 2; days 14-16, and C) visit 3; days 19-24. Data shown for three experimental replicates.

Supplementary Figure 2: More analyte fluctuations were observed in samples collected from the vaginal compartment (●) across the menstrual cycle compared to matched samples collected from the serum compartment (●). Scatter dots are representative of three experimental replicates with bars and lines showing median values and range. p-values <0.05 were considered to be significant.
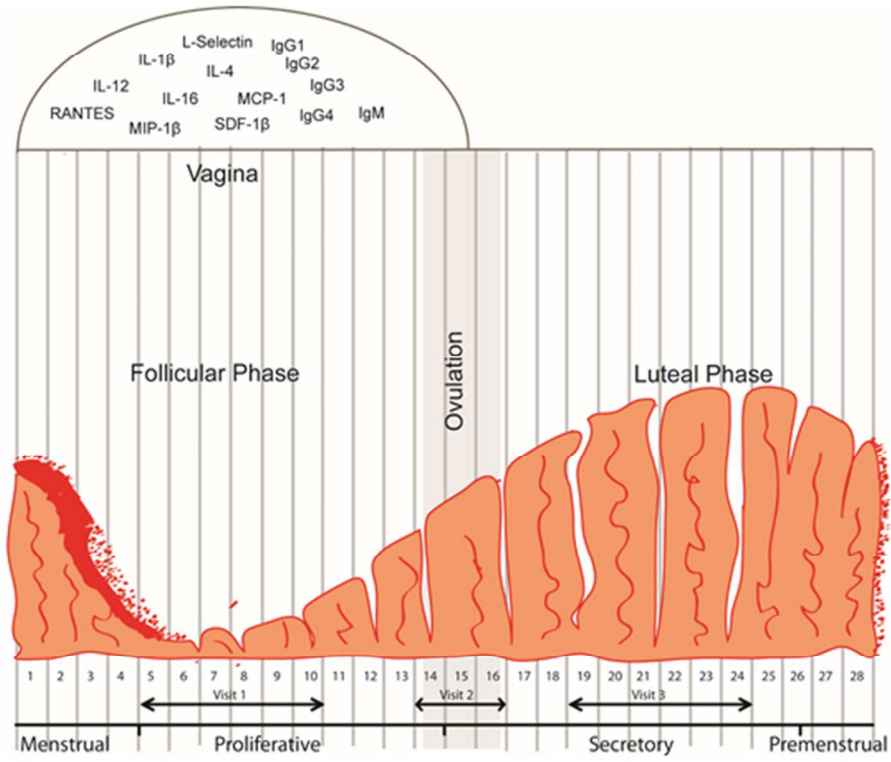
The female mucosal secretome is distinct from serum with characteristic inflammatory and migratory signatures that reflect the local cyclical hormonal changes.

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