Measurement of Calprotectin (S100A8/S100A9) and S100A12 in serum: method development, analytical validation, and clinical application

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A thesis submitted in partial fulfilment of the requirements of the University of Wolverhampton for the degree of Doctor of Biomedical Science

This research programme was carried out in collaboration with the Department of Clinical Chemistry, New Cross Hospital, at the Royal Wolverhampton NHS Trust, 321 Wolverhampton Road, Heath Town, Wolverhampton WV10 0QP, West Midland, U N I T E D K I N G D O M

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DECLARATION & STATEMENTS

This work or any part thereof has not previously been presented in any form to the University or to any other body whether for the purposes of assessment, publication or for any other purpose (unless otherwise indicated). Save for any express acknowledgments, references and/or bibliographies cited in the work, I confirm that the intellectual content of the work is the result of my own efforts and of no other person.

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Michael Mmaduka Udegbune (Candidate)

Date 01 December 2022

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DEDICATION

This thesis is dedicated to the evergreen memory of my beloved father, late (Nze) Bernard Obinani Umeononetiti Udegbune, a.k.a Osondu Agwuike (1935 – 2004), a consummate disciplinarian in his day who through his usual catechism of papal sermons (homily), counselled me very early in life to always stand by the side of the road but never on it; ensure there is a thought behind every action I take and to always bear in mind that the secret of progress in life lies in being able to repay evil with kindness.

And to the loving memory of my dear uncle, late (Nze) Boniface Uchegbu Udegbune (1943 – 2019), who through his words and deeds, admonished me never to be an accomplice in perpetrating evil against my fellow human being no matter the circumstance, reward, motivation or provocation; and who staked his very being to ensure that I, unlike other mortals, must go far beyond the average route in life!

Your vision, words of inspiration, solemn belief in my capability to pursue excellence, by demonstrating an irresistible personal challenge of testing my intellectual ability and determination to achieve a doctoral qualification, encouraged me beyond measure to embark on this mission of an in–depth study of a specific field.

I, your son and nephew love you. May the ever–merciful Almighty God, the God of the universe, the Omnipotent, Omnipresent and Omniscient God, the creator of all peoples of the earth, the creator of Biafrans, the creator of Ndi Igbo, bless and reward you abundantly. AMEN.

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'If I have seen further than others, it is by standing upon the shoulders of giants' – Isaac Newton.

'The mind that opens to a new idea never returns to its original size' – Albert Einstein.

'Never regard study as a duty, but as the enviable opportunity to learn, for your own personal joy and the profit of the community to which your later work belongs' – Albert Einstein.

'Anyone who stops learning is old, whether at twenty or eighty. Anyone who keeps learning stays young. The greatest thing in life is to keep your mind young' – Henry Ford.

From the beginning, I very much wanted to have a professional doctorate experience in biomedical science, although I realised it wasn't going to be easy. A number of people have been kind enough to enrich my knowledge of undertaking a work–based research project through encouragement by sharing their research experiences with me. I well remember Dr Mohsen Mahmoud once telling me how difficult it was, even for him who conduct research all the time, to get back into research after a hiatus of only few months. That thought kept recurring when I considered whether or not to study for a professional doctorate degree in biomedical science whose core course content will reflect my many years of bench–level practical experience as a biomedical scientist in professional practice. But because I wanted this journey to be a personal experience, I knew I had to put myself through it.

If I have succeeded at all, it is due to three people: my supervisory team led by Professor Rousseau Gama, Dr Clare Ford and Dr Simon Dunmore. I thank Rousseau most sincerely for sharing his ideas, his time, and his enthusiasm for this work; for being a patient and inspirational thesis advisor, for teaching

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Life has taught me more lessons than one: life has taught me to be very humble and patient. Life has also taught me that hard work does not kill, rather, it pays. Life has taught me to be very honest, avoid cutting corners and have confidence in one's self. Life has also taught me that I should live every day of my life as if it will be the last one. Therefore, although as mere mortal I have tried, words cannot express the magnitude of my gratitude. I have never forgotten, but have always remembered that whatever I have, whatever I have received and whatever I will have, is by the grace of God and not by my might, wisdom, or diligence but God's abundant grace.

The professional cum academic journey of this research has demonstrated to be an incredibly positive personal experience. I hope this acknowledgement motivates others who may be contemplating a professional doctorate qualification as a pathway for their career progression in whatever field of learning, or the more academic PhD, to cease thinking about it and embark on what will certainly turn out to be a life–changing experience. You will benefit far more than the capability to broaden your knowledge; there are never–ending opportunities to meet so many inspiring people along the way.

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Like the blessed virgin Mary's hymn to the Lord in the words of Magnificat, otherwise known as the 'canticle of Mary in the liturgy of the hours' (Luke 1: 46 – 55), I thank Chukwu Okike Abiama most graciously for a very small favour, undeservedly though.

Finally, I hope the thesis justifies the good judgement I made in embarking on the doctoral experience.

SCIENTIFIC ENVIRONMENT

The present work was carried out in the Department of Clinical Chemistry, Directorate of Pathology, New Cross Hospital, at the Royal Wolverhampton NHS Trust, 321 Wolverhampton Road, Heath Town, Wolverhampton WV10 0QP, West Midlands, United Kingdom. Parts of the study involved the categorisation of the serum samples collected from the twice weekly (Tuesday and Wednesday) IBD Clinic run by Consultant Gastroenterologists at the Department of Gastroenterology, Division of Internal Medicine, New Cross Hospital Wolverhampton, West Midlands, United Kingdom into disease state, diagnosis/monitoring, and disease activity.

The cooperation from the Department of Medical Microbiology, Directorate of Pathology, New Cross Hospital Wolverhampton, West Midlands, United Kingdom, was necessary for the use of Diamedix^{TM} Dynex DS2^{TM} Automated ELISA System (Diamed Corporation, Hialeah, Florida, USA) for the measurement of serum calprotectin and serum S100A12.

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PUBLICATION AND PRESENTATIONS

This work has been the subject of the following publication and presentations

Publication (Abstract)

(Where more than one version of an abstract has been published on several occasions, only one reference is given).

Townsend S; Udegbune M M; Molyneux R; Steed H; Shah A; Gama R; Allen B; Sharrod–Cole H; Kathawala MS; Maddirala V; Ford C; Hussain Y; Brookes M (2019) The efficacy of two commercially available serum calprotectin assays to assess disease activity in IBD. *Gut,* 68 (Suppl 2): A100.

Presentations (Oral)

Udegbune MM (2016) Oral presentation of 'An outline of project & portfolio development and proposal for the professional doctorate in biomedical science based on Module 8BM001' at the Quarterly Seminar/Workshop/ Meeting of DBMS Students to Discuss Study Progression and Feedback, University of Wolverhampton, Wulfruna Street, Wolverhampton, UK; 7th December 2016.

Udegbune MM (2017a) Oral presentation of 'Evaluation of serum and faecal assays for inflammatory bowel disease (IBD) markers I' at the Quarterly Seminar/Workshop/Meeting of DBMS Students to Discuss Study Progression and Feedback, University of Wolverhampton, Wulfruna Street, Wolverhampton, UK; 15th March 2017.

Udegbune MM (2017b) Oral presentation of 'Evaluation of serum and faecal assays for inflammatory bowel disease (IBD) markers II' at the Quarterly Seminar/Workshop/Meeting of DBMS Students to Discuss Study Progression and Feedback, University of Wolverhampton, Wulfruna Street, Wolverhampton, UK; 14th June 2017.

Udegbune MM (2017c) Oral presentation of ‘Faecal calprotectin as a diagnostic marker for inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) I’ at the Quarterly Seminar/Workshop/Meeting of DBMS Students to Discuss Study Progression and Feedback, University of Wolverhampton, Wulfruna Street, Wolverhampton, UK; 13th September 2017.

Udegbune MM (2017d) Oral presentation of ‘Faecal calprotectin as a diagnostic marker for inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) II’ at the Quarterly Seminar/Workshop/Meeting of DBMS Students to Discuss Study Progression and Feedback, University of Wolverhampton, Wulfruna Street, Wolverhampton, UK; 6th December 2017.

Udegbune MM (2018a) Oral presentation of 'Development and validation of Immunodiagnostik™ faecal S100A12 and Bühlmann FCAL™ turbo faecal calprotectin assays for the respective measurement of serum S100A12 and serum calprotectin in inflammatory bowel disease (IBD) I' at the Quarterly Seminar/Workshop/Meeting of DBMS Students to Discuss Study Progression and Feedback, University of Wolverhampton, Wulfruna Street, Wolverhampton, UK; 15th March 2018.

Udegbune MM (2018b) Oral presentation of 'Development and validation of Immunodiagnostik™ faecal S100A12 and Bühlmann FCAL™ turbo faecal calprotectin assays for the respective measurement of serum S100A12 and serum calprotectin in inflammatory bowel disease (IBD) II' at the Quarterly Seminar/Workshop/Meeting of DBMS Students to Discuss Study Progression and Feedback, University of Wolverhampton, Wulfruna Street, Wolverhampton, UK; 13th June 2018.

Udegbune MM (2018c) Oral presentation of 'Development and validation of Immunodiagnostik™ faecal S100A12 and Bühlmann FCAL™ turbo faecal calprotectin assays for the respective measurement of serum S100A12 and serum calprotectin in inflammatory bowel disease (IBD) III' at the Quarterly Seminar/Workshop/Meeting of DBMS Students to Discuss Study Progression and Feedback, University of Wolverhampton, Wulfruna Street, Wolverhampton, UK; 13th September 2018.

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AAV	Antineutrophil cytoplasm antibody–associated vasculitis
Abbott–Turbo	Abbott architect c16000 turbidimetric chemistry analyser
Abs.	Absorbance
ABTS	2,2'–azino–bis(3–ethylbenzothiazoline–6–sulfonic acid)
a.c.	Alternating current
Anti–OmpC	Anti–Outer membrane protein C
ACCA	Antibodies against chitobioside
ALCA	Antibodies against laminaribioside
APP	Acute phase protein
APPR	Acute phase protein response
APR	Acute phase response
AMCA	Antibodies to mannobioside
ANCA	Antineutrophil cytoplasmic antibodies
cANCA	cytoplasmic antineutrophil cytoplasmic antibodies
pANCA	perinuclear antineutrophil cytoplasmic antibodies
5–ASA	5–Aminosalicylic acid
ASCA	Anti–saccharomyces cerevisiae antibodies
AUC	Area under the curve
BMI	Body mass index
BMN®	Bühlmann ELISA kit
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin solution
fCAL®	Bühlmann fCAL™ ELISA calprotectin
fCAL®-Turbo	Bühlmann fCAL® turbo kit
BMN®-Cp	Bühlmann MRP8/14 S100A8/S100A9
CAAF–1	Calcium binding protein in amniotic fluid
cGL	Canine gastric lipase
CI	Confidence interval
Cp	Calprotectin
CD	Crohn's disease
CDAI	Crohn's disease activity index
CDEIS	Crohn's disease endoscopic index of severity
CLIA	Chemiluminescence immunoassay
CRC	Colorectal cancer
CRP	C–reactive protein
CVD	Cardiovascular diseases
DS2™	Diamedix™ Dynex DS2™ automated ELISA system
DF	Dilution factor
EF	Elongation factor hand
EIA	Enzyme immunoassay
ELISA	Enzyme–linked immunosorbent assay
EN–RAGE	Extracellular newly identified receptor for advanced glycation end products

ESR	Erythrocyte sedimentation rate
FEIA	Fluoro–enzyme immunoassay
FN	False negatives
FP	False positives
fA12	Faecal S100A12
fCAL	Faecal calprotectin
fmol	Fentomole
GIT	Gastrointestinal tract
GWAS	Genome–wide association studies
hsCRP	High sensitivity C–reactive protein
H_2O_2	Hydrogen peroxide
HBI	Harvey–Bradshaw Index
HC	Healthy control
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IBDU	Inflammatory bowel disease unclassified
IBS	Irritable bowel syndrome
IDK®	Immunodiagnostik™ ELISA kit
IDK®-A12	Immunodiagnostik™ K6938 S100A12
IDK®-Cp	Immunodiagnostik™ K6935 calprotectin
IgE	Immunoglobin E
IIM	Idiopathic inflammatory myopathies
IL-1β	Interleukin–1 beta
IL-6	Interleukin–6
IQ	Interquartile
IQC	Internal quality control
IQR	Interquartile range
ISO	International standard organisation
IUPAC	International union of pure and applied chemistry
K_2EDTA	Diamine potassium ethylene tetra–acetic acid
kDa	Kilodalton
KS	Kolmogorov–Smirnov
k_w	Kappa coefficient statistic
LoB	Limit of the blank
LoQ	Limit of quantitation
LLM	Lower limit of the measuring range
LLoD	Lower limit of detection
LR	Likelihood ratio
mAb	Monoclonal antibody
mOsm/L	Milliosmolarity per litre
MMA	Methylmalonic Acid
MMPs	Metalloproteinases
M2PK	M2–pyruvate kinase
MPOs	Myeloperoxidases
MPV	Mean platelet volume
MRP	Myeloid–related proteins
$NaHCO_3$	Sodium bicarbonate
Na_2CO_3	Sodium carbonate

NICE	National institute for health and care excellence
NPT	Near–patient testing
NOD2	Nucleotide–binding oligomerization domain–containing protein 2
OD	Optical density
PAB	Pancreatic autoantibodies
P25	25th Percentile
PETIA	Particle–enhanced turbidimetric immunoassay
PBS	Phosphate buffered saline solution
PMN	Polymorphonuclear elastase
pANCA	Perinuclear antineutrophil cytoplasmic antibodies
***p*NPP**	*p*–nitrophenyl phosphate
PLR	Positive likelihood ratio
POCT	Point–of–care testing
PPV	Positive predictive value
PVC	Polyvinyl chloride
µg/mL	Microgram per millilitre
µL	Micro litre
mg/L	Milligram per litre
mL	Millilitre
mm	Millimetre
ng/mL	Nanogram per millilitre
nm	Nanometre
N	Number
NLR	Negative likelihood ratio
NPV	Negative predictive value
NSAIDs	Non–steroidal anti–inflammatory drugs
OD	Optical density
p–Cp	Plasma calprotectin
PLR	Positive likelihood ratio
PPV	Positive predictive value
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
RDW	Red blood cell distribution width
RI	Rachmilewitz Index
RIA	Radioimmunoassay
ROC	Receiver operating characteristics
rpm	Revolution per minute
RSD	Relative standard deviation
A12	S100A12
SAA	Serum amyloid A
SD	Standard deviation
SES–CD	Simple endoscopic score for Crohn's disease
SIR	Systemic inflammatory response
SLE	Systemic lupus erythematosus
SOP	Standard operating procedure
SPSS.v25	Statistical package for social sciences version 25
SS	Sjögren syndrome

SSI	Study specific index
SW	Shapiro–Wilks
H_2SO_4	Sulphuric acid
TCZ	Tocilizumab
TMB	Tetramethylbenzidine
TN	True negatives
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumour necrosis factor alpha
TNFi	Tumour necrosis factor inhibitors
TP	True positives
UC	Ulcerative colitis
ULM	Upper limit of the measuring range
ULT	Ultra low temperature

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ABSTRACT

Background

Faecal biomarkers of intestinal inflammation, in particular faecal calprotectin and to a lesser extent faecal S100A12, are used to discriminate between inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) and categorise active from inactive disease in established IBD. Faecal biomarkers have limitations including intra–individual and inter–individual variability, spot variability in the same sample and reluctance of some patients to provide stool samples. These issues may be overcome by using serum samples for the measurement of calprotectin and S100A12. This offers the prospect that serum calprotectin and serum S100A12 could replace or supplement faecal calprotectin and faecal S100A12 in the identification and assessment of IBD. The measurement in serum calprotectin and serum S100A12, however, requires method development, validation of assays for serum and evaluation of the validated assays for their diagnostic and prognostic utility in IBD.

Method development switches between two processes. It may necessitate adapting an existing method to ensure its suitability for application in a new assay or devising a suitable method by integrating the expertise and experience of the personnel undertaking the task of method development. Assay validation process for commercially available serum immunoassay kits is necessary to underpin assay measurement in order to confirm accuracy of test results, cut costs of undertaking unnecessary and repeat testing procedures, reinforce analytical claim that assay measurement is devoid of uncertainty to justify 'fit for purpose' and confer additional benefit of good reputation to a clinical laboratory. Validation of an assay in serum confirms or disproves kit manufacturer's analytical claim to robust assay performance characteristics that include accuracy, precision, dilution linearity/parallelism, recovery, sensitivity, interference and stability.

Aim/Objectives

This project was designed to

(1) Develop and analytically validate a faecal S100A12 assay (Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) for measurement of S100A12 in serum. Analytically validate serum calprotectin assays provided by Bühlmann (serum BMN®-Cp; Bühlmann Laboratories AG, Baselstrasse 55, CH – 4124 Schönenbuch, Switzerland) and Immunodiagnostik™ (serum IDK®-

Cp; Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany).

(2) Assess whether serum BMN®-Cp, serum IDK®-Cp and serum S100A12 could replace or supplement faecal calprotectin and faecal S100A12 in excluding IBD in patients presenting with chronic diarrhoea.

(3) Evaluate the utility of serum BMN®-Cp and serum IDK®-Cp in discriminating between active and inactive IBD.

(4) Study the effect of the acute phase response (APR) on serum calprotectin determined with two different immunoassays kits (i.e., serum BMN®-Cp and serum IDK®-Cp), and to assess and compare the diagnostic performance of the two assays in APR.

Methods

(1) ELISA assays for faecal S100A12 were developed and optimised for measurement of S100A12 in serum. The serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 were validated by determining analytical sensitivity, functional sensitivity, dilution linearity/parallelism, recovery, precision, and interference.

(2) The diagnostic performances of the serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 assays were compared against faecal calprotectin, as the diagnostic 'gold standard', in 40 patients with IBD and 5 control patients.

(3) Serum BMN®-Cp and serum IDK®-Cp and other conventional inflammatory blood biomarkers (serum CRP and platelets) were compared to faecal calprotectin in discriminating between active and inactive disease in a cohort of 175 patients with IBD.

(4) The effect of APR, as determined by serum CRP, and serum calprotectin was assessed by measuring serum BMN®-Cp and serum IDK®-Cp before and after elective knee or hip surgery in 30 patients.

Results

Analytical validation

Analytical validation of the assays showed a dynamic working range in serum of 10 to 25000 ng/mL, good precision (%CV for intra– and inter–assay variability for the kits were < 10% respectively, for each assay) and good reproducibility. There was no interference from bilirubin, haemoglobin, or lipid in the assays. There was no significant carryover or cross–reactivity across the assays. Assay kits were stable over 12 months. Analytical sensitivity ranged from 0.673 to 577 ng/mL for limit of the blank (LoB), and 1.119 to 597 ng/mL for lower limit of detection (LLoD). Functional sensitivity or limit of

quantitation (LoQ) ranged from 522 to 3615 ng/mL. Measured to Expected ratios for dilution linearity/parallelism and recovery for the kits ranged from 98.4% to 103.7%, and from 82.1% to 126.5% respectively. Method comparison showed 19% positive proportional bias of the BMN®-Cp assay compared to the IDK®-Cp assay.

Serum BMN®-Cp, serum IDK®-Cp and serum S100A12 in identifying IBD

Using faecal calprotectin as the 'gold standard' for identifying IBD, the AUC from ROC curves for serum IDK®-Cp (AUC = 0.793) was greater than that for serum BMN®-Cp (AUC = 0.771) and these were greater than that for serum S100A12 (AUC = 0.700). Faecal calprotectin correlated best with serum IDK®-Cp (r = 0.69), then serum BMN®-Cp (r = 0.66) and least with serum S100A12 (r = 0.44).

Serum BMN®-Cp and serum IDK®-Cp in discriminating between active and inactive IBD.

The cohort of 175 patients with IBD consisted of 101 (57.7%) patients with Crohns disease (CD), 71 (40.6%) with ulcerative colitis (UC) and 3 (1.7%) inflammatory bowel disease unclassified (IBDU). The clinical classification of disease activity was largely based on faecal calprotectin which indicated that the disease was quiescent in 99 (56.6%) patients, active in 73 (41.7%) patients and in 3 (1.7%) patients were IBDU. Faecal calprotectin was, therefore, higher ($p < 0.0001$) in active CD than in quiescent CD, and similarly higher ($p < 0.0001$) in active UC compared to quiescent UC.

Serum BMN®-Cp and serum IDK®-Cp in 175 IBD patients were highly correlated (r = 0.97). Serum BMN®-Cp and serum IDK®-Cp were higher ($p < 0.006$) in active CD than in quiescent CD but were similar ($p > 0.1$) in active and quiescent UC. Serum CRP was higher ($p = 0.0095$) in active CD compared to quiescent CD but similar ($p = 0.0638$) in active and quiescent UC. Platelets were similar ($p = 0.0579$) in active and quiescent CD and similar ($p = 0.8055$) in active and quiescent UC. Serum BMN®-Cp and serum IDK®-Cp concentrations were higher ($p < 0.05$) in active CD than quiescent CD at the ileal and upper GI, and the colonic and ileo–colonic sites of the ileum. Serum BMN®-Cp and serum IDK®-Cp concentrations were similar ($p > 0.05$) in active UC and quiescent UC involving the rectum, distal colon and pancolon.

Based on ROC curve analysis, the performance of serum CRP (AUC = 0.699) was marginally superior to that of serum BMN®-Cp (AUC = 0.662) and serum IDK®-Cp (AUC = 0.656), and these were superior to platelets (AUC = 0.547) in all patients with IBD. In patients with CD, none of the blood biomarkers performed well; serum CRP (AUC = 0.585), serum BMN®-Cp (AUC = 0.585), serum IDK®-Cp (AUC = 0.556) and platelets (AUC = 0.609). In patients with

UC, the performance of serum CRP (AUC = 0.752) was superior to that of serum BMN®-Cp (AUC = 0.670) and serum IDK®-Cp (AUC = 0.660), and these were superior to platelets (AUC = 0.487).

The effect of an APR on serum BMN®-Cp and serum IDK®-Cp

Following elective knee and hip surgery in 30 patients, serum CRP, serum BMN®-Cp, serum IDK®-Cp and blood neutrophils increased ($p < 0.0001$); serum albumin and serum total protein decreased ($p < 0.0001$). The mean (SD) post–operative increase in serum BMN®-Cp (3.0 (1.9) fold) and serum IDK®-Cp (2.8 (1.8) fold) were similar ($p = 0.6575$) but these were both lower ($p < 0.0001$) than serum CRP (82.0 (60.8) fold). Logarithmically transformed serum CRP correlated positively with serum BMN®-Cp ($r = 0.64$), serum IDK®-Cp ($r = 0.65$) and neutrophil count ($r = 0.66$), and negatively with serum total protein ($r = –0.43$) and serum albumin ($r = –0.70$). Serum BMN®-Cp correlated positively with serum IDK®-Cp ($r = 0.97$) and neutrophil count ($r = 0.68$), and negatively with serum albumin ($r = –0.54$). Serum IDK®-Cp correlated positively with neutrophil count ($r = 0.67$), and negatively with serum albumin ($r = –0.55$; $p < 0.0001$). There was no correlation between serum total protein and either serum BMN®-Cp or serum IDK®-Cp.

Conclusions

The developed and optimised serum IDK®-Cp, serum BMN®-Cp and serum S100A12 assays have good analytical performance and compared favourably to manufacturer stated performance characteristics, where available. The large numerical difference between serum BMN®-Cp and serum IDK®-Cp values indicate that results and any derived cut–offs between assays are not directly inter–changeable.

The serum BMN®-Cp and serum IDK®-Cp assays have acceptable diagnostic accuracy for the identification of IBD and these were superior to serum S100A12. Although serum BMN®-Cp results were 1.7–fold higher than matched serum IDK®-Cp results, for diagnostic purposes this was accounted for by their manufacturer provided cut–offs of >3900 ng/mL and >3000 ng/mL respectively. Serum BMN®-Cp and serum IDK®-Cp, however, are unlikely to replace faecal calprotectin but may have a role supplementing faecal calprotectin in the identification of IBD. An elevated serum calprotectin in patients with chronic diarrhoea would be an indication for endoscopy since it has a low false positive rate, but a normal serum calprotectin does not exclude IBD.

In the cohort of 175 patients with IBD, serum BMN®-Cp and serum IDK®-Cp were significantly associated with disease activity in patients with CD irrespective of site of disease. There was, however, no significant association between serum BMN®-Cp and serum IDK®-Cp and disease activity in patients

with UC. ROC curves analyses indicated that serum CRP performed better than serum BMN®-Cp and serum IDK®-Cp in discriminating between active and inactive disease in patients with CD and UC. In this patient cohort, serum calprotectin offers no advantages over serum CRP in discriminating between active and inactive IBD, particularly since serum CRP is easily available and less expensive.

Serum calprotectin is a positive acute phase protein, and both the serum BMN®-Cp and serum IDK®-Cp assays perform equally well during an APR elicited by orthopaedic surgery. The increase in serum calprotectin elicited by trauma and previously reported increase in sepsis indicates that serum calprotectin is a non–specific biomarker of inflammation. At two days following an inflammatory insult, serum CRP may be a better discriminatory biomarker of the APR than serum calprotectin based on a much greater incremental response.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Definition and aetiology of inflammatory bowel disease

Inflammatory bowel disease (IBD) consists largely of two major disorders, Crohn's disease (CD) and ulcerative colitis (UC). CD is characterised by transmural inflammation and skip lesions which may involve any part of the gastrointestinal tract (GIT) from mouth to anus but is more likely to involve the small and large intestines (especially the ileocecum) and the perianal region. The transmural inflammation in CD may cause fistulae and also lead to fibrosis, strictures and bowel obstruction that are not seen in UC (Agrawal et al., 2007). UC is characterised by recurring and often self–limiting inflammation confined to the mucosal layer of the colon and almost invariably involves the rectum and may extend proximally and in a continuous fashion to involve the colon (Baumgart and Carding, 2007; Baumgart and Sandborn, 2007; Xavier and Podolsky, 2007).

Although the aetiology of CD and UC remains largely unknown, some studies have suggested that genetic factors, environmental influences, intestinal microbiota, and immune response are involved in their pathogenesis (Khor et al., 2011; Ek et al., 2014; Bianco et al., 2015; Tsianos et al., 2012; Ye et al., 2015).

There is a 5– to 20–fold increased risk of developing IBD in those with family history of IBD. The peak age of presentation of IBD is between 15 and 40 years with a possible second peak between 50 and 80 years (Ek et al., 2014).

There is a slight female predominance in CD, whereas there may be a slight male predominance in UC. The prevalence of IBD is higher in Jews than non–Jews and lower in blacks and Hispanics than whites. These ethnic and racial differences may be related to environmental and lifestyle factors as well as due to underlying genetic differences (Bianco et al., 2015). Smoking appears to have either no effect or may even be protective against UC, whereas it increases the risk of CD. High fibre diet and high consumption of fruits and vegetables protects against IBD while high intake of protein from fish or meat predisposes to a higher risk of the disease (Ye et al., 2015). Other risk factors which have been implicated include reduced physical activity, gastrointestinal infections and medication including antibiotics, isotretinoin, nonsteroidal anti–inflammatory drugs (NSAIDs), hormone replacement therapy (HRT) and the oral contraceptive pill (Khor et al., 2011; Tsianos et al., 2012).

1.1.1 Crohn's disease: clinical features

The clinical features of CD are variable, and patients are often symptomatic for several years prior to diagnosis. Common presenting symptoms are lethargy, diarrhoea with or without blood, abdominal pain, anorexia, weight loss, and fever, but patients may also experience constipation, nausea and vomiting. Extra–intestinal manifestations include disorders of the joints, eyes, skin, lung and liver. Complications of CD include bowel obstruction, fistulae, osteoporosis and malabsorption (Roda et al., 2020; WGO, 2009; Ballester Ferré et al., 2018; Baumgart and Sandborn, 2012; Torres et al., 2017).

1.1.2 Crohn's disease: management

Management of CD involves lifestyle changes, drug treatment for induction and maintenance of remission and the management of complications (Loftus, 2017; Lichtenstein et al., 2009; Amre et al., 2007). Lifestyle changes include smoking cessation and avoidance of NSAIDs. NSAIDs are associated with mucosal damage, exacerbation of IBD and inhibition of prostaglandin synthesis that triggers the mechanism of CD relapse (Forrest et al., 2004; Kefalakes et al., 2009; Takeuchi et al., 2006).

In general, there are two approaches to drug therapy; step–up therapy or top–down therapy. These are usually based on severity of disease and risk of long–term complications. Assessment of disease severity is determined by clinical scoring systems. The most used scoring systems are the Crohn's disease activity index (CDAI) and the Harvey–Bradshaw index (HBI) (Baran and Karaca, 2013; Nguyen et al., 2020). A simplified version of the CDAI is shown in Table 1.1. Patients are classified into either a low or high–risk category by assessing inflammatory status with endoscopic evaluation for mucosal ulcerations, strictures and disease extent, use of laboratory tests such as serum C–reactive protein (CRP) and/or faecal calprotectin, and whether there is upper gastrointestinal involvement (Colombel et al., 2010).

Step–up therapy is initiated in patients with mild CD who are low–risk. Step–up therapy typically starts with less potent medications associated with fewer side effects such as 5–aminosalicylates (5–ASA) and glucocorticoids. More potent and potentially more toxic medications are used only if the initial therapies are ineffective (Veauthier and Hornecker, 2018; Colombel et al., 2010).

In contrast, top–down therapy is used in high–risk patients with moderate to severe CD. These patients are usually initiated on more potent biologic (e.g., infliximab) or immunomodulatory (e.g., azathioprine) therapy to achieve a rapid onset of clinical remission to avoid the long–term complications of glucocorticoids and prevent steroid dependence (Gomollón et al., 2017; Colombel et al., 2010; Baran and Karaca, 2013).

In addition to assessing patients clinically, monitoring response to treatment and during remission may involve endoscopy and also use of non–invasive systemic and gut biomarkers of inflammation such as serum CRP and faecal calprotectin respectively (Wilkins et al., 2011; Caprilli et al., 2006).

Severity of Symptoms	CDAI	Description
Clinical Remission	< 150	Spontaneous or post treatment remission
Mild to Moderate CD	150 – 220	Good oral intake, absence of dehydration, abdominal tenderness/mass, obstruction, or weight loss of >10%. Ambulatory follow–up is sufficient.
Moderate to Severe CD	220 – 450	Patients with mild to moderate Crohn's disease irresponsive to first line therapy; presence of 2 or more of the following systemic symptoms: fever, weight loss, abdominal pain, nausea and vomiting, and anaemia.
Severe–Fulminant CD	> 450	Ambulatory patients with persisting symptoms despite optimal therapy, presence of high fever, or obstruction symptoms as refractory nausea/vomiting, peritoneal signs, cachexia, or intra–abdominal abscess.

Table 1.1 – A simplified classification of severity of Crohn's disease based on Crohn's disease activity index (CDAI). Modified from: Baran B; Karaca C (2013) Practical medical management of Crohn's disease. *ISRN Gastroenterology, 12.*

1.1.3 Ulcerative colitis: clinical features

Patients with UC usually present with diarrhoea, which may be associated with passage of blood and mucus. Associated symptoms include abdominal pain, urgency, tenesmus, and incontinence. If severe, patients may have systemic symptoms such as fever, weight loss and those associated with anaemia (Keshteli et al., 2019; Hoffmann et al., 2004; Podolsky, 2002). As in CD, there may be extra–intestinal manifestations involving the joints, eyes, skin, lung and liver. Complications of UC include toxic megacolon, bowel perforation, fulminant colitis and colorectal cancer (Rubin et al., 2019; Hoffmann et al., 2004).

1.1.4 Ulcerative colitis: management

Patients may present with mild, moderate, or severe disease. Stratification based on severity is important in guiding management. Severity of disease is a composite of clinical findings, test results of laboratory biomarkers (e.g., haemoglobin and erythrocyte sedimentation rate (ESR)) and colonoscopy (Ko et al., 2019; Singh et al., 2019; Hoffmann et al., 2004).

Topical 5–ASA suppositories and/or enemas induce remission in more than 90% of patients with mild to moderate disease, and maintain remission in 75% of these patients. Other step–up options include topical steroid, oral 5–ASA and oral steroids for persistent disease (Carvello et al., 2020; Hoffmann and Zeitz, 2005; Hoffmann et al., 2004). The management of patients with severe disease and moderately active disease unresponsive to local treatment entails high dose oral steroids, high dose oral 5–ASA, as well as continuing topical 5–ASA. If unresponsive to treatment or progression to

fulminant colitis, patient management involves hospitalisation for intravenous fluid and electrolyte repletion, antibiotics and intravenous steroids stepping up to additive biologics and immunosuppressants with total colectomy as the last but curative option (Wehkamp and Stange, 2018; Hoffmann and Zeitz, 2005; Caprilli et al., 2006).

In addition to assessing patients clinically, monitoring response to treatment during remission may involve endoscopy and use of laboratory biomarkers of inflammation such as serum CRP and faecal calprotectin. UC carries an increased risk for colorectal cancer and therefore surveillance colonoscopy for colorectal cancer screening is based on the extent and duration of the disease (Rubin et al., 2019; Hoffmann et al., 2004).

1.2 Laboratory biomarkers and inflammatory bowel disease

The diagnosis, severity and monitoring of IBD are usually based on a combination of clinical features, endoscopy, radiology, laboratory biomarkers and, if appropriate histology. Laboratory biomarkers should be non–invasive, cheap, simple, objective, rapid, easy to perform and reproducible (Table 1.2). An ideal biomarker for IBD that would combine all these characteristics is not available (Vermeire et al., 2006; BDWG, 2001). Serum biomarkers of systemic inflammation are shown in Table 1.3 and faecal biomarkers of gastrointestinal inflammation are shown in Table 1.4.

Performance	Characteristics
Simple	Disease specific: Identify individuals with IBD; Able to differentiate IBD from non–IBD cases; Able to predict the remission or relapse; Monitor the effect of treatment; Prognostic value in assessing morbidity/mortality
Ease of performance	Ability to objectively measure disease activity without ambiguity
Invasiveness	Not invasive or minimally invasive
Cost	Affordable
Rapid	Quick turnaround time
Reproducibility	Assay results not showing discrepancies between individuals and clinical laboratories.

Table 1.2 – Characteristics of an ideal biomarker for use in the assessment of IBD (Modified from: Vermeire S; van Assche G; Rutgeerts P (2006) Laboratory markers in IBD: useful, magic or unnecessary toys? *Gut,* 55 (3): 426 – 431.

Acute Phase Protein	Increased	Decreased
Proteinase Inhibitors	α_1–Antitrypsin, α_1–Antichymotrypsin, α_2–Macroglobulin*****	
Coagulation and Fibrinolytic Proteins	Fibrinogen, Prothrombin, Factor VIII, Plasminogen, Tissue Plasminogen Activator Antithrombin	Factor XII
Complement System	C1s, C2, C3, C4, C5, C1 Inhibitor, C9	Albumin, Transferrin
Transport Proteins	Haptoglobin, Haemopexin, Caeruloplasmin	Insulin–like Growth Factor, α– Fetoprotein, Cholinesterase
Other Acute Phase Proteins	C–reactive protein, Serum Amyloid A, Ferritin, Fibronectin, Orosomucoid (α_1–Acid Glycoprotein).	

Table 1.3 – Serum acute phase proteins and their responses to IBD and other inflammatory processes. *α_2–macroglobulin shows a different response in animals in comparison to humans, both in positive and negative acute phase protein respectively (Modified from Vermeire S; van Assche G; Rutgeerts P (2006) Laboratory markers in IBD: useful, magic or unnecessary toys? *Gut,* 55 (3): 426 – 431.

Faecal Biomarker	Major Source or Origin
Calprotectin (S100A8/S100A9)	Neutrophils, Monocytes and Epithelial Cells.
Calgranulin C or EN–RAGE (S100A12)	Neutrophils
Lactoferrin	Mucosal Epithelial Cells and Neutrophils
M2–Pyruvate Kinase (M2PK)	Expressed by rapidly dividing cells
Neopterin	Activated Macrophages
Metalloproteinases (MMPs)	Different Cell types including Activated Neutrophils
Myeloperoxidases (MPOs)	Activated Neutrophils
Polymorphonuclear Elastase (PMN)	Activated Neutrophils

Table 1.4 – Faecal biomarkers in clinical use for the diagnosis and differential diagnosis of IBD including its discrimination from IBS. Modified from: Lehmann FS; Burri E; Beglinger C (2015) The role and utility of faecal markers in inflammatory bowel disease. *Therapeutic Advances in Gastroenterology,* 8 (1): 23 – 36.

1.2.1 Blood autophagy genes and nucleotide–binding oligomerization domain–containing protein 2 (NOD2)

Family history of CD is a risk factor for CD (Khor et al., 2011). Genome–wide association studies have reported that nucleotide–binding oligomerization domain–containing protein 2 (NOD2) and Autophagy genes are associated with CD risk while IL23/17 is associated with increased risk of both CD and UC (Cho and Brant, 2011).

1.2.2 Serum antineutrophil cytoplasmic antibodies and anti–saccharomyces cerevisiae antibodies

It may be difficult to differentiate CD from UC due to their overlapping pathological, endoscopic and clinical features. Biomarkers like antineutrophil

cytoplasmic antibodies (ANCAs), which are antibodies against granules of neutrophil cytoplasm and anti–saccharomyces cerevisiae antibodies (ASCAs), which are antibodies against mannan found in the cell walls of saccharomyces cerevisiae (S. cerevisiae) have been used to help differentiate CD from UC (Ferrante et al., 2007).

The sensitivity and specificity of the perinuclear ANCA (pANCA) for the diagnosis of ulcerative colitis is 63% and 86% respectively. ASCA for diagnosing CD had a sensitivity of 72% and a specificity of 82% (Linskens et al., 2002). The utility of these serological tests in differentiating UC from CD is therefore limited but may be of value in studying disease heterogeneity and disease epidemiology.

Other serologic tests investigated to improve the diagnosis and differential diagnosis of IBD include: antibodies to Escherichia coli outer membrane porin (anti–OmpC), antibodies against laminaribioside (ALCAs), antibodies against chitobioside (ACCAs), antibodies to mannobioside (AMCAs), pancreatic autoantibodies (PAB), Pseudomonas fluorescens–associated sequence 12, and anti–flagellin CBir1 but all are of limited diagnostic utility (Prideaux et al., 2012; Lakatos et al., 2011; Bogdanos et al., 2011; Zholudev et al., 2004; Dotan et al., 2006; Mendoza and Abreu, 2009).

1.2.3 Serum C–reactive protein and cytokines

C–Reactive Protein (CRP), a pentameric protein synthesized predominantly in the liver but also in vascular walls and adipose tissue, is a well–established biomarker of acute inflammation. During an acute phase response, hepatocytes rapidly increase production of CRP under the influence of

interleukin 6 (IL–6), tumour necrosis factor alpha (TNF–α), and interleukin 1β (IL–1β). Serum CRP, therefore, rises rapidly up to 100 to 1000–fold peaking in two days and then decreases rapidly with the resolution of the acute insult as it has a half–life of 19 hours. Functionally, CRP is recruited into the complement activation process when it binds to organisms or particles that contain phosphocholine which enables it to opsonise infectious agents and damaged cells (Ballou and Kushner, 1992; Young et al., 1991; Mold et al., 2002). In healthy individuals, CRP circulates in low concentrations (about 1 mg/L). CRP concentrations around 10 to 40 mg/L may be seen in chronic inflammation (Rudolph et al., 2002; Abdelrazeq et al., 2005).

CRP is generally increased in IBD, but appears to respond differently to inflammation in UC and CD. A rise in serum CRP level correlates well with the disease activity in CD. CRP, however, may not be raised or mildly raised in the presence of increased disease activity in UC even though increased levels of IL–6, IL–1β or TNF–α are observed (Saverymuttu et al., 1986a). Other inflammatory markers such as β_2–microglobulin correlate better with histology scores in UC (Sidorofff et al., 2010; Yamamoto–Furusho et al., 2010). The main reason given for this differential CRP response in IBD is that inflammation is limited to the mucosa in UC and, less likely to provoke a systemic response to inflammation when compared to transmural inflammation in CD. Other possible reasons include increased IL–6 levels in CD compared to UC (Gross et al., 1992), and CRP gene polymorphism (Szalai et al., 2002; Russell et al., 2004; Carlson et al., 2005) in UC and CD. CRP levels in IBD patients, however, are not associated with CRP gene polymorphism (Willot et al., 2005).

1.2.4 Circulating haemopoietic biomarkers: erythrocyte sedimentation rate, platelet count, mean platelet volume and red cell distribution width

ESR is an indirect measure of systemic inflammation. ESR measures the rate of migration of erythrocytes through the plasma, and is increased in the presence of increased proteins including acute phase proteins. ESR, compared to CRP, is slow to peak in response to inflammation and slow to decline after resolution of inflammation (Danese et al., 2004a), and is therefore of limited value in IBD assessment (Vermeire et al., 2006).

Inflammation processes, including IBD, cause an increase in platelets and changes in their morphology. Platelet count increases in patients with IBD, particularly in UC when reticulated platelets are taken into account (Collins and Rampton, 1995; Dong et al., 2004). It, however, is not a useful biomarker of IBD, given the wide range of a normal platelet counts (Vermeire et al., 2006; Danese et al., 2004b),

Mean Platelet Volume (MPV) has been reported as decreased in active IBD and has a negative correlation with CRP (Kapsoritakis et al., 2001), while other studies have found no correlation between fall in MPV and the disease activity (Kayahan et al., 2007; Kapsoritakis et al., 2001). Likewise, leucocytes lack specificity as a biomarker for IBD (Zubcevic et al., 2010), and are influenced by treatments in IBD with drugs such as glucocorticoids or azathioprine (increased) and 6–mercaptopurine (decreased) (Vermeire et al., 2006).

Red Blood Cell Distribution Width (RDW) is a measure of size variability and heterogeneity of erythrocytes in the peripheral blood. RDW increases in active IBD, and particularly in CD compared to UC. RDW at a diagnostic cut–off value

of 13.8% in non–anaemic UC patients has a sensitivity and specificity of 76% and 86% respectively, and in non–anaemic CD patients at a diagnostic cut–off value of 14.1% has a sensitivity and specificity of 82% and 83% respectively (Zubcevic et al., 2010; Song et al., 2012).

1.2.5 Other serum acute phase proteins

Other acute phase reactants (Table 1.3) such as fibrinogen, sialic acid, α_1–acid glycoprotein (orosomucoid), α_1–antitrypsin, α_2–globulin, β_2–microglobulin, serum amyloid A (SAA) and albumin as biomarkers of IBD have not been widely studied because of their apparent inferiority to CRP. α_1–acid glycoprotein, shows good correlation with IBD disease activity but its half–life of 5 days makes it unsuitable as IBD biomarker (Danese et al., 2004a; Vermeire et al., 2006).

1.2.6 Faecal lactoferrin

Lactoferrin is an iron–binding protein found in neutrophil granulocytes. In acute intestinal inflammation, the increased mucosal infiltration of neutrophils and subsequent secretion of lactoferrin into the intestinal tract results in increased faecal lactoferrin (Baveye et al., 1999; Kane et al., 2003a). Lactoferrin has anti–bacterial activity by limiting availability of iron, and causes direct damage to bacterial cell membrane. Faecal lactoferrin is resistant to degradation and proteolysis, although less so than calprotectin, making it a useful biomarker of intestinal inflammation. The significant proportion of faecal lactoferrin in stool is, therefore, a reflection of intestinal inflammation (Desai et al., 2007; Joishy et al., 2009; Sugi et al., 1996).

Lactoferrin is stable in stool for up to 5 days and does not deteriorate with repeated freezing and thawing. It can be quantitatively measured by enzyme–linked immunosorbent (ELISA) technique. It is a non–specific intestinal biomarker being raised not only in active IBD but in other inflammatory intestinal disorders including infective diarrhoea, colon cancer and NSAIDs enteropathy. Faecal lactoferrin correlates well with the clinical, endoscopic and histological grading of IBD disease activity and therefore, may distinguish between active and inactive IBD, and between IBD and healthy controls (Kane et al., 2003a; Jones et al., 2008; Walker et al., 2007; Dai et al., 2007; Sipponen et al., 2008a; Schoepfer et al., 2008; Langhorst et al., 2008; Sidhu et al., 2010). For predicting relapse, faecal lactoferrin has 46% sensitivity and 61% specificity for UC, and 77% sensitivity and 68% specificity for CD (Gisbert et al., 2009a). D'Inca et al reported comparable diagnostic accuracy of lactoferrin and calprotectin in patients with IBD and in patients with irritable bowel syndrome (IBS) and healthy controls (D'Inca et al., 2007). Calprotectin and lactoferrin had similar sensitivity (78% versus 80%), specificity (83% versus 85%), overall diagnostic accuracy of 80% versus 81% (D'Inca et al., 2007) and are both significantly elevated in children with IBD (Moishy et al., 2009).

1.2.7 Faecal neopterin

Neopterin is a metabolite of biopterin released by activated macrophages and monocytes. Faecal neopterin correlates well with disease activity in IBD particularly with the severity of mucosal lesions. Faecal neopterin shows better correlation with endoscopic scores in UC ($r = 0.72$; $p < 0.0001$) than in CD ($r = 0.47$; $p < 0.0001$). Faecal neopterin is significantly higher in clinically and endoscopically active IBD compared to inactive IBD. The diagnostic accuracy

of faecal neopterin to predict endoscopic activity in IBD compares favourably with faecal calprotectin in CD (74%) and in UC (87%) and, like calprotectin and lactoferrin, release of neopterin is non–specific as it could be triggered in response to disease conditions such as viral infection and cell–mediated immune response during the early phase of inflammation (Nancey et al., 2013; Husain et al., 2013).

1.2.8 Faecal metalloproteinases

Metalloproteinases (MMPs) are zinc–dependent endopeptidases secreted by various cell types. MMP–9 is the MMP released in highest concentrations by activated neutrophils during intestinal inflammation such as in IBD. While MMP–1, MMP–2 and MMP–3 are detected in significantly higher amount in UC, only MMP–9 levels are significantly higher in active UC than in IBS or healthy controls thereby making it a reliable biomarker in distinguishing between UC and IBS based on 85% sensitivity and 100% specificity (Annahazi et al., 2013). Studies by Kolho et al (2014) reported that faecal MMP–9 correlates well with other faecal biomarkers including calprotectin, lactoferrin and neopterin in endoscopically assessed disease activity in IBD and also demonstrates comparable diagnostic accuracy with faecal calprotectin.

1.2.9 Faecal myeloperoxidases

Myeloperoxidases (MPOs) are lysosomal proteins released by activated neutrophils in response to inflammation. The diagnostic accuracy of faecal MPOs to assess endoscopic disease activity in IBD is inferior to calprotectin and polymorphonuclear (PMN) elastase (Silberer et al., 2005).

1.2.10 Faecal polymorphonuclear elastase

Polymorphonuclear (PMN) elastase is released by activated neutrophils during inflammation and is stable for up to 4 days in stool at ambient temperature. Faecal PMN elastase may be a useful biomarker in IBD as it is significantly higher in patients with active IBD compared to inactive IBD. This is reflected in its diagnostic accuracy of 84% sensitivity and 87% specificity for IBD that increases to 96% sensitivity and 100% specificity when combined with calprotectin and lactoferrin (Schroeder et al., 2007; Langhorst et al., 2008).

1.2.11 Faecal M2–pyruvate kinase

Faecal levels of M2–pyruvate kinase (M2PK), a multi–functional protein found in undifferentiated and proliferating cells, are associated with active IBD and correlate well with calprotectin in distinguishing IBD from IBS (Czub et al., 2007; Turner et al., 2010). M2PK is a superior biomarker to calprotectin, lactoferrin and S100A12 in predicting steroid refractoriness in severe paediatric UC (Chung–Faye et al., 2007). It also holds promise as potential screening biomarker for colorectal cancer (CRC) in UC (Fengming and Jianbing, 2014).

1.2.12 Faecal and serum S100A12

S100A12, shown in figure 1.1, belongs to a family of low molecular weight S100 proteins or calgranulins (figure 1.2). S100A12 is a 10.4 kDa molecular weight 91 amino acids, calcium–binding proinflammatory protein predominantly expressed and secreted by neutrophil granulocytes, represents 5% of total cytosolic protein (Donato et al., 2013). The name 'S100 protein' was derived from their ability to be 100% soluble in a saturated solution of

ammonium sulphate at neutral pH. These proteins bind calcium and are characterized by two calcium–binding motifs called the elongation factor (EF) hand. There are over 600 members of EF–hand super family (Fritz et al., 2010; Santamaria–Kisiel et al., 2006; Donato, 2001; Heizmann et al., 2002). The S100 proteins (calgranulins) are expressed in myeloid cells including neutrophils, monocytes, and dendritic cells (Ye et al., 2004). The S100 proteins have several functions including roles in cellular inflammation, proliferation, differentiation, apoptosis, signal transduction, calcium homeostasis and energy metabolism (Pietzsch and Hoppmann, 2009; Schafer and Heizmann, 1996; Heizmann and Cox, 1998; Santamaria–Kisiel et al., 2006; Donato, 2003). In chronic inflammation, S100 proteins are actively expressed in activated granulocytes (Vogl et al., 1999; Karl et al., 2008; Foell et al., 2008).

The cytokine–like extracellular functions of S100 proteins such as chemotactic activities related to inflammation and the acute phase response are exhibited mainly by S100A8, S100A9 and S100A12, and these are often referred to as the calgranulins or myeloid–related proteins (MRP) (Ravasi et al., 2004; Dell'Angelica et al., 1994; Guignard et al., 1995).

S100A12 consists of 91 amino acids with molecular weight of 10.4 kilodaltons (kDa), and is predominantly expressed and secreted by neutrophil granulocytes (Manolakis et al., 2011). S100A12 is a ligand for the receptor for advanced glycation end products (RAGE) and therefore, also named as extracellular newly identified receptor for advanced glycation end products binding protein (EN–RAGE). Other alternative names for S100A12 are calgranulin C, migration inhibition factor–related protein 6 (MRP–6) and

calcium–binding protein in amniotic fluid (CAAF–1) (Ravasi et al., 2004; Hoffmann et al., 1999; Hitomi et al., 1996). The binding of S100A12 to RAGE modulates these extracellular functions in disease (Schmidt et al., 2000). In inflammatory disease, serum S100 correlates with disease activity parameters and in these conditions S100 proteins may be superior to conventional laboratory biomarkers of inflammation including CRP and ESR (Foell et al., 2004a; Foell et al., 2004b).

Serum S100A12 have been reported in increased concentrations in various biological materials including synovial fluid, synovial tissue and serum of patients with inflammatory arthritis (Yang et al., 2001; Rouleau et al., 2003; Foell et al., 2003a; Liao et al, 2004). Serum S100A12 is also raised in neurodegenerative diseases, diabetes mellitus (Kosaki et al., 2004), rheumatoid arthritis (RA), osteoarthritis (Nakashima et al., 2012), cancerogenesis (Thierolf et al., 2008), familial mediterranean fever (Kallinich et al., 2010), idiopathic pulmonary fibrosis (Miniati et al., 2011), cardiovascular diseases (CVD) (Hofmann–Bowman et al., 2008) and atherosclerosis (Mori et al., 2009). S100A12 levels were also elevated in patients with peripheral radiographic features (p = 0.036), but did not correlate with clinical variables of disease activity in psoriatic arthritis (Madland et al., 2007).

In haemodialysis patients, levels of S100A12 are linked to cardiovascular disease (Kalousova et al., 2012; Shiotsu et al., 2011). Elevated concentrations of S100A12 and its receptors are found in pulmonary tissue and broncho–alveolar lavage fluid in acute lung injury (Kikkawa et al., 2010; Wittkowski et al., 2007). Both S100A8 and S100A9 hetero–complexes are actively expressed preceding prostate tumour genesis and subsequent development,

progression and enlargement of prostate carcinomas. Alone, serum S100A9 is increased significantly in prostate cancer patients compared to healthy controls or patients with benign prostatic hyperplasia (BPH); thereby underlining its role as a useful biomarker in differentiating prostate carcinoma and BPH (Hermani et al., 2005).

Studies by Sidler et al (2008) and de Jong Naomi et al (2006) have shown faecal S100A12 to be a novel non–invasive biomarker of IBD in paediatric populations. S100A12 levels in stool can be used as an indicator of disease activity in chronic IBD and to gauge the degree of gastrointestinal tract inflammation. As a biomarker of neutrophil activation, faecal S100A12 could play a significant role as a non–invasive biomarker of intestinal inflammation.

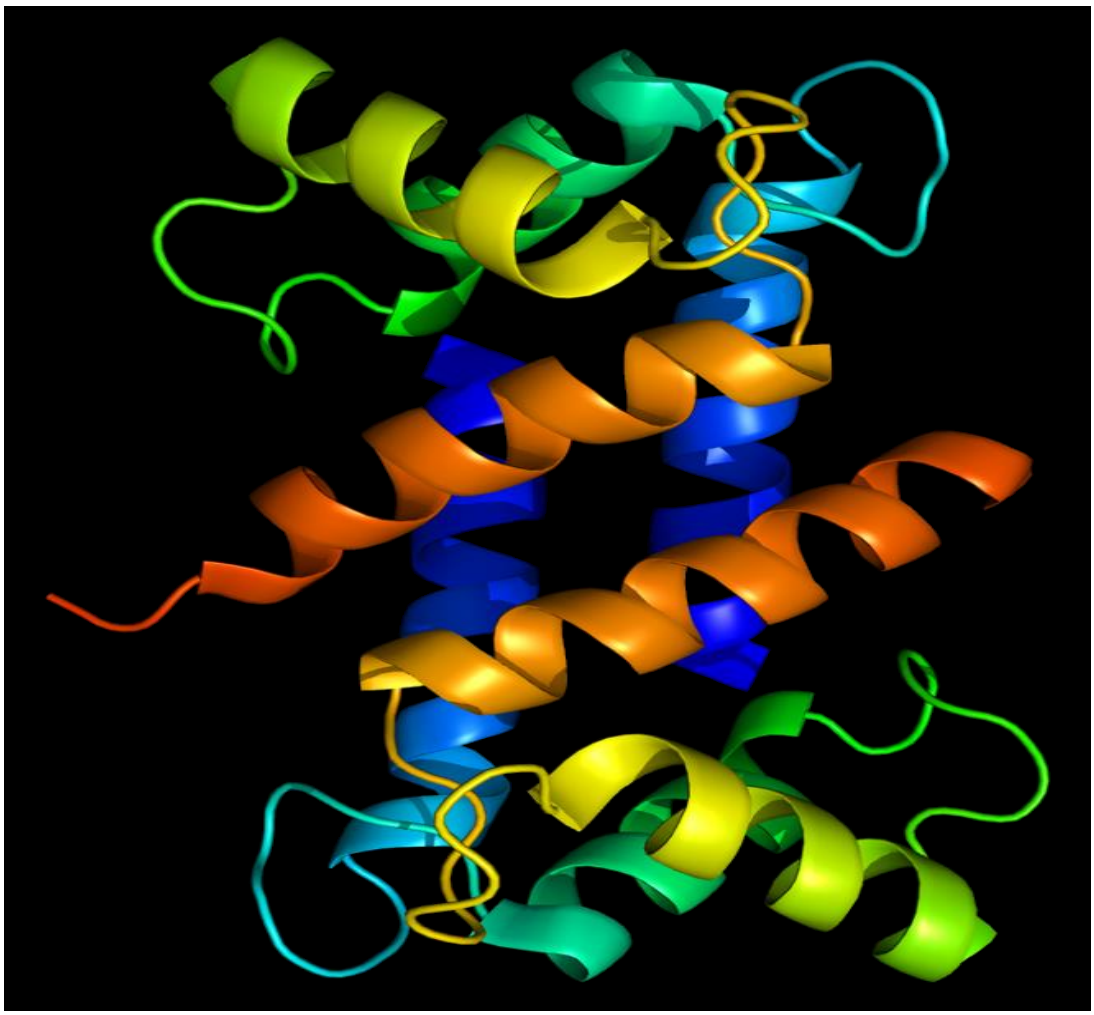

Figure 1.1 – Structure of S100A12. A three–dimensional model of the crystal structure of the S100A12 protein in a hexameric form and its proposed role in receptor signalling. The stereo view of the Ca^{2+} and Cu^{2+}–S100A12 dimer in ribbon representation, with individual subunits shown in red and blue. The chain topology, subunit arrangement, and juxtaposition of metal–binding sites are typical of metal–bound S100 proteins. Modified from: Moroz et al (2001); Moroz et al (2002); Moroz et al (2003a) and Moroz et al (2003b).

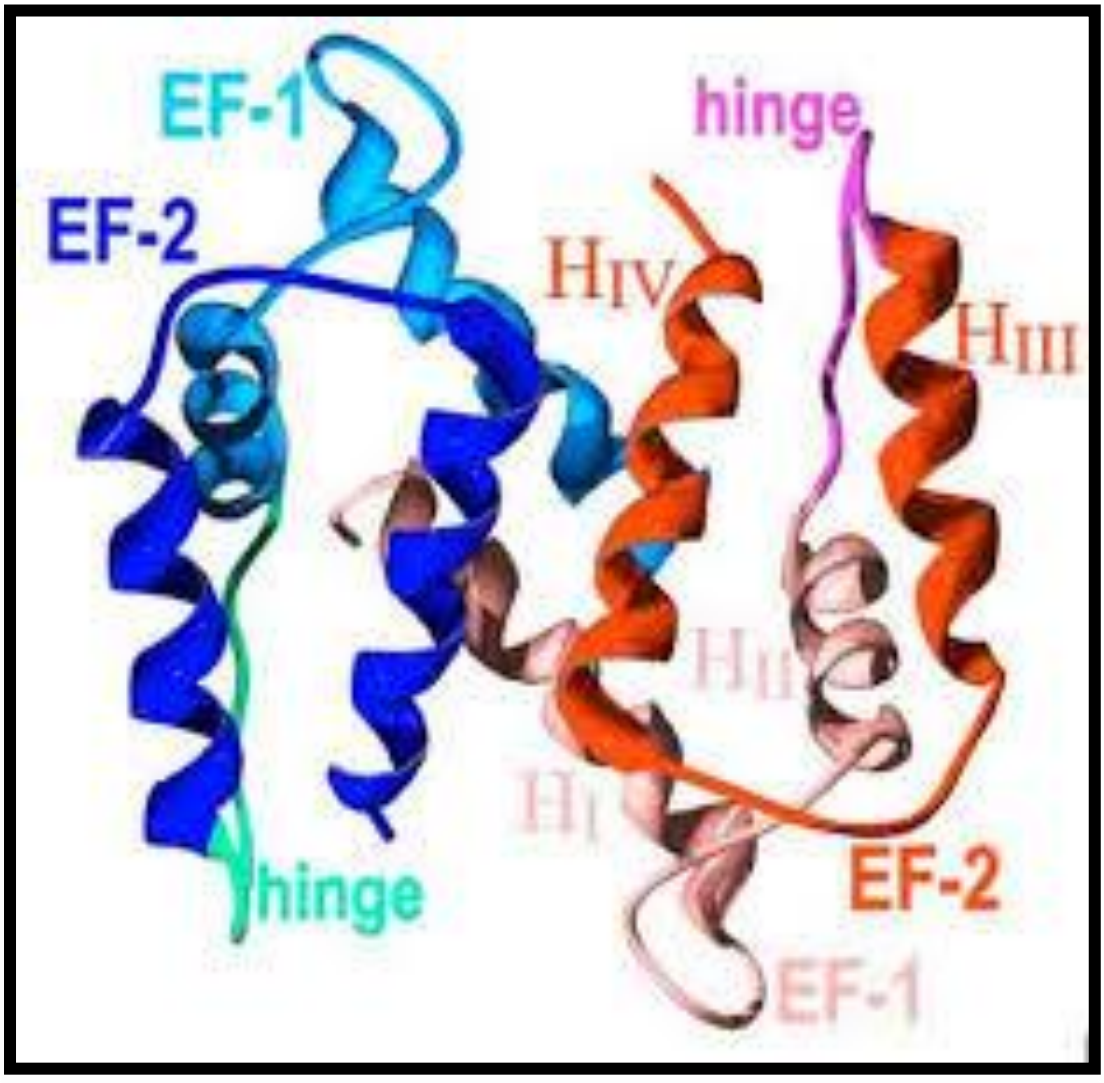

Figure 1.2 – Dimer structure of S100 protein. S100 proteins are small proteins with a molecular weight of 10–12 kDa. Each S100 protein consists of two EF–hand helix–loop–helix structural motifs, which are arranged in a back–to–back manner and linked with a flexible hinge. Modified from: Fritz G; Botelho HM; Morozova–Roche LA; Gomes CM (2010) Natural and amyloid self–assembly of S100 proteins: structural basis of functional diversity. *FEBS Journal*, 277: 4578–90. doi:10.1111/j.1742–4658.2010.07887.x

1.2.13 Faecal and serum calprotectin

Calprotectin is a 36 kDa calcium and zinc–binding protein composed of two heavy 14 kDa S100A9 (MRP14) and one light 8 kDa S100A8 (MRP8) subunits which are members of the EF–hand motif containing S100 family of proteins. Like all S100 proteins, the genes that code for calprotectin are located within the gene cluster on chromosome 1q21 region. Calprotectin is expressed predominantly in neutrophils and monocytes – in which it constitutes up to 60% of the cytosolic protein. It is also expressed in macrophages, keratinocytes, epithelial cells and endothelial cells (Donato et al., 2013; Fagerhol et al., 1980; Dale et al., 1985; Dale and Brandtzaeg, 1989; Brandtzaeg et al., 1987a; Brandtzaeg et al., 1987b).

Calprotectin was first characterised in 1980 as the Leucocyte protein candidate 1 (L1), to reflect *in vivo* granulocyte turnover (Berntzen and Fagerhol, 1990; Berntzen et al., 1991a; Berntzen et al., 1991b; Dale, 1990). L1 was renamed calprotectin in recognition of its antimicrobial activity (Santhanagopalan et al., 1995), calcium–binding roles and subsequent involvement in intracellular signal transduction and regulatory functions in acute phase response and inflammatory processes (Fagerhol et al., 1980; Steinbakk et al., 1990; Johne et al., 1997). Neither S100A8 nor S100A9 subunits in isolation have anti–microbial characteristics. It is possible that the high affinity of calprotectin subunits, S100A8 and S100A9, for zinc binding site could explain the reduction of zinc concentration sufficiently to allow calprotectin to inhibit microbial growth (Sampson et al., 2002; Kerkhoff et al., 1999; Steinbakk et al., 1990; Isaksen and Fagerhol, 2001; Sohnle et al., 2000).

Alternative names for calprotectin include: MRP–8 and MRP–14 (MRP8/ MRP14), p8/14, p34 and S100A8/S100A9. Calprotectin is composed of two heterocomplexes: S100A8 (also known as MRP–8, Calgranulin A and CP–10 in mouse) and S100A9 (also known as MRP–14 and Calgranulin B).

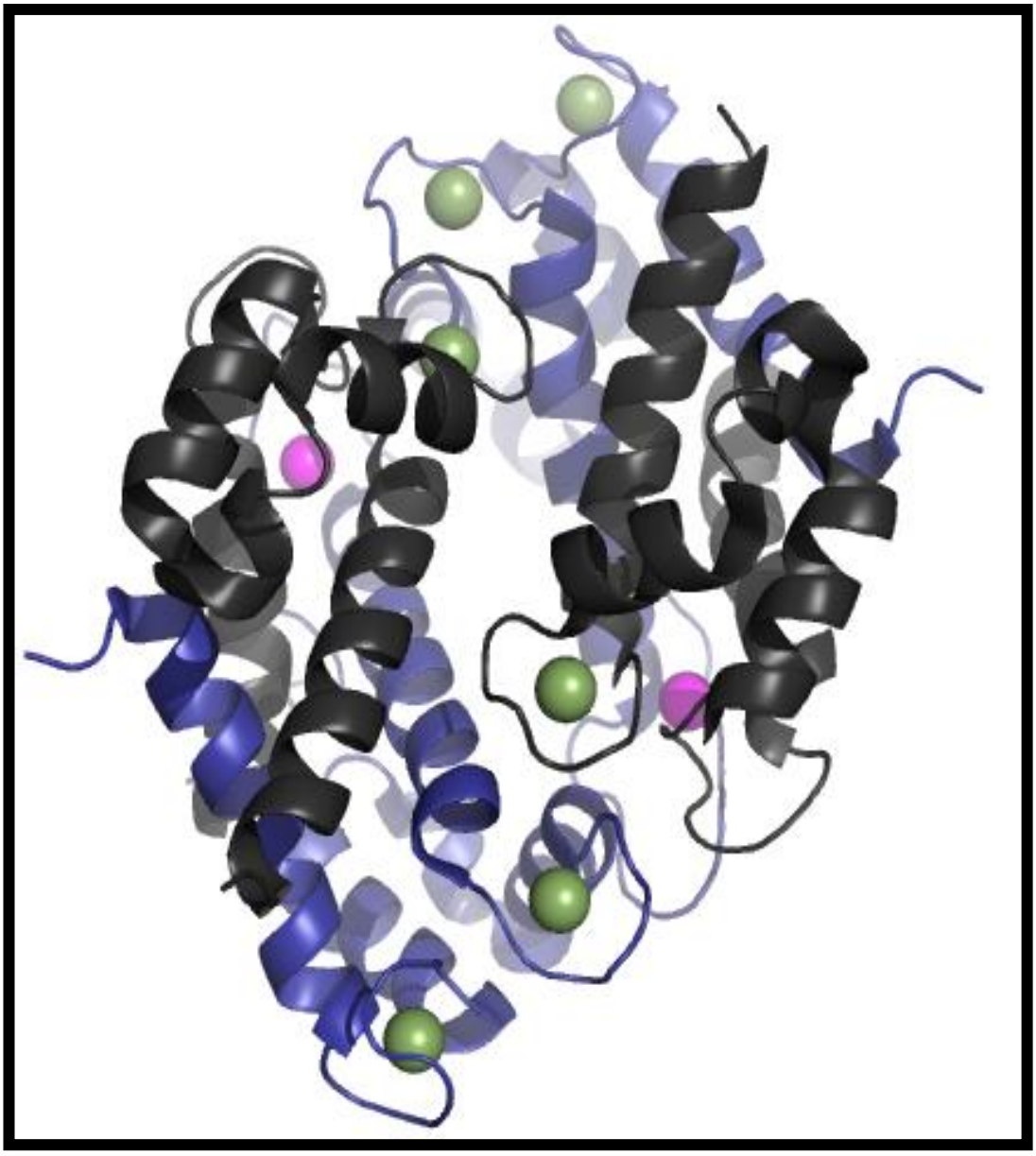

Figure 1.3 – Structure of calprotectin. A three–dimensional model of the crystal structure of Mn^{2+} and Ca^{2+} loaded calprotectin protein, showing two heterodimers: S100A8 and S100A9 as determined by X–ray diffraction. The grey and blue chains represent S100A8 and S100A9, respectively. The purple spheres represent Mn^{2+} and green spheres represent Ca^{2+}. Only one manganese ion can bind per calprotectin heterodimer. The modified figure was taken from the protein data bank (PDB) at: http://www.rcs b.org/pdb

Calprotectin is found in plasma, saliva, cerebrospinal fluid (CSF), urine and faeces (Tibble and Bjarnason, 2001; Larsen et al., 2004). Circulating calprotectin is commonly raised in inflammatory diseases including acute coronary syndromes (Healy et al., 2006; Altwegg et al., 2007), cystic fibrosis (Wilkinson et al., 1988), multiple sclerosis (Yui et al., 2003; Striz and Trebichavsky, 2004), human immunodeficiency virus (HIV) infections (Müller et al., 1994), rheumatoid arthritis (Madland et al., 2002; Berntzen et al., 1989; Brun et al., 1992), reactive arthritis (Hammer et al., 1995), juvenile chronic arthritis (Berntzen et al., 1991b), juvenile idiopathic arthritis (Wittkowski et al., 2008), psoriatic arthritis (Brun et al., 1994; Kane et al., 2003b) polymyalgia rheumatica (Brun et al., 2005), systemic lupus erythematosus (SLE) (Haga et al., 1993) and acute rejection in kidney allograft transplantation (Burkhardt et al., 2001; Striz et al., 2001).

Calprotectin expression correlates with microglial activation in cerebral malaria; and serum levels are prognostic biomarkers in recurrent infection and survival in alcoholic liver disease (Heller et al., 2011; Lönnkvist et al., 2011; Börekci et al., 2009).

Early studies by Røseth et al (1997), Røseth et al (1999) and Røseth (2003) reported increased calprotectin concentrations in faecal samples of patients with IBD, and this observation reflects granulocyte migration through the inflamed intestinal wall. Measurement of faecal calprotectin now enjoys wide application in the diagnosis and monitoring of IBD, and an increased concentration indicates organic intestinal disorders (Guardiola et al., 2018). Calprotectin is an extremely stable protein, and remains unaltered in stool

samples left unprepared for longer than seven days (Røseth et al., 1992; Tøn et al., 2000).

Studies have focused on the accuracy of faecal calprotectin in the diagnosis and monitoring of IBD. A meta–analysis by von Roon and colleagues, reported 95% sensitivity and 91% specificity of faecal calprotectin in the identification of IBD. They also reported that faecal calprotectin was superior to CRP and ESR (von Roon et al., 2007).

The National Institute for Health and Care Excellence (NICE) recommends faecal calprotectin as a diagnostic tool to help in the differential diagnosis of IBD and IBS (NICE, 2013). A normal faecal calprotectin excludes IBD, whereas an elevated faecal calprotectin is an indication for colonoscopy, thereby reducing referrals for unnecessary endoscopic evaluation. A meta–analysis of 13 studies concluded that faecal calprotectin testing would result in a 67% reduction in the number of adults requiring endoscopy, but with a delayed diagnosis in 8% of adults because of false negative results (van Rheene et al., 2010). One area of controversy surrounding faecal calprotectin testing is the determination of an appropriate cut–off value, above which the result is deemed as positive. In most centres, a relatively low level of 50 µg/g is used. Pavlidis et al (2013) studied a cohort of adult patients undergoing faecal calprotectin testing in primary care. At a cut–off value of 50 µg/g, faecal calprotectin had a negative predictive value (NPV) of 98% and positive predictive value (PPV) of 28%. Increasing the cut–off value to 150 µg/g gave a very comparable NPV of 97%, but a much higher PPV of 71%. Given these values, it was calculated that increasing the cut–off value to 150 µg/g, would

reduce colonoscopy and flexible sigmoidoscopy bookings by 10% at the cost of 4 missed cases of IBD (Pavlidis et al., 2013).

1.3 Faecal biomarkers as 'gold standards' in intestinal inflammation

There is no gold standard diagnostic test for IBD. A combination of any or all of clinical, biochemical, radiological, endoscopic, and histological investigation are used (Soubières and Poullis, 2016). As previously alluded, anaemia and blood inflammatory markers (thrombocytosis, CRP and ESR) may indicate disease activity but lack sensitivity and specificity.

Faecal biomarkers of intestinal inflammation, although sensitive for inflammation, are not specific for the causal disease. A combination of two or more biomarkers could optimise diagnostic efficiency. A combination of calprotectin with lactoferrin or neopterin or S100A12, however, does not increase its diagnostic accuracy (Schroeder et al., 2007). More data are required to justify the use of more than one faecal biomarker as this may not increase diagnostic performance but has increased cost implications.

Faecal calprotectin is used in most IBD studies as the 'gold standard' against which most other faecal biomarkers are benchmarked. (Lehmann et al., 2015). Studies by Tibble et al (2000a), Schoepfer et al (2008) and Henderson et al (2014) have demonstrated that faecal calprotectin, with a higher sensitivity and specificity compared to certain blood parameters like CRP, ESR and other leucocytic indices is consistently a better diagnostic biomarker in the assessment of mucosal inflammation.

The quantitative measurement of faecal calprotectin using ELISA technique is well established (Røseth et al., 1992), and commercially prepared ELISA

kits are now available for routine laboratory use. The manufacturers stated performance characteristics, however, must be confirmed by the user–laboratory prior to being rolled for routine use.

Variations in ELISA kit performance for faecal calprotectin have been reported by Burri et al (2013) with kits that employ monoclonal antibodies having superior analytical performance in terms of accuracy, imprecision, functional sensitivity, recovery, linearity of dilution, assay drift, stability and interference compared to those with polyclonal antibodies (Coorevitis et al., 2013; Dolci and Panteghini, 2012).

Like other faecal biomarkers, calprotectin is subject to limitations including:

(1) Lack of specificity for IBD. Faecal biomarkers for mucosal inflammation will be raised not only in IBD but in other inflammatory bowel disorders including colorectal carcinoma, post pelvic radiation, diverticulitis, gastroenteritis (either bacterial or viral), non–steroidal enteropathy and food intolerance (Manz et al., 2012).

(2) Levels of calprotectin in stool samples are dependent on diverse physiological considerations such as age and clinical co–morbidities. There is significant within–patient variability particularly with high faecal concentrations (Røseth et al., 1992; Naismith et al., 2013; Lasson et al., 2014). Faecal calprotectin also has considerable biological variability and spot variability during multiple sampling from the same stool sample (Husebye et al., 2001; Moum et al., 2010; Hare et al., 2013).

(3) The lack of validated, optimal and varying assay cut–offs makes it difficult to characterize active inflammatory disease, distinguish IBD from IBS

(Whitehead et al., 2017; Foell and Roth, 2005), predict clinical remission and mucosal healing, and assess response to treatment. This also makes it difficult to directly compare results from different studies using different assays (Coorevitis et al., 2013; Damms and Bischoff, 2008).

D'Inca et al (2007), however, suggested a higher cut–off value for IBD assessment and a lower cut–off value for screening of IBD. Furthermore, a lower cut–off value will help to improve assay sensitivity for distinguishing IBD from IBS but increase the number of false–positive patients referred for unnecessary endoscopies. A broad range of assay for faecal calprotectin cut–offs such as 52 to 274 µg/g have been suggested for predicting endoscopic remission. The optimal cut–off value such as less than 50 µg/g (negative), 50 to 100 µg/g (weakly positive or indeterminate) and greater than 100 µg/g (positive) in screening for IBD is still dependent on different parameters recommended by the manufacturers (Burri and Beglinger, 2012).

(4) Significant overlap in calprotectin levels (50 to 150 µg/g) in both IBD and IBS patients presents an ambiguous situation regarding the decision to refer a patient for endoscopy. While repeat testing may enable decision on endoscopy referral, it is critical to note that calprotectin levels cannot be relied on to localize IBD as well as to differentiate between CD and UC because of paucity of data to support the correlation of calprotectin levels to specific disease locations. Calprotectin and lactoferrin levels could be normal or higher in both clinically and endoscopically active CD, especially in ileal disease (Sipponen et al., 2008b). This makes it difficult to appropriately categorize the existence of validated characterization of mucosal healing in endoscopically quiescent IBD.

(5) Patient reluctance to collect stool samples for initial diagnosis and particularly for monitoring disease activity is a limitation of faecal biomarkers in the diagnosis and serial assessment of IBD (Tibble et al., 2002; Lehmann et al., 2014).

1.4 Serum acute phase proteins in the assessment of inflammatory bowel disease

Shine et al (1985) and Beattie et al (1995) reported CRP to be a sensitive blood biomarker for the identification of IBD and for differentiating IBD from IBS. Compared to ESR and α_1–glycoprotein, CRP was increased in most patients with active CD, about 50% of those with active UC and none with functional bowel disorders.

Similarly in a paediatric cohort, increased CRP was reported in 100% of children with CD, 60% of children with UC and none in healthy controls. A raised ESR was reported in 85% and 23% of children with CD and UC respectively, whereas a normal result was reported in children with normal investigations. Improvement in CRP sensitivity was achieved through adjusting the cut–off threshold: between 70% and 100% increase was reported in CD patients while between 50% and 60% was reported in UC patients (Tibble et al., 2000b; Fagerberg et al., 2005; Thjodleifsson et al., 2003).

CRP, ESR, albumin, haematocrit, cholinesterase, β_2–macroglobulin and α_1–proteinase inhibitor have good correlation with endoscopic activity in IBD. Increased inflammatory biomarkers are common among patients with severe disease compared to patients without IBD; and the increased levels correlate well with severity of the inflammatory process (Tromm et al., 1992; Fagan et

al., 1982). There are variations in correlations with disease activity among these blood biomarkers; but only CRP show significant correlation with clinical activity status in IBD and this is much stronger for CD than for UC.

There is a paucity of data on serum laboratory biomarkers in assessing the disease course and outcome in UC. A prospective study by Brignola et al (1986) reported that laboratory biomarkers including CRP, ESR, albumin, white blood cells, haemoglobin, α_1–glycoprotein, α_2–globulin, α_2–antitrypsin and serum iron predict clinical relapse in CD based on CDAI of less than 150 in patients with clinically inactive disease. The authors reported that ESR, α_1–glycoprotein and α_2–antitrypsin were the best differentiators of patients in whom clinical relapse occurred from those in whom it did not.

Boirivant et al (1988) reported that the probability of a clinical relapse occurring after 24 months in patients was increased in those with increased CRP levels compared with those with normal CRP levels. CD patients, however, may present with clinically active disease despite having normal CRP levels, and raised CRP levels may be present in patients with clinically inactive disease. Cosigny et al (2001) reported that only CRP and ESR from a host of laboratory biomarkers that included full blood count, α_1–antitrypsin and orosomucoid showed good promise in predicting clinical relapse in patients with CD, defined as a CDAI of greater than 150 with an increase of greater than 100 points from baseline. Raised CRP values correlate well with disease activity; but normal CRP and ESR values did not rule out clinical relapse.

1.5 Faecal calprotectin: Inflammatory bowel disease and irritable bowel syndrome

It is vital to distinguish non–inflammatory disorders from inflammatory, malignant or infectious gastrointestinal tract diseases. Distinction between these diseases necessitates the use of clinical, radiology, endoscopy, histology and serological techniques that are inherently invasive, expensive, time–consuming and/or hindered by imprecisions that arise from subjective components (Däbritz et al., 2014; Soubières and Poullis, 2016). Similar clinical symptoms, such as abdominal pain, diarrhoea, nausea and general malaise in patients presenting with IBD and IBS makes their clinical differentiation difficult, if not impossible. The application of reliable, non–invasive biomarkers that could differentiate IBD from IBS becomes critical (Alibrahim et al., 2015; Sydora et al., 2012; Wassell et al., 2012; Damms and Bischoff, 2008).

IBD can be distinguished from IBS using inflammatory biomarkers which have traditionally been detected in faeces (Figure 1.4) and more recently in serum. The use of these biomarkers (Table 1.4) avoids subjecting patients with IBS to invasive, expensive and unnecessary endoscopies (Cury et al., 2013).

S100A12 and calprotectin are phagocyte–specific proteins of the S100 family that are promising faecal biomarkers of inflammation. Biomarkers of faecal leukocyte degranulation such as lactoferrin, PMN and MPOs have also been suggested for use in differentiating IBD from IBS (Däbritz et al., 2014). Calprotectin is the most widely studied sensitive and specific faecal

biomarker of intestinal inflammatory activity for differentiating IBD from IBS (figure 1.5). The high diagnostic accuracy of faecal calprotectin in ruling out intestinal inflammation accounts for its use as a non–invasive screen for IBD and many disorders that present with symptoms similar to IBS (Seenan et al., 2015; Furman and Cash, 2011; Jahnsen et al., 2008).

The pioneering study by Røseth et al (1992) indicated the usefulness of increased faecal calprotectin levels in both CD and UC, and in the differentiation between IBD and IBS. Based on a PPV of 70% to 100% and an NPV of 51% to 91%, studies by Carroccio et at (2003), Konikoff and Denson (2006) and Schoepfer et al (2008) confirmed faecal calprotectin to be a very useful biomarker in discriminating between IBD and IBS.

van Rheenen et al (2010) and van Roon et al (2007) reported that faecal calprotectin had 93% sensitivity and 96% specificity for the identification of IBD, and was able to discriminate between IBD and IBS based on 95% sensitivity and 91% specificity. This was subsequently confirmed by Gisbert and McNicholl (2009) with a sensitivity of 83% and specificity of 84% to discriminate between these patient groups.

The diagnostic accuracy of faecal calprotectin in discriminating between IBD and IBS is different in children and adults. van Rheenen et al (2010) reported a high sensitivity of 92% in children versus 93% in adults, and a lower specificity of 76% in children versus 96% in adults. Henderson et al (2014) similarly reported a sensitivity of 98% with a specificity of 68% for faecal calprotectin in distinguishing IBD and IBS in a paediatric population.

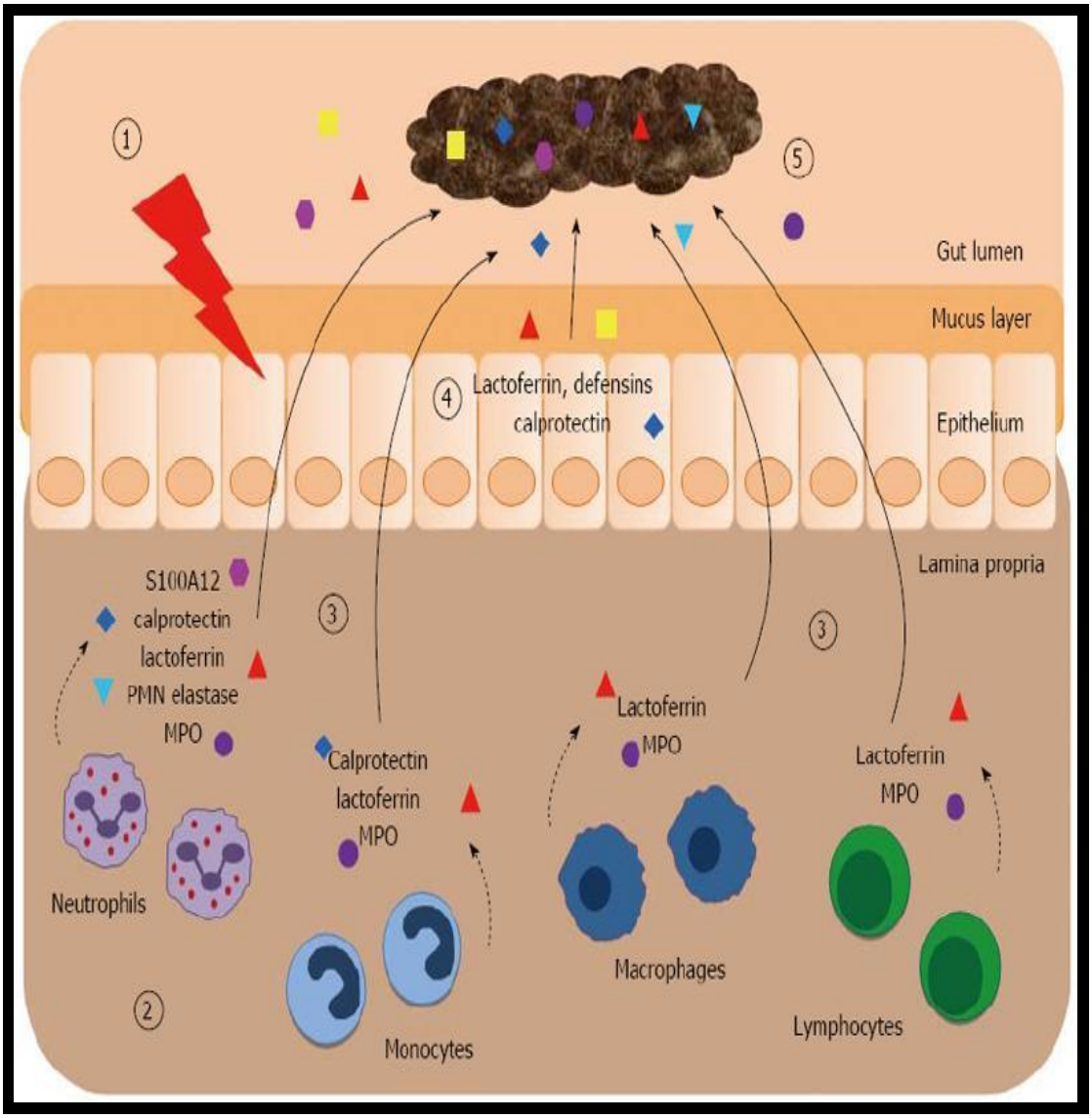

Figure 1.4 – Faecal biomarkers of intestinal inflammation are linked to the innate immune system and expressed in granulocytes, monocytes/ macrophages and epithelial cells. (1) Initially, unidentified triggers affect the epithelium and lead to an activation of the intestinal immune system; (2) The initiated immune response involves the influx of different innate immune cells (e.g., granulocytes, monocytes, macrophages) and cells of the adaptive immune system (e.g., T cells) into the affected mucosa. These cells actively secret inflammatory mediators or release granule proteins by cell degranulation. The contents of neutrophil granules [▲Lactoferrin; ▼Polymorphonuclear (PMN) elastase; ● Myeloperoxidase (MPO)] have antimicrobial properties. The cytosol is the source of the damage associated molecular pattern proteins S100A8/A9 (Calprotectin and S100A12) (3) During early stages of intestinal inflammation these released proteins spill over from the mucosa into the gut lumen; (4) Some of these factors (including defensins) are also released from the epithelium and the mucus layer; (5) In direct contact with the intestinal mucosa, the faecal stream contains the specific proteins of mucosal disease. The detection of these markers in faeces indicates the presence and degree of intestinal inflammation. The modified structure was taken from: Däbritz J; Musci J; Foell D (2014).

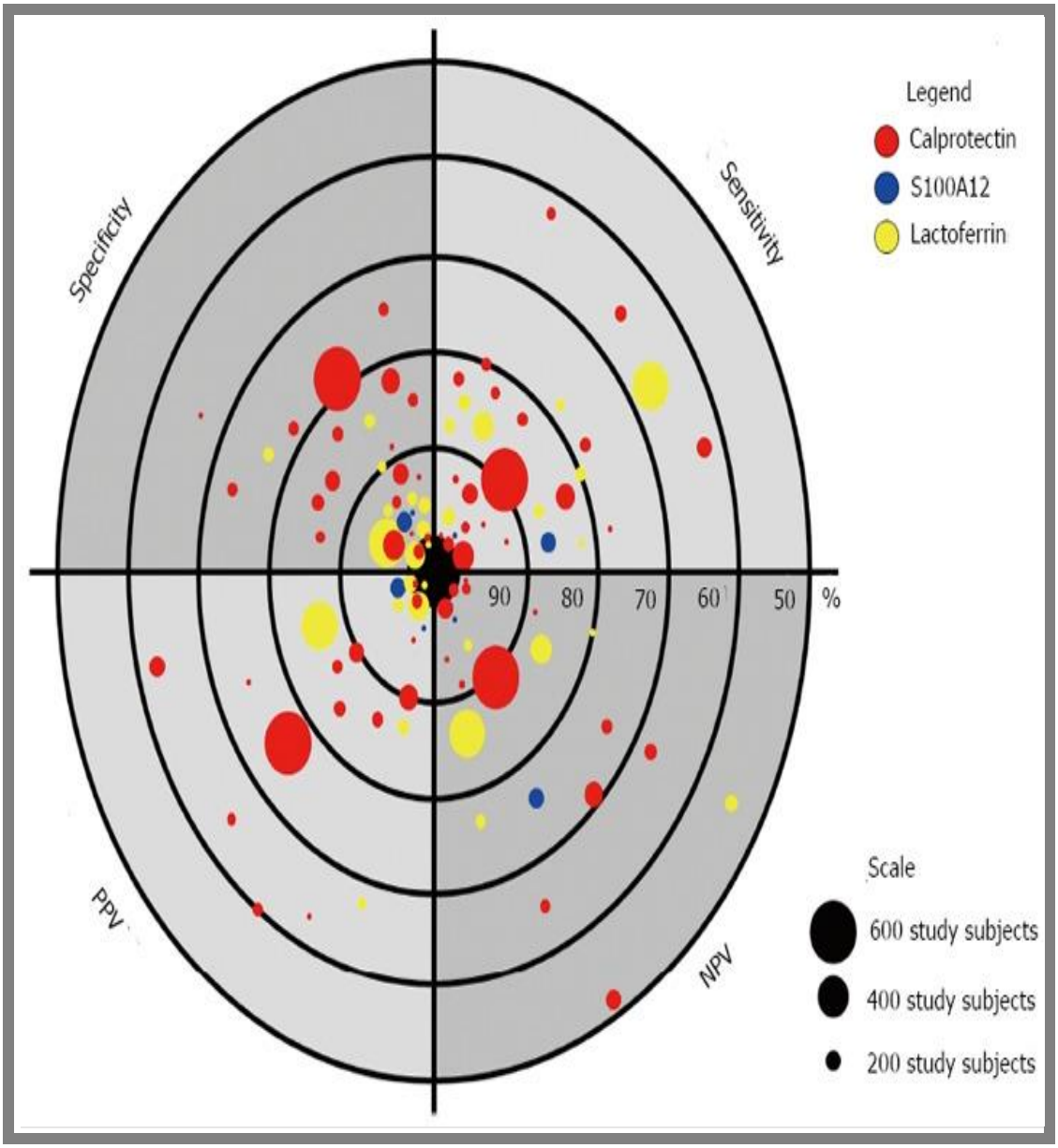

Figure 1.5 – Diagnostic accuracy of faecal markers in the differentiation of organic gastrointestinal disease vs irritable bowel syndrome (IBS). The figure illustrates statistical measures of the diagnostic performance of different studies on the role of faecal markers in the diagnosis of irritable bowel syndrome. Sensitivities, specificities, positive predictive values (PPV), and negative predictive values (NPV) of different biomarker studies are represented with highest values close to the center of the 'dartboard' (i.e., 100%). Each dot represents a biomarker study and different colours represent the type of the faecal marker (see legend). The size of each dot represents the number of included study subjects (see scale). Modified from: Däbritz J; Musci J; Foell D (2014) Diagnostic utility of faecal biomarkers in patients with irritable bowel syndrome. *World Journal of Gastroenterology,* 20 (2): 363 – 375.

1.6 Inflammatory and serological biomarkers in differential diagnosis of inflammatory bowel disease

The differential diagnosis of IBD includes colon cancer, ischaemic colitis, diverticulitis, bacterial and viral infection. These alternative or co–existing disorders must be ruled out prior to confirmation of intestinal inflammation as a first step in the differential diagnosis of IBD (Figure 1.6).

Accurate diagnosis of CD and UC is therefore dependent on not only laboratory test results but also on the patient's clinical history and examination, histology, imaging results (X–ray, CT and/or MRI scans) and endoscopy (Soubières and Poullis, 2016; Iskandar and Ciorba, 2012).

Inflammation in UC is relatively superficial because it affects the mucosa. It is confined to the colon starting at the rectum, with rare extension to the terminal ileum. There is, however, a 10 to 15% chance of misdiagnosis due to difficulty in distinguishing UC from CD. Accuracy in differential diagnosis is critical because while inflammation in CD extends deeper into the tissues and could affect any portion of the gastrointestinal tract, associated symptoms like abdominal pain, fever, malnutrition and severe bloody diarrhoea are common to both (Savige et al., 2003).

1.6.1 Inflammatory biomarkers

The initial step in the identification of IBD is exclusion of other organic disorders. Serum CRP, ESR, faecal calprotectin and faecal lactoferrin form the available inflammatory biomarkers.

Many laboratory biomarkers have been evaluated as the ideal replacement for or supplement to faecal calprotectin in IBD studies but CRP and ESR

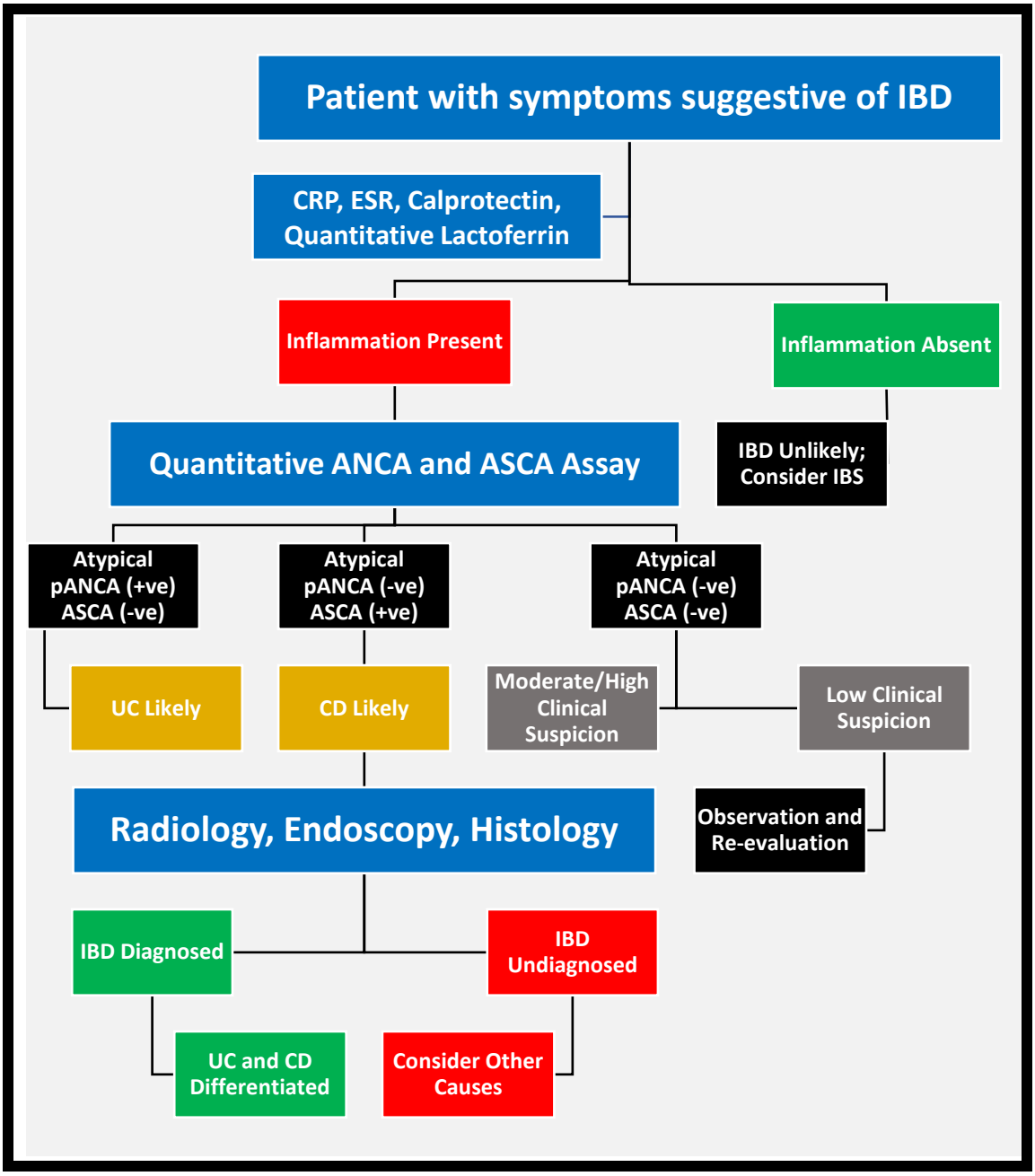

Figure 1.6 – The interplay of inflammatory (CRP, ESR, Calprotectin and Lactoferrin) and serological (pANCA and ASCA) biomarkers in the differential diagnosis of inflammatory bowel disease. The likelihood of CD, UC and Moderate/High Clinical Suspicion are subjected to further radiographic, endoscopic and histological investigations to complete the differential diagnosis. While the above illustration provides the necessary information to aid in test selection, interpretation, diagnosis and overall patient management decisions, it is not a substitute for the clinician's underpinning knowledge of IBD in patient assessment based on clinical expertise. Modified from: Iskandar and Ciorba (2012), Lewis (2011), Kornbluth and Sachar (2010) and Reese et al (2006).

remain the most widely used and assessed tests (Vermeire et al., 2006). Other biomarkers used include leucocyte counts, platelet counts, albumin levels and orosomucoid concentrations (Cioffi et al., 2015), but there is no evidence that they are of greater benefit compared to CRP in the identification of IBD and monitoring its disease activity (Vilela et al., 2012).

CRP is routinely available as it is rapid, inexpensive and a simple testing technique. CRP's reliability as a biomarker of choice in routine clinical practice is well established (Vermeire et al., 2004). Serum CRP may help in differentiating IBD from other functional bowel disorders (Shine et al., 1985). However, normal CRP levels (≤ 5 mg/L) at diagnosis occur in majority of UC patients and in 25% of CD patients (Henriksen et al., 2008). There is, however, a correlation between CRP and endoscopic activity in IBD and this may have a useful role in monitoring responses to therapy (Solem et al., 2005; Reinisch et al., 2012).

In summary, increased CRP is more common in CD than in UC, thereby supporting CRP to be a useful biomarker of disease activity in CD than in UC (Vermeire et al., 2006; Henriksen et al., 2008; Solem et al., 2005). CRP, however like most serologic biomarkers, is a non–specific biomarker of systemic inflammation.

1.6.2 Serological biomarkers

In the presence of inflammation, ANCA and ASCA may be useful in the identification of IBD (Iskandar and Ciorba, 2012; Lewis, 2011). A positive result will necessitate radiology, endoscopy and histological investigation to confirm the diagnosis of IBD and differentiation of CD and UC. If serological

markers are negative in the presence of a high clinical suspicion of IBD, then radiologic, endoscopic and histological findings could be used to diagnose or exclude IBD and, if present, to distinguish CD and UC.

ASCA and atypical pANCA are the two serologic markers most commonly used to distinguish CD and UC (Zhou et al., 2010). A positive ANCA result is reflexed to determine the relevant pattern(s): cytoplasmic (cANCA), perinuclear (pANCA) or atypical pANCA patterns and their titres. Whereas cANCA and pANCA are found in vasculitis, atypical pANCA is present in IBD. While atypical pANCA is detected in only 5% to 25% of patients with CD, it occurs in about 55% to 80% of those with UC (Reese et al., 2006; Bossuyt, 2006). Conversely, ASCA is detected in about 60% to 70% of CD patients and only in about 6% to 15% of UC patients (Bossuyt, 2006; Abreu et al., 2001).

A combination of pANCA and ASCA has a sensitivity of 53% and specificity of 93% for both CD and UC; and may be of value in assessing IBD in patients that cannot be distinguished as CD or UC on the basis of indeterminate colitis or established criteria (Reese et al., 2006; Kornbluth and Sachar, 2010).

Atypical pANCA and ASCA may help stratify CD. Positive atypical pANCA in CD indicate colonic involvement and an association with a clinical phenotype analogous to UC or UC–like CD, while a positive ASCA result is linked with non–UC–like CD (Abreu et al., 2001; Klebl et al., 2003).

Serologic biomarkers may be of value in children. In children at high risk of IBD, pANCA and ASCA may help identify children with IBD and avoid

invasive assessment (Viennois et al., 2015; Van der Vijvers et al., 2012; Reese et al., 2006; Bartůnková et al., 2002; Ruemmele et al., 1998).

1.7 Management of disease activity in inflammatory bowel disease

Achieving mucosal healing with subsequent improvement in natural course of disease state in patients with IBD remains the overall goal of treatment, including immunomodulators and biological agents. Monitoring for efficacy of treatment and relapse of disease activity, therefore, becomes an important consideration (De vos et al., 2012; Dignass et al., 2010)

No single test procedure or examination has been able to satisfy all the necessary requirements for the clinical management of IBD patients. Assessing disease activity for the foreseeable future involves the use of laboratory tests, radiology, endoscopy, clinical examination and symptoms (Soubières and Poullis, 2016). This should not, however, detract from the search for a reliable, non–invasive, highly sensitive and reproducible biomarker of disease activity (Vrabie and Kane, 2014; Vermeire et al., 2006).

The faecal excretion of ^{111}Indium–labelled (^{111}In) granulocytes is considered to be the gold standard method for measuring the degree of neutrophilic infiltration into the intestinal mucosa in IBD and hence the disease activity particularly in patients with small bowel CD (Bjarnason et al., 1995; Saverymuttu et al., 1983). The unwieldy nature of radio–labelling techniques in addition to being expensive and involving exposure to radiation restricts their routine clinical use.

Endoscopic assessment of disease activity in IBD, particularly via ileo–colonoscopy is regarded as the 'gold standard' because it has the added

advantage of enabling biopsy sampling for histopathological examination apart from offering the opportunity for direct mucosal visualization, endoscopic management of complications, assessing the success of various treatments and predicting the course of disease (Matsuura et al., 2021; Spiceland and Lodhia, 2018; Chang et al., 2015; Carter and Eliakim, 2014; Carter et al., 2013; Dignass et al., 2012; Van Assche et al., 2010). Ileo–colonoscopy, however, is invasive, time–consuming and is limited by expense, risks of complications, patient discomfort and variation in interpretation between endoscopists (Mitselos et al., 2021; Zhou et al., 2021). This underscores the desirability for an easier method to monitor disease activity.

1.7.1 Active versus inactive disease state

Increased serum CRP is an indication of active disease in CD patients and it compares favourably with endoscopic disease activity (Jones et al., 2008). Endoscopic assessment should be considered in patients with UC if elevated serum CRP concentrations fail to normalise with or without underlying symptomology (Peyrin–Biroulet et al., 2015).

In the patients with endoscopically confirmed active IBD, faecal calprotectin and lactoferrin levels correlate with endoscopic disease activity in both CD and UC patients (Jones et al., 2008). When compared to serum CRP (49%), both faecal calprotectin (88%) and lactoferrin (82%) are more sensitive because of their ability to correlate better with colonic than ileal disease activity (Iskandar and Ciorba, 2012; Lewis, 2011).

The relatively high sensitivity of both faecal biomarkers (i.e., calprotectin and lactoferrin) in patients with active disease underscores their usefulness in managing patients with IBD (Mosli et al., 2015) and thus avoiding endoscopy in patients with a high clinical suspicion of active disease with raised faecal biomarkers. As a corollary, a negative faecal biomarker result may not rule out active disease and therefore endoscopy may be required if clinically indicated (Mosli et al., 2015). Similarly, with CRP, endoscopic assessment should be considered in patients with UC if elevated faecal biomarkers fail to normalise irrespective of underlying symptoms (Peyrin–Biroulet et al., 2015).

1.7.2 Relapse, remission, and disease course

An increase in serum CRP levels predict relapse in disease activity in patients with CD following medically induced remission (Lewis, 2011; Peyrin–Biroulet et al., 2015).

Faecal biomarkers, particularly calprotectin, play a crucial role in predicting relapse in IBD particularly in UC than in CD (Costa et al., 2005). Elevated faecal calprotectin level is a common feature in patients with CD who relapse compared to those who remain in remission over a 12–month follow–up (Gisbert et al., 2009b). Low faecal calprotectin levels help identify IBD patients who remain in stable remission during follow–up (Mooiweer et al., 2015).

Based on a cut–off value of 167 µg/g, faecal calprotectin with a sensitivity of 69% and specificity of 75% appears to be the best biomarker for predicting IBD relapse following remission (Iskandar and Ciorba, 2012; Gisbert et al., 2009b). Faecal calprotectin levels may also be particularly valuable in

predicting relapse in patients with CD who have undergone surgical resection (Iskandar and Ciorba, 2012; D'Incà et al., 2008).

Faecal lactoferrin also appears to have a role in predicting IBD relapse. Elevated faecal lactoferrin levels have a sensitivity of 62% and specificity of 65% in predicting early disease relapse in paediatric patients (Gisbert et al., 2009a; Gisbert et al., 2009b). In some paediatric patients with CD, pANCA and ASCA levels also predict complicated disease courses (Iskandar and Ciorba, 2012; Lewis, 2011).

1.7.3 Response to therapy

A normal CRP level in CD patients undergoing treatment is linked to a favourable response to therapy (Peyrin–Biroulet et al., 2015). Whereas normalization of CRP levels may serve as a reliable biomarker to measure the response to therapy, there are currently a paucity of studies investigating the roles of faecal calprotectin, lactoferrin, pANCA and ASCA in predicting response to therapy.

1.8 Structure of the dissertation and order of presentation

The dissertation consists of eight chapters, an appendix and a list of references and bibliographies cited in the main text. Each of the chapters as illustrated in figure 1.7 discusses a different aspect of the study.

Chapter One provides a general introduction to the study, outlining a brief definition and aetiology of IBD, clinical features and management of both CD and UC, and laboratory biomarkers and IBD. It also discusses a brief background to faecal biomarkers as 'gold standards' in intestinal inflammation, acute phase proteins and assessment of IBD, the distinction

between IBD and IBS, inflammatory and serological biomarkers in differential diagnosis of IBD and general management of disease activity in IBD, including active versus inactive disease state, relapse, remission and disease course and response to therapy.

Chapter Two sets out the main aims and objectives of study.

Chapter Three provides an introduction to the development and optimisation of ELISA technique used in the study, identifying the principles of ELISA, rationale for the development of a two–site sandwich ELISA method, optimisation of a two–site sandwich ELISA components and finally discusses the implications and significance of the two–site sandwich ELISA on the rest of the study.

Chapter Four attempts to analytically validate faecal S100A12 K 6938 ELISA (Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) and fCAL® turbo (Bühlmann Laboratories AG, Baselstrasse 55, CH – 4124 Schönenbuch, Switzerland) assays for the respective measurement of serum S100A12 and serum calprotectin. The chapter discussed the principles and importance of assay validation in ELISA in relation to the experimental procedures for imprecision, spiking recovery, linearity of dilution or parallelism, carry over and assay drift, analytical sensitivity (i.e., limit of the blank or LoB, and lower limit of detection or LLoD), functional sensitivity or limit of quantitation (LoQ), analytical interference and stability.

The chapter concludes with the findings that serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 assays are suitable assays for routine application in a clinical laboratory; performance characteristics were robust and sensitive

based on results of LoB, LLoD, LoQ and carry over studies, and reliable from the results of imprecision, reproducibility, spiking recovery and linearity of dilution or parallelism experiments that compared favourably to manufacturer provided performance characteristics. The large difference in numerical values between serum BMN®-Cp and serum IDK®-Cp indicate that results and any derived cut–offs between the assays are not directly inter–changeable.

Chapter Five aims to evaluate the utility of serum S100A12 and serum calprotectin as alternatives to faecal S100A12 and faecal calprotectin in the exclusion of IBD in patients presenting with chronic diarrhoea. The chapter evaluated the analytical performance and diagnostic accuracy of serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 assays in 40 patients with IBD by comparing their results with faecal calprotectin regarded as the 'gold standard' biomarker for IBD diagnosis. Also, it analysed correlations between the three assays, and used ROC curve analyses and AUC values to establish serum IDK®-Cp assay as the best discriminator for IBD diagnosis relative to faecal calprotectin. Concordance between faecal calprotectin and serum calprotectin analysed with the BMN®-Cp and IDK®-Cp assays in 40 patients with IBD further established agreements between the methods.

The chapter concludes that: both the serum IDK®-Cp and serum BMN®-Cp assays have good analytical performance and diagnostic accuracy with serum IDK®-Cp having marginally the best diagnostic performance. Serum calprotectin, irrespective of the assay method, is unlikely to replace faecal calprotectin but may have a role supplementing faecal calprotectin in the diagnosis of IBD. Elevated serum calprotectin in patients with chronic

diarrhoea would be an indication for endoscopy since it has a low false positive rate but a normal serum calprotectin does not exclude IBD. The diagnostic performance of serum S100A12, in this study, was poor as a biomarker for IBD assessment.

Chapter Six explores the potential of serum calprotectin as a biomarker to replace or supplement faecal calprotectin and other conventional inflammatory blood biomarkers in discriminating between active and inactive disease using a common set of 175 IBD samples. The chapter evaluated baseline patient characteristics and medical information, compared levels of serum calprotectin determined with the Bühlmann and Immunodiagnostik™ ELISA kits. Statistical analysis of data to evaluate the levels of relationship (correlations), and to ascertain the limits of agreement between the methods was carried out. An assessment of the disease activity based on the results of faecal calprotectin, serum calprotectin, serum CRP and platelets in active and quiescent CD and UC, and according to site of disease was presented. The utility of serum calprotectin, serum CRP and platelets to discriminate between active and inactive IBD, CD and UC with the aid of the ROC curves and AUC values in the light of the sensitivity and specificity of serum BMN®-Cp, serum IDK®-Cp, serum CRP and platelets was discussed.

The chapter concludes with the findings that significant relationship exist between serum calprotectin (measured with both BMN®-Cp and IDK®-Cp assays) and disease activity in patients with CD irrespective of site of disease, but not in patients with UC. The correlation of faecal calprotectin was best with serum CRP and less with platelets and serum calprotectin, irrespective of assay. Serum calprotectin, irrespective of assay, correlated

best with serum CRP and both correlated less with platelets. Serum CRP demonstrated a modest benefit over serum calprotectin, irrespective of assay, based on ROC curves analyses for CD and UC. Serum calprotectin offers no advantages over serum CRP, which is easily available and less expensive.

Chapter Seven concludes the clinical study phase with an investigation of the role of serum calprotectin in acute phase response (APR). It specifically studied the effect of APR on serum calprotectin by measuring it before and 48 hours after elective knee or hip surgery. The level of agreement between serum calprotectin measured with the BMN®-Cp and IDK®-Cp assays were assessed by the Bland–Altman plot.

The chapter concludes with a confirmation that serum calprotectin is a positive acute phase protein. The Bühlmann and Immunodiagnostik™ serum calprotectin assays perform comparably during the course of the APR. At two days following an inflammatory insult, serum CRP may be a better discriminatory biomarker of APR than serum calprotectin based on a much greater incremental response.

Chapter Eight presents the summary, conclusions and future perspectives of the entire study. The chapter opens with a concise commentary on analytical study involving the development and optimisation of ELISA techniques, followed by a brief discussion on the analytical validation of Bühlmann Laboratories AG and Immunodiagnostik™ AG ELISA kits for the measurements of serum calprotectin and serum S100A12 as examples of an evaluation of fitness for purpose of analytical methods. It summarises the

findings of the three key clinical studies on the diagnosis of IBD, evaluation of utility of serum calprotectin to discriminate between active and inactive IBD, and an investigation of the role of serum calprotectin in APR.

The chapter concludes with a succinct discussion of the implications of the findings of the whole study upon professional clinical laboratory practice as a field, as the nature of the study demands, including recommendations drawn therefrom before pointing the direction for future perspectives.

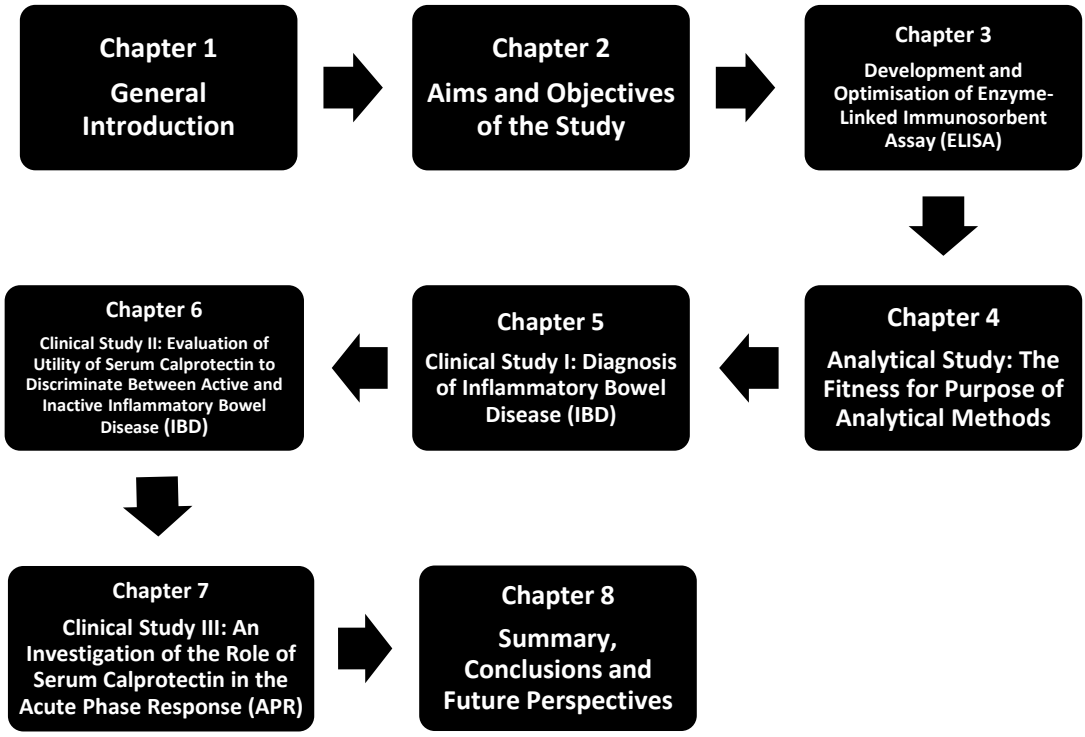

Figure 1.7 – The structure of the dissertation. The ideal, well–developed, standardised and constructed layout is delivered in eight chapters that covers the scope of the dissertation, with each chapter dealing with a specific but altogether interrelated aspects of the study. The layout helps to present a perfect 'framework' for a seamless understanding of the purpose of the individual chapter as discussed in this general introductory chapter.

CHAPTER 2

AIMS AND OBJECTIVES OF THE STUDY

Hitherto, the assessment of IBD – a group of lifelong gastrointestinal tract disorders characterized by prolonged symptomatic episodes of risk of relapse, remission and erratic frequent disease flare–ups – is contingent on the combination of clinical presentation, radiological feature, endoscopic evaluations, biochemical analyses and histological investigations of the disease in secondary care. Faecal calprotectin and to a lesser extent faecal S100A12 play major roles in IBD assessment, and there is the potential that serum calprotectin and serum S100A12 could replace or supplement faecal calprotectin and faecal S100A12 in IBD assessment.

The main aim of the study was:

To develop and analytically validate faecal S100A12 ELISA K 6938 (Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) for the measurement of S100A12 in serum.

To analytically validate serum calprotectin assays: MRP8/MRP14 Calprotectin S100A8/S100A9 ELISA (serum BMN®-Cp) provided by Bühlmann (Bühlmann Laboratories AG, Baselstrasse 55, CH – 4124 Schönenbuch, Switzerland) and IDK® Calprotectin ELISA K 6935 (serum IDK®-Cp) provided by Immunodiagnostik™ (Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany).

If successful, it was intended to use these serum assays to achieve the following objectives:

(1) Evaluate the usefulness of serum S100A12, and serum BMN®-Cp and serum IDK®-Cp as alternatives to faecal S100A12 and faecal calprotectin in the exclusion of IBD in patients presenting with chronic diarrhoea.

(2) Assess if serum BMN®-Cp and serum IDK®-Cp could replace or supplement faecal calprotectin in monitoring patients with IBD by discriminating between active and inactive disease.

(3) Study the effect of the APR as provoked by elective knee and hip replacement surgery to prospectively evaluate the diagnostic performance of serum calprotectin determined with commercially available immunoassay kits from two different manufacturers: Bühlmann Laboratories AG, Schönenbuch, Switzerland and Immunodiagnostik™ AG, Bensheim, Germany, and compare their results with CRP – a sensitive, reliable and commonly used diagnostic biomarker for APR. This will widen the scope of the study, and further explore a possible role for serum calprotectin in a different inflammatory phenomenon other than IBD.

CHAPTER 3

DEVELOPMENT AND OPTIMISATION OF ENZYME–LINKED IMMUNOSORBENT ASSAY

3.1 Introduction

3.1.1 What is enzyme–linked immunosorbent assay?

Enzyme–linked immunosorbent assay (ELISA) is a plate–based analytical technique designed for the detection and quantitation of a specific antigen or antibody in a biological sample.

An unknown amount of an antibody/antigen is immobilised onto a solid surface and then a specific antibody is applied (complexed) over the surface so that it can bind to the analyte of interest. The antibody is linked to an enzyme, and in the final step the enzyme converts an added substrate to a detectable signal, most commonly a colour change in a chemical substrate (Hornbeck, 2015; Gan and Patel, 2013). The most critical element of the detection strategy is a highly specific antigen–antibody interaction.

The quantitation of a specific marker using an enzyme–linked immunosorbent assay–based technique has advantages when compared to more a qualitative or semi–quantitative techniques such as western blotting (Meftahi et al., 2021; Pillai–Kastoori et al., 2020), and other related techniques that include dot blot analysis (Cheng et al., 2019; Bunsanong et al., 2018), quantitative dot blot (Zhang et al., 2019; Qi et al., 2018; Tian et al., 2017), immunocytochemistry (Kanber et al., 2021; Jain et al., 2019; Tan et al., 2020; Dupré and Courtade–

Saidi, 2012) and immunohistochemistry (Sukswai and Khoury, 2019; Torlakovic et al., 2015; De Smet et al., 2021; Taylor and Levenson, 2006) .

ELISA is highly sensitive, quicker, reproducible, flexible and has high throughput with the ability and capacity to analyse a mixture of diverse sample types of different origin (Konstantinou, 2017; Gan and Patel, 2013).

3.2 The principles of enzyme–linked immunosorbent assay

3.2.1 ELISA Components

The basic components of ELISA kit are a flat–bottomed, 96–well polystyrene or polyvinyl chloride (PVC) microtiter plate (figure 3.1), calibrators, controls, diluent (sample or incubation buffer), wash buffer, conjugate, substrate and stop solution.

The microtiter plate is the solid support (immunosorbent) coated with antigens and antibodies and can be stored at low temperature ($4^{o}C$) for up to six months. The plate is a special adsorbent plate (e.g., NUNC Immuno plates) to ensure the antibody or antigen sticks to the surface. Polystyrene or PVC are strong in adsorbing and capturing antibodies, which retain their immunological characteristics after being immobilised on to it (Lai et al., 2004).

The calibrators, controls, diluent, wash buffer, conjugate, substrate and stop solutions are supplied by kit manufacturer and are ready for use, although some items may need to be reconstituted with deionised water, physiological saline or solutions provided by kit manufacturer prior to use. The calibrators and controls are supplied in lyophilised forms and in different concentrations.

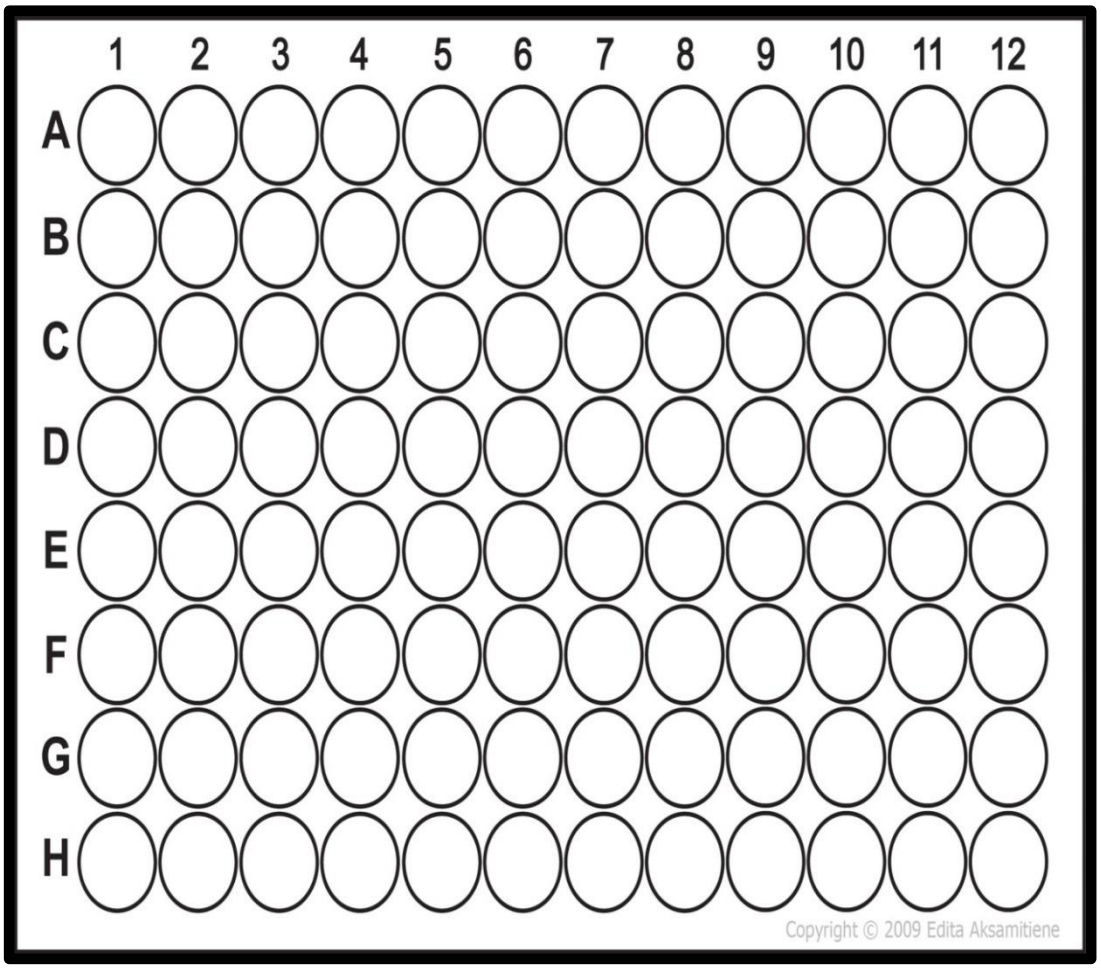

Figure 3.1 – A 96–well microtitre plate being used for ELISA template. The first two wells (A1 + B1) of the plate were allocated as reagent blanks (incubation or sample buffer alone). Note: the lowest standard (0 ng/mL) in the immunodiagnostik™ kit (incubation buffer for Bühlmann kit) served as the reagent blank. Subsequent wells were used for the kit standards, each assayed in duplicate. Control materials supplied by Bühlmann and Immunodiagnostik™ were run in duplicate in two positions on the plate respectively: at the beginning of the plate in the wells immediately after the standards and at the end after the patient samples. This arrangement is crucial because it helps to check for assay drift which is a known potential artefact in automated ELISA processors. The remaining wells between the two sets of control materials were used for study samples which were, unless otherwise stated, assayed in duplicate.

3.2.2 ELISA Controls

The most basic control material is the blank sample control. Lyophilised controls are needed to provide a comparison to physiological conditions and

a control mechanism for quality assurance so that the assay continues to provide accurate results. Control materials whose analyte concentrations have been validated by another analytical method are used. These are set up in the assay protocol as positive and negative controls (Rissin et al., 2010).

Control materials are spiked samples containing known amount of the standard analyte in a matrix (usually plasma or serum) used for the test samples on ELISA. This helps track assay performance over time. Spiked controls may monitor assay performance by calculating percent recovery from ELISA readout (Kaneko et al., 2019). If recombinant proteins are employed, their equivalent functionality to the endogenous wild type versions needs to be confirmed. A spiked sample, where a known amount of analyte is added to samples, is an alternative way to obtain calibration points in the ELISA.

3.2.3 ELISA Formats

ELISA can be performed with modifications into different formats such as direct, indirect, sandwich and competitive methods (figure 3.2) to the basic procedure (Balsam et al., 2013). The first step is to immobilise the antigen of interest in a sample to the walls of the wells of a microtiter plate. This is accomplished by either direct adsorption to the plate's surface or indirectly by using a capture antibody that has been attached to the plate. In the highly sensitive, highly specific, and robust immunometric or sandwich ELISA format (figure 3.3), the capture antigen is specific to the target antigen and the analyte to be measured is bound between two primary antibodies – the capture antibody and the detection antibody.

Addition of a detection antibody, after immobilisation, to the adsorbed antigen results in the formation of an antigen–antibody complex. The detection antibody (a labelled primary antibody) is either directly conjugated to an enzyme such as horseradish peroxidase (HRP), or indirectly provides a binding site for a labelled secondary antibody (Konstantinou, 2017; Hornbeck, 2015; Gan and Patel, 2013).

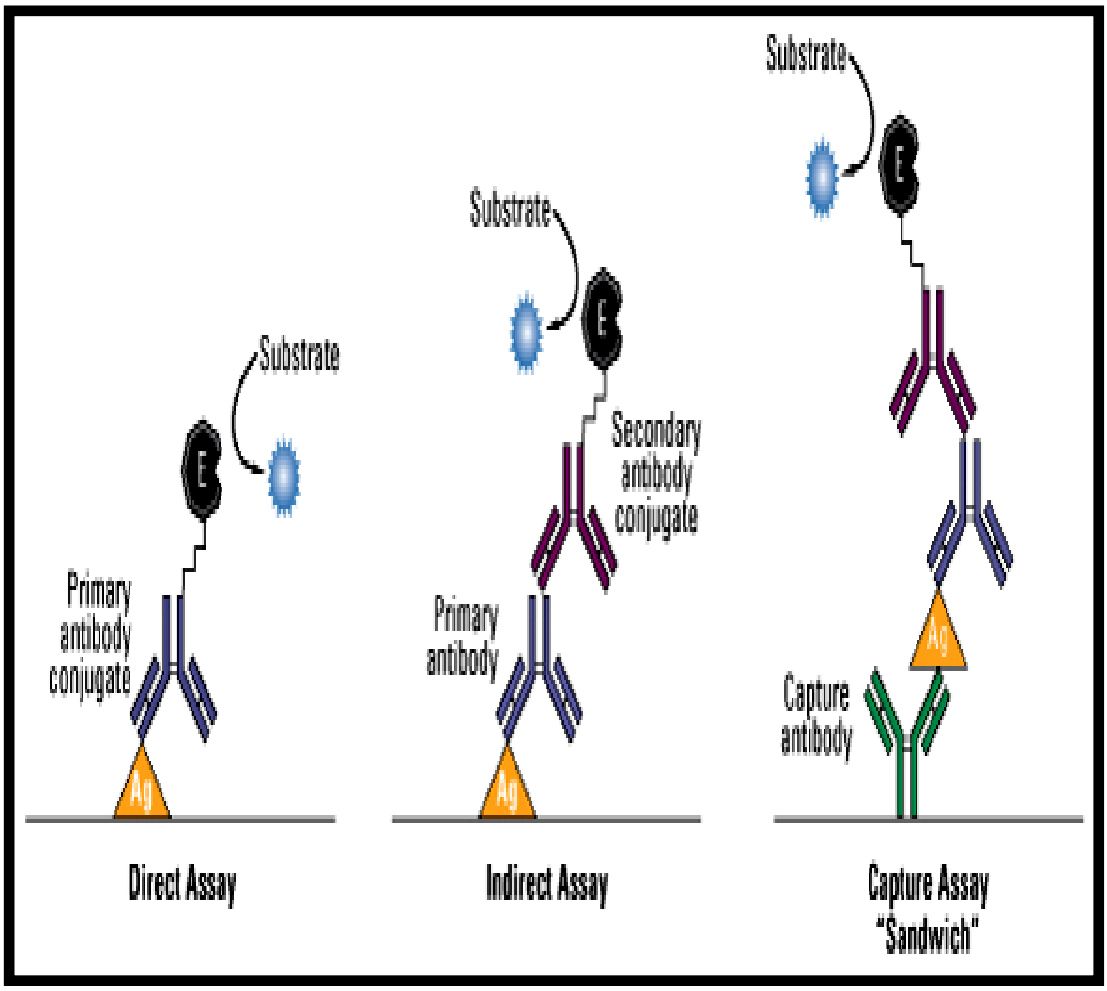

Figure 3.2 – Major formats of ELISA. Diagram to illustrate common ELISA formats: direct, indirect and sandwich assays. In the assay, the antigen of interest is immobilized by direct adsorption to the assay plate or by first attaching a capture antibody to the plate surface (capture assay or sandwich detection). Detection of the antigen can then be performed using an enzyme–conjugated primary antibody (direct detection) or a matched set of unlabelled primary and conjugated secondary antibodies (indirect detection). Modified figure taken from: https://www.thermofisher.com/uk/en/home/lif e–science.

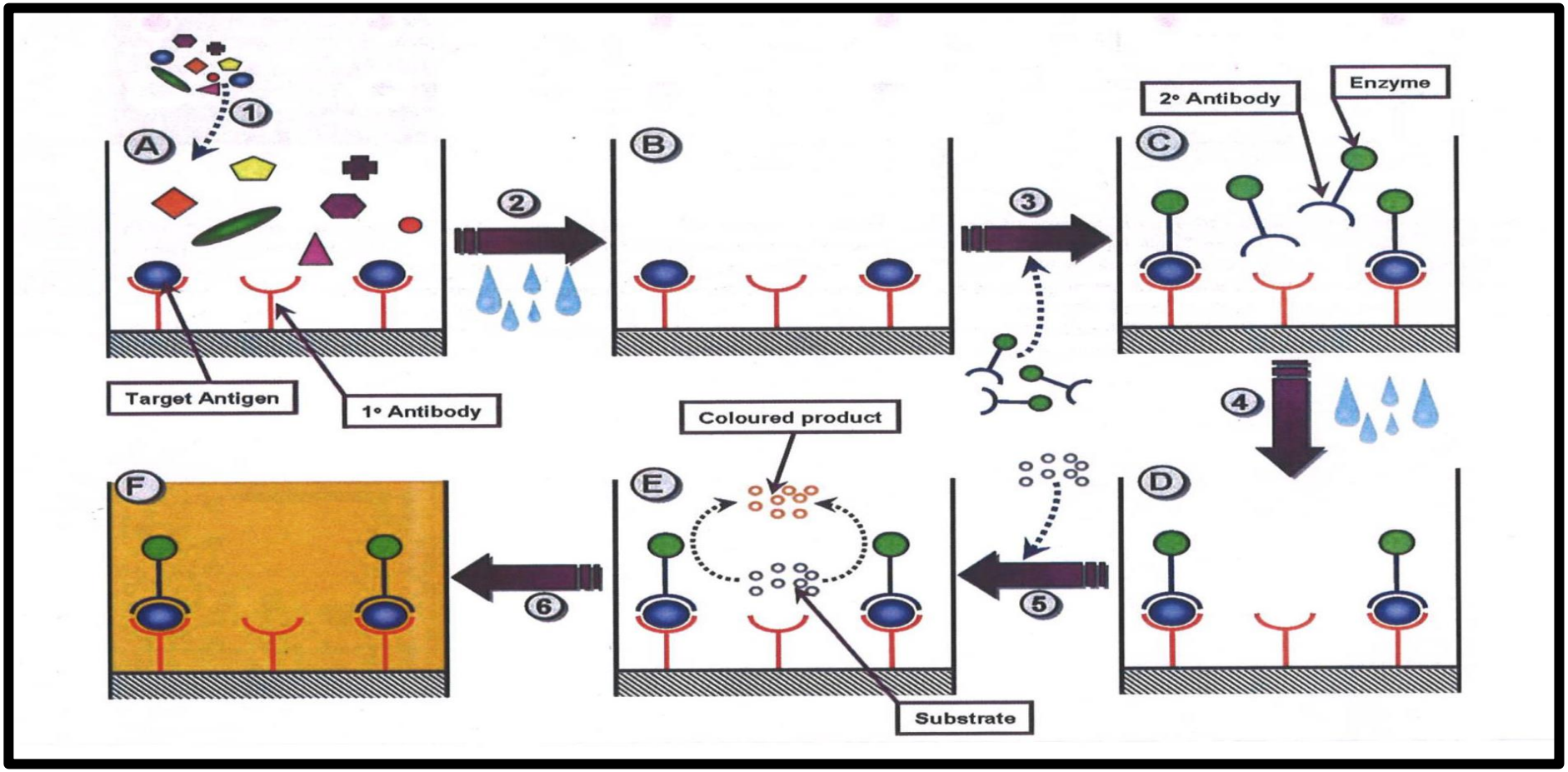

Figure 3.3 – A basic outline of a sandwich ELISA protocol. The sample is added (Step 1) containing a mixture of different antigens. During the incubation of the sample in the well (Panel A), the primary (1°) capture antibody (Ab), which is coated on to the well surface, binds the target antigen (Ag). Unbound antigens are removed by washing (Step 2) to leave the 1° Ag–Ab complex (Panel B). The secondary (2°) Ab–Enzyme conjugate is subsequently added (Step 3) and incubated with the previously formed 1° Ag–Ab complex. (Panel C); the secondary Ab recognises a different epitope on the antigen resulting in the formation of a 1° Ag–Ab–2° Ab–Enzyme complex. A second wash step (Step 4) removes any unbound 2° Ab to leave the 1° Ag–Ab–2° Ab–Enzyme complex bound to the solid support (Panel D). A substrate is then added (Step 5) which is converted to a coloured product by the enzyme conjugated to the 2° Ab (Panel E). After a defined incubation period an acidic stop solution is added (Step 6) to prevent any further colour development. Finally, the absorbance is measured using a spectrophotometric plate reader. Modified from: Bais, R (2006). Princples of Clinical Enzymology.

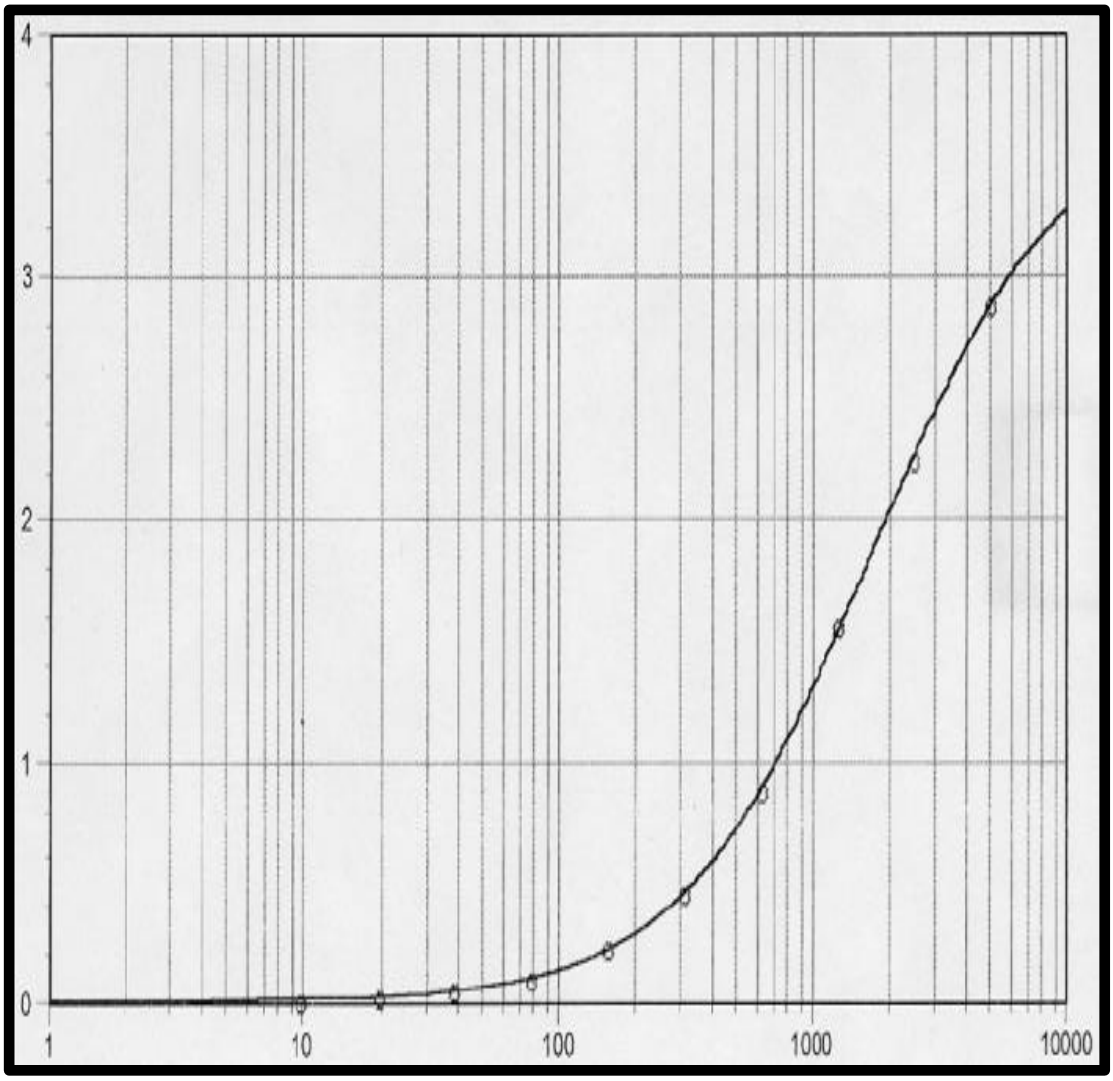

Figure 3.4 – A representative calibration curve for the estimation of calprotectin and S100A12 by the two–site sandwich ELISA. This method utilises two selected monoclonal antibodies that bind to human calprotectin and S100A12 respectively. The standard curve was calculated using the 4–parameter curve fit: $y = (A - D)/(1 + [x/C]^B) + D$; where A = 0.002, B = 1.055, C = 18.669 and D = 3.531. The y axis displays the absorbance at a dual wavelength mode of 450 nm(s) and 620 nm(s). R–Squared (r^2) = 1.000

3.2.4 ELISA Calibration curve

Data obtained from ELISA technique is characteristically graphed with absorbance or optical density (OD) versus log concentration to generate a sigmoidal curve (figure 3.4). Known concentrations of the antigen are used to generate a calibration curve and then these data are used to measure the

concentration of unknown samples by comparison to the linear portion of the calibration curve. This can be done directly on the graph or with curve fitting software that is typically found on ELISA plate readers (Hornbeck, 2015; Lai et al., 2004)

3.2.5 Calibration curve representation

For quantitative results, the mean of duplicate readings of the calibrators is obtained from the reading of the blank control sample. The calibration curve is then plotted, as a line of best fit or a point–to–point curve to enable concentration of the samples to be determined. Any dilutions made are adjusted for at this stage (Gan and Patel, 2013). The data may be plotted using semi–log, log/log, log/logit, and its derivatives: 4 or 5 parameter logistic representations. Using a software–based or automated resolution enables more complicated graphing methods; and linear regression within software package adds more checks such as the square of the regression (r^2) value to ascertain overall quality of fit (Hornbeck, 2015; Lai et al., 2004)

For the section of curve where the relationship between concentration and readout is linear, r^2 is greater than 0.99, and represents a very good fit. Using further calibration concentrations within this range will further enhance the accuracy (Hornbeck, 2015). Linear plots tend to compress the data points at the lower concentrations of the calibration curve, thereby making this portion of the curve the most accurate range or area most likely to achieve the desired r^2 value.

Attempt to counteract the effects of compressed data points will necessitate the use of semi–log chart. This involves plotting the log concentrations on *x*–axis against the absorbance on *y*–axis, which gives an S–shaped (sigmoid)

calibration curve that permits the distribution of more data points into the more user–friendly sigmoidal pattern (Konstantinou, 2017; Hornbeck, 2015; Gan and Patel, 2013; Lai et al., 2004).

A plot of the log concentrations against log absorbance (log/log) attempts to incorporate more data points to present a linear curve. The low to medium calibration concentration range is normally linear in this representation; it is only at the higher end of the range that linearity tends to slope off (Konstantinou, 2017).

The log/logit and its derivatives: 4 or 5 parameter logistic representations are more complicated as, they require more intricate calculations and estimations of the minimum, maximum and slope values and EC50 or the half maximal effective concentration – which refers to the concentration of an antigen or antibody that induces a response halfway (i.e., where 50% of the maximal effect of the concentration of the antigen or antibody is observed) between the baseline and maximum after a specified exposure time (Chen et al., 2013) (figure 3.5). The 5–parameter representation requires additional asymmetry value.

Whilst these calibration curve representations give improved performance; it is important to use the log/log plot with verification on the analyte recovery from spiked samples or recovery percentage as a starting point (Lai et al., 2004; Gan and Patel, 2013). Alternatively, 'back–fitting' the calibration curve absorbance values could be attempted. This involves a back calculation of the calibration values and verifying that they fall within 20 per cent of the nominal absorbance value.

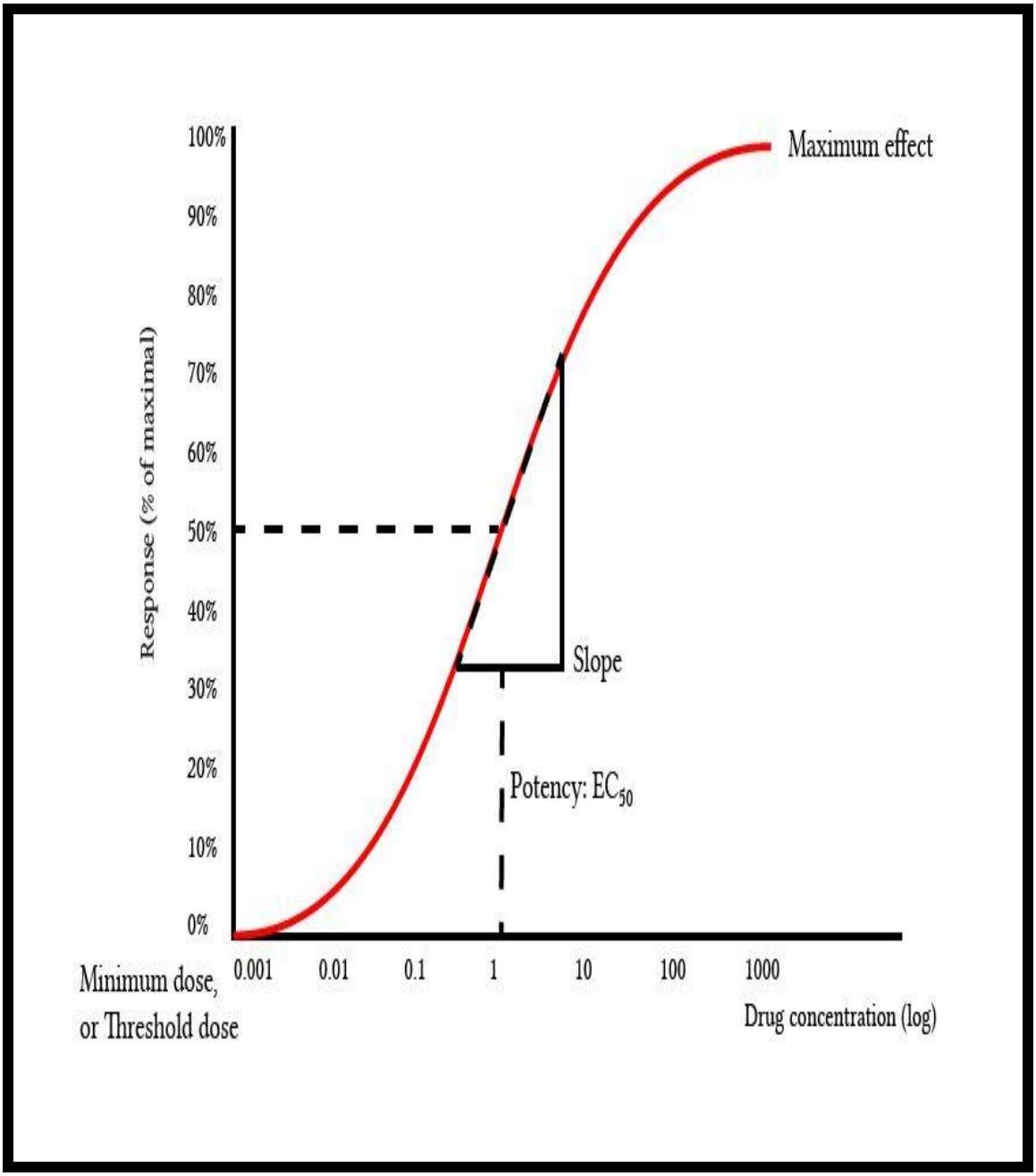

Figure 3.5 – A representative dose–response curve of the relationship between the dose or concentration of a drug (i.e., antigen or antibody) and the effect or response it elicits. The inflection point at which the increase in response with increasing ligand concentration begins to slow is the EC50 (i.e., the potency), which can be mathematically determined by derivation of the best–fit line. While relying on a graph for estimation is more convenient, this typical method yields less accurate results and less precise. The quotient of response divided by concentration represents the slope.

3.2.6 ELISA Sensitivity

ELISA is regarded as a very sensitive immunoassay method available for routine use in clinical laboratories. This is because the typical detection range for most ELISAs are 0.1 to 1.0 Fentomole (fmol) ((Rissin et al., 2010) or 0.01 to 0.1 nanogram (ng) (Dobrovolskaia et al., 2006). The sensitivity depends upon the particular characteristics of the antigen–antibody reaction. Assay results can be improved using some substrates that generate enhanced chemiluminescent or fluorescent signals (Kaneko et al., 2019). Indirect ELISA format tends to be more sensitive because it produces higher signal levels. However, higher signal levels could cause higher background signal that could reduce the net specific signal levels.

3.2.7 Aim of this chapter

The aim of the study presented in this chapter was to develop and/or optimise a two–site sandwich ELISA method to measure serum S100A12 and serum calprotectin that is amenable to a high throughput clinical laboratory. ELISA method will be developed using dual (i.e., capture and detection) monoclonal antibody profile with a direct enzyme–labelled detection reagent (i.e., Anti–MRP8/14 Antibody or Anti–S100A12 Antibody conjugated to horseradish peroxidase (HRP)).

3.3 Development of a two–site sandwich ELISA method

The diagrammatic representation of the two–site sandwich ELISA method for Bühlmann AG and Immunodiagnostik™ calprotectin and S100A12 assays (Bühlmann, 2012; Immunodiagnostik™, 2017) are shown in figure 3.6. These include antibody matrix and antibody labelling, preparation of buffers

(incubation or sample and wash), conjugate (enzyme label), substrate, stop solution and reconstitution of lyophilised materials (controls and calibrators) and assay optimisation. This is followed by the process of method evaluation or validation to assess the performance characteristics of the method.

3.3.1 Antibody matrix

Affinity, avidity and specificity of antibodies for, and their interaction with antigens occur in two–site sandwich ELISA in diverse ways to influence assay (ELISA) optimisation, e.g., antibody concentration and required buffers.

Specificity is the binding of an antibody exclusively to a unique epitope from a single antigen in a single specie or if it binds to similar epitopes that are present on numerous molecules from a few diverse species. Cross–reactivity occurs when specificity of the antibody is not guaranteed or absent.

Affinity is the binding strength of an antibody to a single epitope. It is reversible, and therefore determines the amount of antigen bound by antibody, and the rapidity and duration of the binding process. Affinity antibodies, therefore, are the best choice for two–site sandwich ELISA assay development because they quickly produce the maximum number of stable immune complexes to provide optimum detection.

Avidity is total stability of the antibody–antigen reaction. Although based on the binding capacity of the antibody to a single epitope (affinity), avidity is also dependent on the valency of the antibody, or the numerical strength of the antigen binding sites. Avidity fluctuates with isotype, whether the antibody is unbroken or splintered, and the involvement of spatial configuration of the entire immune complex.

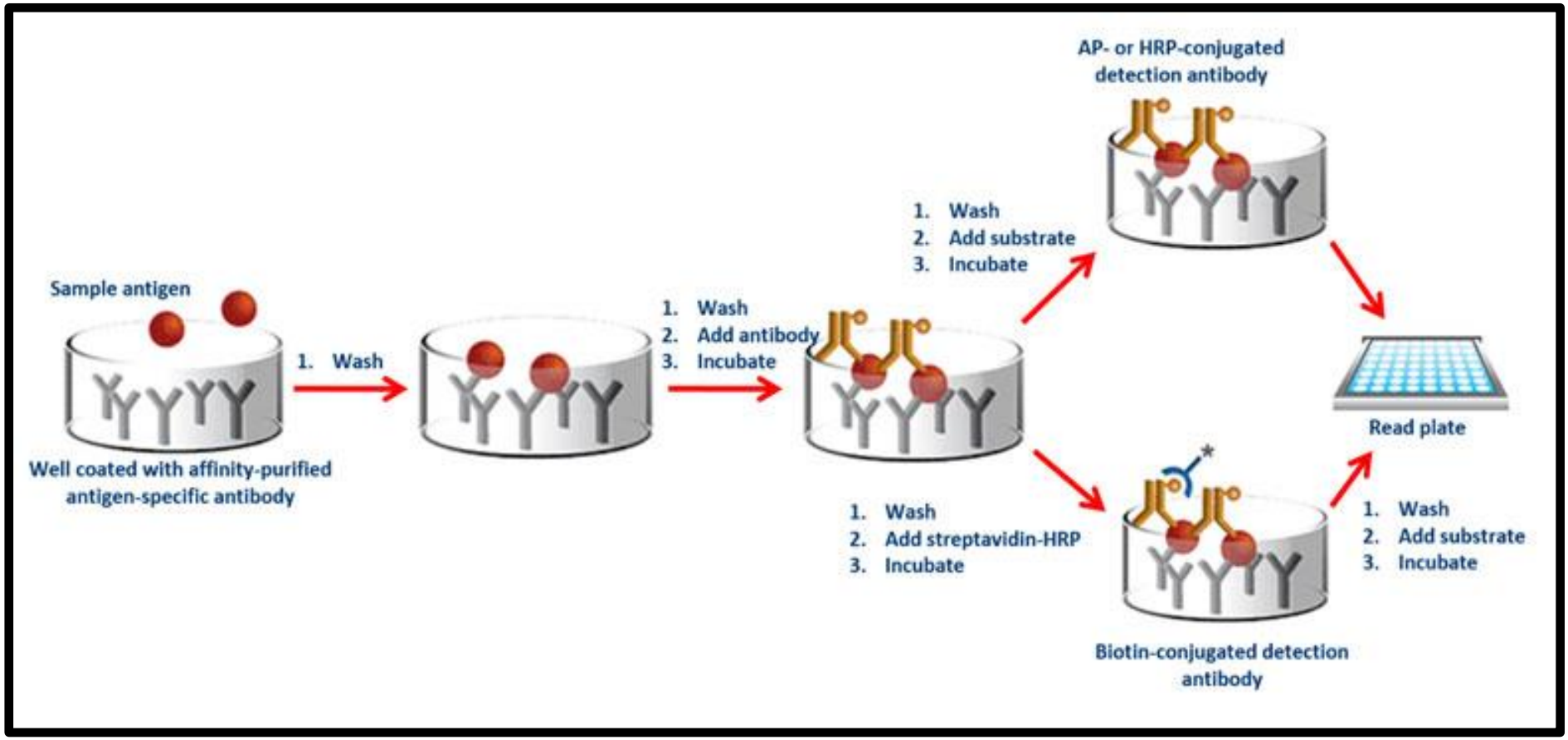

Figure 3.6 – Illustration of a two–site sandwich ELISA format. The method is stepwise in the order shown from left to right. The first step is to coat the ELISA micro plate with capture antibody, any excess, unbound antibody is then washed from the plate. The capture antibody is an antibody raised against the antigen of interest. The second step is to add the sample (e.g., serum, urine or cell supernatant). Any antigen found in the sample will bind to the capture antigen already coating the plate. Samples are usually added in duplicate (to allow for statistical analysis), and in varying concentrations to guarantee it falls within the levels of detection of the assay. Again, any excess sample is washed from the plate. The third step is to add the detection antibody labelled with an enzyme, usually horseradish peroxidase (HRP) or alkaline phosphatase. The detection antibody binds to a target antigen already immobilised on the plate. Finally, a substrate is added to the plate. Two–site sandwich ELISA assays are usually chromogenic using a reaction that converts the substrate (e.g., tetramethylbenzidine [TMB] or 2,2'–azino–bis[3–ethylbenzothiazoline–6–sulfonic acid] (ABTS)) into a coloured product which can be measured using a plate reader. The determination of antigen concentration in a sample requires generation of a calibration curve (figure 3.3) using antigens of a known concentration. The concentration of antigen in a sample can then be calculated using the absorbance. Modified figure taken from: https://www.enzolifesciences.com/ science–center

3.3.2 Monoclonal antibodies

Monoclonal antibodies are used in a two–site sandwich ELISA assays for the measurement of calprotectin (Bühlmann, 2012; Immunodiagnostik™, 2017) and S100A12. Other ELISA methods could use polyclonal antibodies or a combination of polyclonal and monoclonal antibodies (Immunodiagnostik™, 2015). Either type of antibody offers unique advantages for the development of the particular ELISA assay that it is used for; and this is based on the desired analyte to be measured.

Monoclonal antibodies used in the two–site sandwich ELISA assays are homogenous and highly specific for single epitope or small region of a protein. They, therefore, are most unlikely to react with closely related proteins or trigger non–specific signals during the assay process.

The Bühlmann MRP8/MRP14 Calprotectin S100A8/S100A9 ELISA assay uses a monoclonal capture antibody (mAb) that is highly specific to the MRP8/MRP14 heterodimeric and polymeric complexes for measurement of calprotectin. The Immunodiagnostik™ Calprotectin ELISA K 6935 kit method utilises a high–affinity monoclonal anti–human calprotectin for measurement of calprotectin.

A monoclonal antibody specific for S100A12 is used in the Immunodiagnostik™ S100A12 ELISA K 6938 kit method. These antibodies may be commonly used as sets of matched pairs for capture or detection of a single antigen for all antibody–linked steps in the two–site sandwich ELISA assays (Zwadlo et al, 1986; Goebeler et al, 1994; Hessian and Fisher, 2001). In combination with polyclonal antibody, they are capable of improving reaction

signal to provide a better chance of capturing antigen from a composite solution.

3.3.3 Antibody labelling

Fully standardized antibodies for antigen capture and detection contained in commercially prepared ELISA kits, are advantageous in that they guarantee quality assurance in terms of consistency in assay performance and convenience for use. Where the antibody is purified in 10 – 50 mM amine–free buffer at a pH range of 6.5 – 8.5, it will be expedient and rapid to label it with HRP or with alkaline phosphatase.

3.3.4 Buffer, conjugate, substrate and stop solutions

Several different buffers are used in a two–site sandwich ELISA assay. The microtiter plate manufactured by both Bühlmann (Bühlmann Laboratories AG, Baselstrasse 55, CH – 4124 Schönenbuch, Switzerland) and Immunodiagnostik^{TM} (Immunodiagnostik^{TM} AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) are pre–coated with their respective monoclonal anti–human antibodies (mAb) and polyclonal anti–human S100A12 antibodies coating buffers. A typical coating buffer is a mixture of 1.5 g Sodium Carbonate and 2.93 g Sodium Bicarbonate dissolved in 1Litre of distilled water at a pH of 9.6. It guarantees stability of antigen or antibody used for coating the plate, maximises adsorption to the plate and optimises the reaction with the detecting antibody.

The wash buffer and sample or incubation buffer to which have been added the necessary preservatives: phosphate buffered saline (PBS) containing 0.05% volume/volume Tween–20 (wash buffer) and PBS containing 1%

weight/volume bovine serum albumin (BSA) solution (incubation or sample buffer) must be reconstituted according to the manufacturer's instruction prior to use.

Conjugate solution (Enzyme label, for Bühlmann MRP8/MRP14 calprotectin S100A8/S100A9 ELISA kit) contains either anti–MRP8/MRP14 heterodimeric–polymeric complexes or polyclonal anti–human S100A12 antibody (for Immunodiagnostik^{TM} S100A12 ELISA K 6938 kit) which has been labelled with streptavidin–HRP (Immunodiagnostik^{TM}, 2015). The conjugate solution detects the MRP8/MRP14 or S100A12 molecules bound to the monoclonal (or polyclonal) antibody coated onto the microtiter plate to form the capture antibody–human calprotectin (or S100A12)–peroxidase conjugate.

Substrate solution contains a high–performance 3,3',5,5'–tetramethylbenzidine (TMB) in citrate buffer with hydrogen peroxide for HRP–enzyme detection system. TMB with hydrogen peroxide has been optimised for increased sensitivity, minimal background signal and quick development to produce a deep blue–coloured solution after incubation at the appropriate temperature. Alternatively, the substrate solution could contain *p*–nitrophenyl phosphate (*p*NPP) in place of TMB for alkaline phosphatase enzyme–based detection system (Gan and Patel, 2013).

Stop solution is a corrosive agent that contains 0.25 M Sulphuric acid, which is added to stop the reaction and to produce a yellow–coloured end–product. The intensity of the yellow colour is directly proportional to the concentration of calprotectin (or S100A12) in the sample. A standard (dose–response) curve (figure 3.4) is constructed by plotting absorbance (at dual wavelengths: 450

nm or primary test filter and 620 nm or primary reference filter) values against concentrations of calprotectin (or S100A12) calibrators. Calprotectin or S100A12 concentrations of unknown (patient) samples are determined from this standard curve.

3.3.5 Calibrators and controls

Calibrators and controls for MRP8/MRP14 calprotectin S100A8/S100A9 and S100A12 were supplied as lyophilised materials from Bühlmann (Bühlmann Laboratories AG, Baselstrasse 55, CH – 4124 Schönenbuch, Switzerland) and Immunodiagnostik^{TM} (Immunodiagnostik^{TM} AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) (table 3.1). These were reconstituted with deionised water (diluent) as recommended by the kit manufacturers. Reconstituted calibrators and controls with their resulting concentrations of MRP8/MRP14 calprotectin S100A8/S100A9 and S100A12 are shown in table 3.1.

3.4 Optimisation of a two–site sandwich ELISA components

The optimisation of the ELISA method for the development of a two–site sandwich ELISA assay includes processes to maintain consistency between wells, suitability and concentration of antibodies, matrix effects, buffer (coating, blocking, washing and incubation/sample), preparation/standardisation and the sandwich ELISA analysis itself.

An important aspect of an ELISA assay is consistency and standardisation of the conditions since these will affect the accuracy and precision of results (Mendoza et al., 1999). During the preliminary stages of assay development, it is very important to investigate a range of parameters by completing a

ELISA Kit	Quality Control (ng/mL)						Standard (Calibrator)						Practical Working Range [ng/mL] & (Dilution Factor)
	Level (SD)	Target	-2SD	-1SD	1SD	2SD	#	Absorbance (nm) 1	2	Mean	CV (%)	Conc. (ng/mL)	
Bühlmann MRP8/MRP14 Calprotectin S100A8/S100A9 (BMN®-Cp)	**Low (200)**	1400	1000	1200	1600	1800	**1**	0.073	0.078	0.075	4.5	**4**	**12 – 240 (Dilution Factor) = 1 in 100 [1,200 – 24,000]**
							2	0.198	0.200	0.199	0.8	**12**	
	High (300)	5600	5000	5300	5900	6200	**3**	0.625	0.646	0.636	2.3	**40**	
							4	1.549	1.608	1.579	2.6	**120**	
							5	2.406	2.409	2.407	0.1	**240**	
Immunodiagnostik™ K 6935 Calprotectin (IDK®-Cp)	**Low (4.0)**	15.4	7.4	11.4	19.4	23.4	**1**	0.047	0.049	0.048	2.9	**0.0**	**3.9 – 250.0 (Dilution Factor) = 1 in 100 [400 – 25,000]**
							2	0.174	0.169	0.172	2.1	**3.9**	
	High (9)	49	31	40	58	67	**3**	0.472	0.485	0.479	1.9	**15.6**	
							4	1.188	1.200	1.194	0.7	**62.5**	
							5	2.037	2.085	2.061	1.6	**250.0**	
Immunodiagnostik™ K 6938 S100A12 (IDK®-A12)	**Low (0.17)**	0.54	0.20	0.37	0.71	0.88	**1**	0.007	0.006	0.007	10.9	**0.00**	**0.66 – 54.00 (Dilution Factor) = 1 in 10 [1.0 – 540.0]**
							2	0.020	0.022	0.021	6.7	**0.66**	
							3	0.063	0.063	0.063	0.0	**2.00**	
	High (1.5)	6.5	3.5	5.0	8.0	9.5	**4**	0.191	0.199	0.195	2.9	**6.00**	
							5	0.675	0.663	0.669	1.3	**18.00**	
							6	1.832	1.841	1.837	0.4	**54.00**	

Table 3.1 – Quality Control Protocol and Datasheet for Bühlmann MRP8/MRP14 Calprotectin S100A8/S100A9, Immunodiagnostik™ K6935 Calprotectin and Immunodiagnostik™ K6938 S100A12. Quality Control Materials (B–CAL–CONSET for BMN®-Cp; K 6935 CTRL1/CTRL2 for IDK®-Cp and K 6938 CTRL1/CTRL2 for IDK®-A12) and Calibrators (Standards) (B–CAL–CASET for BMN®-Cp; K 6935 STD for IDK®-Cp and K 6938 STD for IDK®-A12) Data taken from package inserts provided by kit Manufacturers (Bühlmann Laboratories AG and Immunodiagnostik™ AG) for use in assay validation of their respective kits. All concentrations are reported in nanogram per millilitre (ng/mL) to allow for easy comparison of the respective assay results.

checkerboard dilution series to examine various assay conditions in a systematic manner. It is also crucial to ensure that buffers, operational temperature, and humidity are kept constant between and within experiments in order to produce standardised and reliable results.

3.4.1 Consistency between wells

A typical two–site sandwich ELISA assay incorporates multi–channel pipettes, multi–well plates and plate washers that provide consistent and quicker results in addition to higher throughput (Kohl and Ascoli, 2017; Shah and Maghsoudlou, 2016; Engvall, 2010). It is imperative to ensure that all pipettes used in the assay process are properly calibrated at regular intervals to prevent significant variations in the results. In addition, and as part of the procedure, it is a good laboratory practice to monitor the level of liquid in the pipette tip and the wells during pipetting to ensure sample volumes in all wells are consistent. This precautionary measure is particularly important when multi–channel pipettes are used because of a tendency for the tips in the end rows not to be attached firmly to the pipette (Shah and Maghsoudlou, 2016; Aydin, 2015).

3.4.2 Suitability and concentration of antibodies

The preliminary task is to determine the operational concentration of the monoclonal antibodies to be used in the sandwich ELISA. This is based on the understanding that a matched pair of antibodies should be available to the analyte in order to establish the two–site sandwich ELISA (Joos et al., 2000).

Dilutions of capture antibodies are prepared in coating buffers at different strengths of 0.5, 1, 2 and 5 µg/mL. High and low concentrations of the analyte are placed in their respective wells at the stage that sample addition is required.

Blank (incubation or sample buffer) is included to complete the protocol and prepare the assay for the detection stage. Addition of high and low concentration of the analyte ensures that the dynamic range of the assay is established. The low concentration suggests the sensitivity and specificity of the assay while the blank indicates non–specific binding characteristic of the assay. These are then distributed against dilutions of detection antibody in buffer at strengths of 1:200, 1:1000, 1:5000 and 1:25000 as shown in the protocol of 96–well ELISA microtitre plate (Table 3.2).

The set of antibody concentrations with maximum signal–to–noise ration or largest difference between low and high analyte concentration should be further optimised. In the event of the blank sample showing disproportionate absorbance readings of above 0.2 nm, it should be checked for optimisation by reviewing the ELISA plate type and washing/blocking buffers used. If the background absorbance readings are suitable, but the assay sensitivity is not high enough, then further review of the matrix considerations, buffers and incubation periods becomes necessary. If this situation cannot be improved, then different antibody combinations must be produced.

Detection Antibody	**Capture Antibody**											
	5 µg/mL			**2 µg/mL**			**1 µg/mL**			**0.5 µg/mL**		
1:200	H	L	B	H	L	B	H	L	B	H	L	B
	H	L	B	H	L	B	H	L	B	H	L	B
1:1000	H	L	B	H	L	B	H	L	B	H	L	B
	H	L	B	H	L	B	H	L	B	H	L	B
1:5000	H	L	B	H	L	B	H	L	B	H	L	B
	H	L	B	H	L	B	H	L	B	H	L	B
1:25000	H	L	B	H	L	B	H	L	B	H	L	B
	H	L	B	H	L	B	H	L	B	H	L	B

Table 3.2 – An example of a work sheet of a typical antibody concentration optimisation microtitre plate layout used in two–site sandwich ELISA. H = high concentration of analyte; L = low concentration of analyte and B = blank, usually an incubation buffer or a sample buffer.

3.4.3 Matrix effects

Matrix effects may occur in assay procedures due to biological constituents in plasma and serum (Kaneko et al., 2019). These phenomena may cause interferences that are either cross–reactive or non–reactive depending on the degree of interactions of the substances that constitute the sample matrix or metabolic products that develop during the sample handling processes (Kaneko et al., 2019).

It is possible that matrix effects could be reduced through sample dilution, and this could be confirmed by spiking recovery experiments. This procedure is often practicable in a new application that shares same matrix (i.e., serum) and common broad characteristics, such that the requirements of instrumentation principle, reagents preparation and storage, sample collection and preparation, assay quantification including testing principle and procedure, quality assurance and result reporting format are the same with the subsisting method (i.e., faecal) (Otero–Estévez et al., 2015; Farkas et al., 2015). This approach was employed in this study for the in–vitro measurement of S100A12 in serum from an existing method for the in–vitro determination of S100A12 in stool. The use, however, of specialised buffers for washing, coating, blocking and sample dilution would improve matrix effects and guarantee consistency in assay performance.

3.4.4 Specialist buffers

Most specialist buffers are multifunctional and have concurrent coating, stabilization and blocking properties. By preserving the biological activities of adsorbed proteins during assay processes, specialist buffers help prevent the coating material from being leached, degraded, and denatured. Specialist buffers by their multifunctional nature ensures that assay time is shortened, improve ELISA results by reducing background contrast and generating sharper calibration curves. This helps maximise efficiency of the entire process by extending the lifespan of the coated plates.

3.4.5 Coating buffers

Specific buffers developed for coating microtiter plate wells are optimised for ELISA assays to stabilise adsorbed proteins or other biomolecules, preserve the antigenic regions, and ensure maximum binding reactivity to improve precise signal.

3.4.6 Blocking buffers

Blocking buffers contain preservatives to ensure long–term stability environments of constituent proteins (Haapakoski et al., 2013). Other options are commercially prepared high performance, optimised formulations that contain small protein molecules that block free binding sites and less accessible surfaces of the multiwell plate. Blocking buffers that are non–reactive or are less amenable to cross–reactivity is used in ELISA assays that involve human, porcine and bovine samples. Buffers that guarantee maximal blocking strength are formulations that are inert and contain synthetic materials that reduce non–specific interferences.

3.4.7 Washing buffers

Washing buffers are specially prepared to guarantee greatest consistency in adding and removing the liquid used during the washing stages of the assay. They contain formulations of salts and detergents in an environment of optimised pH stabilisers that enable reduced background noise and improve precise signal.

3.4.8 Sample buffers

Specially prepared sample or incubation buffers are important assay diluents in a two–site sandwich ELISA. They serve to dilute different sample types (plasma, serum, or cell culture supernatant) and detection antibodies, thereby reducing cross–reactivity, matrix effects and non–specific binding (Gan and Patel, 2013). Typical constituents include goat serum proteins to reduce the difference between the sample matrix and the diluent used to produce calibration curve; proprietary chelating agents that block possible interferences from complement and coagulation factors e.g., thrombin in plasma and serum.

3.5 Discussion

The fundamental characteristic of ELISA ensures that the separation of specific and non–specific exchanges take place through serial binding onto a solid surface, usually a polystyrene multiwell plate to enable the quantitative measurement of analytes. This function is the basic difference between an ELISA method and other antibody–based immunoassay techniques such as enzyme immunoassay (EIA) and radioimmunoassay (RIA) (Kricka, 2008).

The ELISA method results in a final–coloured complex of biomolecular end–product which, is equivalent to the concentration of the analyte contained in the primary (original) sample. ELISAs are rapid and easy to perform. They are purposefully designed to quickly cope with large number of samples arranged in parallel; and this makes them a very popular immunoassay technique necessary for diverse diagnostic and research objectives (Leng et al., 2008).

First developed over four decades ago in the 1970s as substitute for RIA, ELISA techniques still retain their wide application in both original and expanded formats with changes that permit the inclusion of multiple analytes per well, extremely sensitive readout device and direct cell–based output (Lequin, 2005).

As a general principle, the ELISA process begins with the coating step that enables the first layer of either an antigen or an antibody to be adsorbed onto a well in the ELISA microtitre plate in coating buffer. This is quickly followed by the blocking and detection steps, whereby a buffer containing unconnected protein is applied to block free sites in the well while an enzyme–conjugated–detection–antibody binds the antigen. Between the coating, blocking and detection steps, several wash cycles are repeated for each ELISA step to remove buffer, unbound materials (i.e., antigens or antibodies), and wash plate to ensure that the assay retains its surface binding for separation (Kricka, 2008).

The efficiency of these steps is necessary to ensure that excess liquid is removed so as to prevent the unintended dilution of solutions that would be added in subsequent steps. Use of specialised microtitre plate washers in the

ELISA process guarantees the greatest consistency and best outcome during the wash cycles.

It must be emphasised that ELISA techniques may be complex, such as the use of several layers of antibodies to magnify reaction signal and optimise detection. The complexity is due to a variety of superseding steps, including the capability to determine protein concentrations in heterogeneous samples such as serum and plasma (Leng et al., 2008; Adler et al., 2009)

As reported by Crowther (2009), ELISA technology is underpinned by four major types: direct, indirect, competitive/inhibition and sandwich ELISAs. The Direct ELISA technique is a straightforward detection by an antibody directly conjugated to an enzyme such as HRP of an antigen that is immobilised in a microtitre well of an ELISA plate. It is best suited for analysing the immune response to an antigen; and is also advantageous in being the fastest ELISA method and less prone to error as it uses less reagents and requires fewer steps (Balsam et al., 2013). However, drawbacks include non–specificity of antigen immobilisation; in which all proteins in the sample including target proteins bind to the plate thereby causing higher background noise. Other drawbacks are inflexibility due to individual target proteins requiring precise conjugated primary antibody – and lack of signal amplification reducing assay sensitivity (Gan and Patel, 2013).

Indirect ELISA is best suited for measuring total amount of antibody in samples (Schmidt et al., 2012). Following the adsorption of antigen to a well in ELISA microtitre plate, the detection phase occurs in two steps: firstly, the binding of unlabelled primary antibody to specific antigen; secondly, the application of an

enzyme–conjugated secondary antibody that is directed against the unlabelled primary antibody of the first step. Indirect ELISA is advantageous in being economical as fewer labelled antibodies are required, highly sensitive because more than one labelled secondary antibody can bind the primary antibody and permits greater flexibility because dissimilar primary antibodies could be applied with a single labelled secondary antibody (Haapakoski et al., 2013). The likelihood, however, of background noise due to cross–reactivity of the secondary antibody and longer duration of the process due to additional incubation step required for the secondary antibody makes Indirect ELISA a disadvantageous method when compared to direct ELISA (Crowther, 2009).

The competitive/inhibition ELISA is best when just one antibody is available to bind the antigen of interest and when detecting small antigens that cannot be bound by two dissimilar antibodies as applicable in the sandwich ELISA technique (Schmidt et al., 2012). The practical aspect of competitive/inhibition ELISA technique involves coating the wells of the microtitre plate with a known, control antigen in coating buffer. After a wash cycle to remove excess liquid, buffers containing unknown antigen are added to block free antigenic sites exposed by the wash cycle. Following another wash cycle, an enzyme–labelled conjugate containing secondary detection antibody and an unknown antigen is added for detection via a relevant substrate such as TMB. The catalytic reaction of the two complexes generates a coloured end–product whose concentration is inversely proportional to the amount of antigen in the sample (Mariani et al., 1998).

Competitive/inhibition ELISA does not require laborious sample processing steps, implying that crude or impure samples could be used (Dobrovolskaia et

al., 2006). It is more robust, i.e., being less susceptible to effects of sample matrix and dilution than sandwich ELISAs; more consistent, i.e., shows less variability between duplicate samples and assays; has maximum flexibility, i.e., can be adapted from direct, indirect and sandwich ELISAs. However, its major weakness is the potential to combine the limitations of other ELISAs as each can be adapted as the base ELISA for the competitive/inhibition format (Crowther, 2009; Schmidt et al., 2012)

The Sandwich ELISA is designed to analyse complex biomolecules as the antigen does not require purification before measurement. Practically, each of matched antibody pair (i.e., capture and detection antibodies), is specific for a different and non–overlapping region or epitope of the antigen in order to achieve accurate result. The capture antibody binds the antigen that could be detected either in a direct or indirect ELISA format (Kragstrup et al., 2013).

First, the wells of the microtitre plate are coated with a capture antibody, and then followed with the analyte or sample. A detection antibody such as an enzyme–labelled conjugate complex is added to configure the whole process as a direct sandwich ELISA or an unlabelled, secondary enzyme–conjugated complex is used as the detection antigen for an indirect sandwich ELISA. Sandwich ELISA enjoy some advantages including high sensitivity 2 to 5–fold greater than the direct or indirect ELISA, high specificity because two antibodies are recruited; and maximum flexibility since it incorporates both direct and indirect formats (Canady et al., 2013). Conversely, it may be difficult to optimise antibody owing to the cross–reactivity occurring between capture and detection antibodies.

3.6 Conclusion

ELISA, a microtitre plate–based immunological assay technique, has wide application in the detection and quantitation of substances like antibodies, antigens, target proteins in serum, plasma, supernatants, cell lysates, glycoproteins, peptides and hormones in biological sample types.

Irrespective of the ELISA format, all measure a specific antigen; and ELISA techniques are practically demonstrated in a 96–well microtitre plate which, permits the measurement of multiple samples in one experiment.

Ranging from the quantitation of soluble receptors or cytokines in serum or cell supernatants, detection of antigen–antibody reactions in pregnancy test to the diagnosis of HIV infection and other viral diseases, basic ELISA technology is configured around the same principle of immobilising an antigen onto a solid surface, followed by the initiation of a complex with an antibody linked to an enzyme. The specificity of the antigen–antibody interaction is vital to the detection phase in the ELISA protocol; and this is achieved with incubation of the conjugated enzyme and a relevant substrate to produce a quantifiable, end–product.

A special adsorbent microtitre plate such as NUNC Immuno plate which, ensures that the antibody sticks to its surface, is a key component of commercially prepared ELISA kits, that are now available for the measurement of an assortment of antigens.

CHAPTER 4

ANALYTICAL STUDY: FITNESS FOR PURPOSE OF ANALYTICAL METHODS

4.1 Introduction

4.1.1 Method validation and its importance

Method validation may be defined as (1) 'confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled' through a qualification process' that demonstrate the ability to fulfil specified requirements' (ISO, 2005a); (2) 'confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled' (ISO, 2005b) and (3) 'verification, where the specified requirements are adequate for an intended use', with verification in this context being the provision of objective evidence that a given item fulfils specified requirements' (IVM, 2007).

A distinction must be made between method validation as a successor to method development and optimisation that was discussed in Chapter 3 under sections 3.3 and 3.4. Method validation involves an evaluation of method performance indices, such as selectivity, analytical sensitivity or detection limits, method (assay) working range including linearity of dilution (parallelism), functional sensitivity, trueness (i.e., measurement of bias and recovery), precision (i.e., measurement of repeatability, intermediate precision, and reproducibility) and robustness, and is an integral component

of quality assurance (Wood, 2005; USEPA, 2009; ISO, 2003; Bressolle et al., 1996).

Commercially manufactured immunoassay kits are examples of validated procedures that have been standardised as a complete measuring system that may be used for specific assay application. It, however, is good laboratory practice to undertake in–house validation to confirm the manufacturer's claim to analytical efficacy (Tiwari and Tiwari, 2010).

A robust method validation process is necessary to underpin assay measurement for a number of reasons (Thompson et al., 2002). First, to confirm that results obtained are correct. Second, that the costs of unnecessary and repeat testing are minimised (Thompson et al., 2002). Third, the end–user laboratory is able to justify 'fit for purpose' with verifiable method validation (Ellison and Williams, 2012). Fourth, it must be recognised that for practical purposes, method validation confers additional benefit of good reputation to a laboratory undertaking the validation (Ellison and Williams, 2012).

4.1.2 Method validation

4.1.2.1 Imprecision

The imprecision of an analytical method expresses the proximity of individual measures of an assay when the process is applied repeatedly to several aliquots of a single homogeneous (or pooled) volume of a biological material. A minimum of five determinations for a pooled biological material e.g., serum is recommended, and this should not exceed 15 per cent of the coefficient of variation (%CV). The intra–batch imprecision explains the variation of results

within a given set of data obtained for a particular run. This is expressed by the intra–batch %CV that was used to check the deviation within the same assay. The inter–batch imprecision describes the variation of results obtained from repeated assay runs. It is expressed by inter–batch %CV that was used to check the variability of results between different assay runs.

4.1.2.2 Recovery

A spiking recovery experiment is undertaken with a known concentration of an analyte spiked into the baseline or natural test sample matrix (biological sample + sample diluent), and thereafter measured (recovered) in the assay by comparing it to an identical spike in the standard diluent. The purpose was to help to maximize signal–to–noise ratio while attaining similar responses for a given concentration of the analyte in standard diluent (the standard curve) and sample matrix.

4.1.2.3 Linearity

Linearity of dilution assesses the extent to which the concentration of an analyte in a dose–response phenomenon of a sample is linear over the desired dilution range of the diluent that contains the analyte (CLSI, 2003).

4.1.2.4 Limit of the blank (LoB), lower limit of detection (LLoD) and limit of quantitation (LoQ).

Limit of the blank (LoB), lower limit of detection (LLoD), and limit of quantitation (LoQ) are terms used to describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure (Armbruster and Pry, 2008; CLSI, 2004a). LoB and LLoD are important to discriminate between the

presence or absence of an analyte. The importance of LoQ is to reliably measure low levels of analytes for clinical diagnosis and management.

LoB is the highest apparent analyte concentration expected to be found when several replicates of a blank sample (usually a sample buffer) containing no analyte are analysed and calculated using the formula:

$$LoB = Mean_{(BLANK)} + 2(SD_{BLANK})$$

LLoD is the lowest analyte concentration likely to be reliably distinguished from the LoB. LLoD is determined by utilising the measured LoB and several test replicates of a sample known to contain a low concentration of analyte.

$$LLoD = LoB + 2(SD_{LOW\ CONCENTRATION\ OF\ SPIKED\ SAMPLE})$$

LoQ, or functional sensitivity, is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. The LoQ may be equivalent to the LLoD, or it could be at a much higher concentration.

The interrelationship between analytical sensitivity (i.e., limit of the blank, LoB and lower limit of detection, LLoD) and functional sensitivity (i.e., limit of quantitation, LoQ) is contained in Appendix A.

4.1.2.5 Carry over and assay drift

Sample to sample carry over may cause erroneously test results as a result of the properties of the sample, presence of cross–reacting substances, anti–analyte antibodies or anti–reagent antibodies (Sturgeon and Viljoen, 2011; Armbruster and Alexander 2006). In practice, however, carry over is a systematic error introduced into a sample by contamination from a preceding

sample (Zeng et al., 2006; Kavsak and Zeidler, 2016). Carry over experiments are, therefore, performed as part of the validation process in immunoassays (Keller and Brinkmann, 2014).

4.1.3 Aim of this chapter

The aim of the study presented in this chapter was to analytically validate new two–site sandwich ELISA methods suitable for routine clinical laboratory application of serum Bühlmann MRP8/MRP14 Calprotectin S100A8/S100A9 (BMN®-Cp), serum Immunodiagnostik™ Calprotectin ELISA K 6935 (IDK®-Cp) and serum Immunodiagnostik™ S100A12 ELISA K 6938 (IDK®-A12) as biomarkers for the diagnosis and monitoring of IBD using faecal calprotectin (BÜHLMANN fCAL® ELISA assay) (see section 4.5.1), as the 'gold standard' reference assay.

4.2 Analytical Materials and Methods

The materials required for the assay validation are the components of individual ELISA kit supplied by the manufacturers: Bühlmann Laboratories AG, Baselstrasse 55, CH – 4124 Schönenbuch, Switzerland and Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany. These can be found in Appendix B.

Other materials include Ultra–pure water (Water type 1; ISO 3696 which is free of undissolved and colloidal ions, and organic molecules free of particles > 0.2 µm with an electrical conductivity of 0.055 µS/cm at 25°C (≥ 18.2 MΩ cm) and saline solution (NaCl 0.45%, 500 mL, pH 5.0 – 7.2, V1204 07176C (for *in–vitro* use only, and supplied by BioMerieux Ltd., Basingstoke, UK)).

Universal container, 20 mL, L0262–7 121090 (for *in–vitro* uses only, and supplied by Sarstedt Aktiengessellschaft & Co., D–51588 Nümbrecht, Germany).

Micro tube, 2 mL, 72.609 6350712 (for *in–vitro* uses only, and supplied by Sarstedt Aktiengessellschaft & Co., D–51588 Nümbrecht, Germany).

Screw caps 65.716–022 6083911 (for *in–vitro* use only, and supplied by Sarstedt Aktiengessellschaft & Co., D–51588 Nümbrecht, Germany),

DSX/DS2™ Sample tips, reagent tips and dilution wells; 65910 16222C0 (for *in–vitro* use only, and supplied by Dynex Technologies, Chantilly, VA 20151, USA).

Liquipette™ disposable Pasteur pipettes; 127–P423–000/127–P511–000 (for *in–vitro* uses only and supplied by Elkay. Precision Laboratory Consumables).

All solvents and other reagents were of analytical grades.

4.3 Apparatus

4.3.1 Alere Diamedix™ Dynex DS2™ Automated ELISA System

A non–competitive or two–site sandwich ELISA measurement of calprotectin and S100A12 was achieved using the Diamedix™ Dynex DS2™ Automated ELISA System (DS2) (Diamed Corporation, Hialeah, Florida, USA).

The workstation is maintained as a two–plate, two–wash platform at ambient temperature (18–25°C) for ease of sample dilution & distribution, incubation,

washing, reagent dispensing, automatic barcode scanning and reading with automatic data reduction and quality control.

The DS2 was graphically interfaced with the LISLINK's TD–Web IT system to deliver accurate sample–in/results–out automation of microplate assays.

A one multi–function robot arm was incorporated to ensure performance of every operation from pipetting to operating the barcode reader for maximum efficiency and reliability. The vertical design and multi–plate carrier feature limits space requirements thereby enabling minimum footprint.

The control monitor provided by Alere™ helped eliminate variations that occur with minimal processes, using advanced automation and precise liquid–handling capabilities to deliver the rigorous, repeatable analyses required in critical applications.

The Alere™ software package (dual wavelength mode) was used for both primary test filter and primary reference filter at 450 and 620 nm respectively, to generate a sigmoid curve fit type.

Prior to commencement of daily assay run, the DS2™ Dynex Technologies 1.24, Serial # 1DSA 1515, Software Version 1.24 and Firmware Version DSM 1.10 carries out a self–test exercise to ensure that the specifications of the module components: operating system, robot arm, micro–plate reader, incubator, washer, and pipette passed the manufacturer's in–built criteria necessary for the optimal performance of the analyser.

4.3.2 Other equipment

Stuart SRT9 tube roller mixer, with 9 roller and fixed speed of 33 rpm measuring 340 mm in length and 30 mm in diameter, connected to a 230V 50 Hz Alternating Current (a.c.), was used for sample freeze–thaw cycle.

Samples were mixed using a VWR VX–2500 multi–tube vortex mixer. For sample centrifugation, a THERMO SCIENTIFIC Sorvall Legend micro 17 centrifuge (DJB Labcare Ltd., Bucks, UK) was used.

4.4 Experimental Procedures

This section sets out the details of patients' recruitment and study design; the study demographics, inclusion and exclusion criteria for both groups of patients with IBD and those categorised as control patients, and procedures for sample collection and general serum preparation for both groups of patients.

4.4.1 Patient and control samples

4.4.1.1 Control Patients

Surplus serum samples from patients in primary care were collected as part of a routine annual general check–up that met the exclusion criteria of IBD, chronic illness and CRP results ≤ 5 mg/L. Serum, which had been collected within two hours of phlebotomy, was aliquoted and stored at –80ºC until thawed for analysis of serum BMN®-Cp, serum IDK®-Cp and serum S100A12. Icteric, lipaemic and haemolysed samples were not included in this study.

4.4.1.2 Patients with IBD

Surplus serum samples were obtained from patients with known active IBD based on a faecal calprotectin concentration of > 200 µg/g stool, who also had a concomitant blood sample collection. Serum, which had been collected within two hours of phlebotomy, was aliquoted and stored at –80ºC until thawed for analysis of serum BMN®-Cp, serum IDK®-Cp and serum S100A12. Icteric, lipaemic and haemolysed samples were not excluded.

4.5 Laboratory Methods

Measurement of serum calprotectin was performed using two different ELISA Kits: Bühlmann MRP8/MRP14 Calprotectin S100A8/S100A9 ELISA and Immunodiagnostik™ Calprotectin ELISA K 6935 manufactured by Bühlmann Laboratories AG, Baselstrasse 55, CH – 4124 Schönenbuch, Switzerland and Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany respectively. The Immunodiagnostik™ S100A12 ELISA K 6938 kit was used for the measurement of serum S100A12. All assays were performed on an automated ELISA instrument following manufacturer's instructions. An overview of the different assay methods is presented in table 4.1. All the assays were, however, CE marked for clinical diagnostic use.

		ELISA KIT Manufacturer		
Assay Characteristics		Bühlmann AG (BMN®-Cp)	Immunodiagnostik™ AG	
			(IDK®-Cp)	(IDK®-A12)[d]
Antibody Profile	Capture	Monoclonal, anti–human	Monoclonal, anti–human	Monoclonal, anti–human
	Detection	Monoclonal, horse	Monoclonal, anti–human	Polyclonal, horse
Dilution Factor [a]		1 in 100	1 in 100	1 in 10
Number of Calibrators or Standards		5	5	6
Number of Quality Control Materials (2 levels)		1 Low, 1 High	1 Low, 1 High	1 Low, 1 High
Principle of the assay		ELISA, sandwich	ELISA, two–site sandwich	ELISA, two–site sandwich
Cross-reactivity with MRP8/14[b] (%)		< 0.1	None observed	Not Relevant
Reference cut-off (ng/mL)		> 3900	> 3000	Not Reported
Measuring Range[c] (ng/mL)		400 – 24,000	0 – 25,000	0 – 540
Lower Limit of Detection (LLoD) (ng/mL)		< 100	78	0.16
Limit of Quantitation (LoQ) (ng/mL)		< 400	78	Not Available
Linearity of Dilution/Parallelism (%)		97.8	95.9	91.9
Spiking Recovery (%)		100.8	95.8	94.4
Precision and Reproducibility expressed as the %CV	Intra–assay	4.3	5.8	5.3
	Inter–assay	5.8	6.3	17.8
Number of tests per Kit		96	96	96

Table 4.1 – An overview of serum calprotectin and serum S100A12 assay characteristics. [a]Using the assay procedure supplied by Bühlmann Laboratories AG, CH – 4124 Schönenbuch, Switzerland and Immunodiagnostik™ AG, D – 64625 Bensheim, Germany. [b]Incubation buffer (BMN®-Cp) or mouse serum (IDK®-Cp) spiked with different amounts of recombinant monomer MRP8 and MRP14 were observed according to assay procedure. [c]The actual concentration of the calibrators A to E are 4, 12, 40, 120 and 240 ng/mL. Serum samples will be diluted 1 in 100, therefore the calibrators A to E are labelled as follows: 0.4, 1.2, 4.0, 12.0 and 24.0 microgram per millilitre (µg/mL) as supplied by Bühlmann Laboratories AG, CH – 4124 Schönenbuch, Switzerland. After 1 in 100 (BMN®-Cp and IDK®-Cp) and 1 in 10 (IDK®-A12) dilutions, concentrations up to 24,000 (BMN®-Cp), 25,000 (IDK®-Cp) and 540 (IDK®-A12) can be obtained. [d]Supplied as S100A12 ELISA Kit for the *in–vitro* determination of S100A12 in stool but was adapted for serum S100A12 assay in this study.

4.5.1 Extraction and measurement of faecal calprotectin

It was vital to mention that measurement of faecal calprotectin was not one of the aims and objectives of the present study. However, the fact that levels of faecal calprotectin was one of the criteria used for patient inclusion (see section 4.4.1.2) in the present study meant that a summarised account of the in–house, 'gold standard' procedure for routine measurement of faecal calprotectin at New Cross Hospital Wolverhampton should be mentioned. Another reason was the fact that results of faecal calprotectin of IBD patients recruited for the present study were used as the dataset against which results of serum calprotectin and serum S100A12 were compared.

Faecal calprotectin extraction was done according to the method described in the published work by Whitehead et al (2013) which, incidentally, is the in–house, 'gold standard' procedure for routine faecal calprotectin analysis at New Cross Hospital Wolverhampton, with great care taken to prevent pre–analytical variation of calprotectin in the stool samples during the extraction process. Following extraction, measurement of faecal calprotectin was performed on an automated ELISA instrument in accordance with the manufacturer's instructions. Analytical performance of the ELISA assay: Bühlmann fCAL^{TM} ELISA Calprotectin EK–CAL–U/EK–CAL–U (Bühlmann Laboratories AG, Baselstrasse 55, CH – 4124 Schönenbuch, Switzerland) was validated as described by Whitehead et al (2013).

4.5.2 Measurement of serum calprotectin in IBD and control patients

The storage and preparation of reagents were performed as instructed by the kit manufacturer. Although the assay protocol according to the instruction from

each kit manufacturer varied slightly in terms of the wash buffer, sample or incubation buffer, incubation timings, washing steps, choice of conjugate, substrate and stop solutions, they were nevertheless based on the same principle framework contained in Appendix C with further details in the kit inserts that can be found in Appendices D and E.

4.5.3 Measurement of serum S100A12 in IBD and control patients

The method developed by Immunodiagnostik™ AG for the *in–vitro* determination of S100A12 in stool was adapted for the *in–vitro* measurement of S100A12 in serum (Appendix F) in this study. A modified version of this method that provided the backdrop to Appendix F is described in sub section 4.5.3.1 below.

4.5.3.1 Modified ELISA method of S100A12 concentration in serum

S100A12 in serum samples were measured using a 96–well flat bottom ELISA microtitre plate manufactured by Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany. The assay was run on the DS2 analyser kept in the Medical Microbiology Department of New Cross Hospital Wolverhampton.

All routine maintenance of the instrument was performed as recommended in the owner's operating manual provided by the manufacturer: Diamed Corporation, Hialeah, Florida, USA. The modified program used to run S100A12 assays on the DS2 analyser for the Immunodiagnostik™'s ELISA kits were kindly made available for the present study by Biohit Healthcare UK Limited.

The assay protocol used disposable tips for sample and reagent pipetting steps. The rest of sample loading and on–board dilution steps used five millilitre (mL) fraction collection tubes (Sarstedt Aktiengesellschaft & Co., Germany). Part of the assay modification process involved reporting S100A12 concentration in serum as nanogram per millilitre (ng/mL). The measuring range for Immunodiagnostik™'s ELISA K 6938 kit for S100A12 assay was quoted in microgram per millilitre (µg/mL). This necessitated the multiplication of the results by a factor of 1000 so that the final results are reported in ng/mL.

S100A12 ELISA kits supplied by Immunodiagnostik™ were two–site sandwich ELISA immunoassay. The principle of this assay allows the binding of the antigen or antibody to a solid surface or a latex particle. The following template is an adjusted protocol for measuring serum S100A12 on an automated platform like the DS2 analyser used in the present study.

4.5.3.1.1 Layout

Mark the well positions of the standards, controls, and unknown serum samples on a protocol (layout) sheet of the microtitre plate before each particular run. This acts as a guide for proper sample and reagent pipetting steps. The standards, controls and blank should always be run alongside samples. The format of the ELISA microtitre plate layout could be varied between different runs based on the requirement for a specific performance characteristic of the assay being investigated. The basic template however remains the same as shown below:

(a) The first two wells (A1 + B1) of the plate were allocated as reagent blanks (sample buffer alone). Note: the lowest standard (0 ng/mL) in the Immunodiagnostik™ S100A12 ELISA K 6938 kit served as the reagent blank.

(b) Subsequent wells were used for the kit standards, each assayed in duplicate.

(c) Control materials supplied by Immunodiagnostik™ were run in duplicate in two positions on the plate: at the beginning of the plate in the wells immediately after the standards and at the end after the patient samples. This arrangement is crucial because it helps to check for assay drift which is a known potential artefact in automated ELISA processors.

(d) The remaining wells between the two sets of control materials were used for study samples which were, unless otherwise stated, assayed in duplicate.

4.5.3.1.2 Reconstitution

Reagents and samples were maintained at room temperature (18–25°C) for at least 3 hours and mixed well prior to running the assay. Any reagents supplied as concentrates (wash buffer and conjugate solution) or lyophilised materials (standards and controls) were prepared (or reconstituted) as instructed. Excess microtiter strips were immediately resealed and stored as instructed by the kit manufacturer.

4.5.3.1.3 Dilution

The conjugate concentrate supplied by Immunodiagnostik™ for measuring S100A12 must be diluted 101–fold in wash buffer already diluted 10–fold (i.e.,

100 µL CONJUGATE + 10 mL Wash Buffer). Note: calibrators and controls are already pre–diluted and run as neat additions.

4.5.3.1.4 Plate pre–wash

First, the ELISA Washer was purged with 9999 µL of IDK® wash buffer (Label: WASHBUF; Cat. No. K6938) and a super sweep action on the last cycle. This was followed by dispensing 350 µL of IDK® wash buffer in each of the plates coated wells to commence a 5–cycle wash in a plate–wise manner with constant timing (as recommended by Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany). Final aspirate cycle was done to make sure that no liquid remained in the wells after this last wash step. The pre–wash step was then completed by cleaning the Washer after use with 5000 µL of deionised water.

4.5.3.1.5 Pipetting

A deep well dilution of the serum samples was carried out by adding 30 µL of sample to 270 µL of IDK® sample buffer (Label: SAMPLEBUF; Cat. No. K6938) and mixed 3 times. Thereafter 100 µL of IDK® sample buffer (blank), calibrator, control material or diluted serum was then added to their respective wells as specified in the protocol sheet while ensuring a maximum post pipette delay of 5 minutes for the entire exercise.

4.5.3.1.6 Incubation

The plate was then covered and incubated at 18–25°C (ambient temperature) for 60 minutes with orbital shaking at medium speed for the entire duration of the incubation process.

4.5.3.1.7 Plate wash

The contents of each well were aspirated after which, the ELISA Washer was purged with 9999 µL of IDK® wash buffer and a super sweep action on the last cycle. This was followed by dispensing 350 µL of IDK® wash buffer in each of the plates coated wells to commence a 5–cycle wash in a plate–wise manner with constant timing (as recommended by Immunodiagnostik™). This was done to separate the free unbound molecules from those that are bound to the *capture antibody–human S100A12–peroxidase conjugate.* A final aspirate cycle was done to make sure that no liquid remained in the wells after this last wash step. The pre–wash step was then completed by cleaning the Washer after use with 5000 µL of deionised water.

4.5.3.1.8 Conjugate

100 µL of secondary antibody–enzyme conjugate (Label: CONJ; Cat. No. K6938 containing *anti–S100A12 antibody conjugated to horseradish* for Immunodiagnostik™ S100A12) was added to each well.

4.5.3.1.9 Incubation

The plate was then covered and incubated at 18–25°C (ambient temperature) for 60 minutes with orbital shaking at medium speed for the entire duration of the incubation process.

4.5.3.1.10 Plate wash

Any unbound secondary antibody–enzyme conjugate was then removed by aspirating the contents of each well after which, the ELISA Washer was purged with 9999 µL of IDK® wash buffer and a super sweep action on the last cycle.

This was followed by dispensing 350 µL of IDK® wash buffer in each of the plates coated wells to commence a 5–cycle wash in a plate–wise manner with constant timing (as recommended by Immunodiagnostik™). This was done to separate the free unbound molecules from those that are bound to the *capture antibody–human S100A12–peroxidase conjugate.* Final aspirate cycle was done to make sure that no liquid remained in the wells after this last wash step. The pre–wash step was then completed by cleaning the Washer after use with 5000 µL of deionised water.

4.5.3.1.11 Substrate

100 µL of 3,3',5,5'–Tetramethylbenzidine (TMB) in citrate buffer with hydrogen peroxide *(IDK® Substrate Solution – Label: SUB; Cat. No. K6938)* was added to each well.

4.5.3.1.12 Incubation

The plate was then covered and incubated at 18–25°C (ambient temperature) for 12 minutes. Unlike in previous incubation process, this incubation step did not require orbital shaking at medium speed.

4.5.3.1.13 Stop

100 µL of 0.25M Sulphuric Acid *(IDK® Stop Solution – Label: STOP; Cat. No. 6938)* was promptly added to the contents of each well.

4.5.3.1.14 Reader

Following an initial orbital shaking that lasted three seconds, the absorbance of each well was measured immediately by the microtitre plate reader on the DS2 analyser at two wavelengths: (1) *the absorbance measured at 450 nm*

served as the primary test filter wavelength and (2) the absorbance measured at 620 nm served as the primary reference filter wavelength. It was important that the absorbance should be read within 5 minutes of developing the colour in step 4.5.3.1.13 taking into consideration that the intensity of the colour change is temperature sensitive.

4.5.3.1.15 Curve fit

A standard curve was generated (4–parameter–algorithm plot) by plotting the mean absorbance for each standard versus the serum S100A12 concentration following the subtraction of the mean blank reading. The mean blank absorbance was subtracted from the other readings (averages if assayed in duplicate) and the corrected absorbance used to determine the S100A12 concentration in serum from the calibration curve (figure 3.4).

Steps 4.5.3.1.4 – 4.5.3.1.15 were performed automatically by the DS2 analyser as instructed by the assay's specific control program. The assay duration for a full plate run ranged from between 2.5 and 3.5 hours.

4.5.4 Assay working range

As a limitation to the general assay protocol based on the template outlined in steps 4.5.3.1.4 – 4.5.3.1.15 above, samples (sera) with concentrations of the assay (S100A12) above the kit's measuring range (as defined by the concentration range of the relevant calibrator shown in table 3.1; Chapter 3 under section 3.3.5) were further diluted and re–assayed. The result obtained was multiplied by the dilution factor used.

Samples with concentrations of the assay below the kit's measuring range cannot be clearly quantified. However, (i) the upper limit of the measuring

range (ULM) can be calculated as: highest concentration of the standard curve multiplied by sample dilution factor to be used (i.e., ULM = highest concentration of standard x DF) while (ii) the lower limit of the measuring range (LLM) can be calculated as: limit of the blank (LoB) multiplied by sample dilution factor to be used (i.e., LLM = LoB x DF).

4.5.5 Statistical Analysis

Data processing and statistical analysis were performed using a combination of software: GraphPad Instat version 3.00 for Windows 95 (GraphPad Software, San Diego, California, USA); Microsoft Excel® XLSTAT (Addinsoft, New York, NY 10001, USA; Available Online at https://www.xlstat.com/en/); Analyse–it Software (Analyse–it Software Ltd., The Tannery, 91 Kirkstall Road, LEEDS LS3 1HS, United Kingdom) and SPSS Statistics version 25 (IBM SPSS Statistics Software, Armonk, New York, USA).

Data collected during the study were recorded in Microsoft Office Excel 2007 Spreadsheets and thereafter imported to SPSS. Diagnostic plots were used within the SPSS and the cut–offs point for individual parameters were applied in accordance with the established guidelines.

4.6 Analytical Validation of Bühlmann Laboratories AG and Immunodiagnostik™ AG ELISA kits for the measurements of serum calprotectin and serum S100A12

4.6.1 Experimental Methods and Results

In the experiments both serum samples and the manufacturer's supplied calibrator and control materials were used to assess a kit's performance characteristics for the same parameter.

In these instances, the serum samples in µg/mL were first diluted serially with either physiological saline (i.e., 0.90% w/v of NaCl, 308 mOsm/L or 9.0 g/L for Bühlmann calprotectin) or sample buffer for linearity of dilution (parallelism) assay prior to being pipetted onto the ELISA plate as part of the assay protocol, whereas the control materials, which came pre–prepared in µg/mL (Bühlmann calprotectin only) or as lyophilised materials in ng/mL were reconstituted according to manufacturer's instructions and used neat without a dilution step.

4.6.2 Imprecision

4.6.2.1 Method

Aliquots of pooled serum samples from patients with IBD and controls were prepared and stored at –80°C in 0.75 mL bottles until analysis. Five to seven aliquots from both groups were used for a particular run based on the availability of wells on the ELISA microtitre plate. The internal quality control (IQC) materials were freshly reconstituted in the case of Immunodiagnostik^{TM} kits for this purpose. The internal quality control materials supplied by Bühlmann are ready for use.

However, it is noteworthy that no reference range for serum S100A12 was supplied by the kit manufacturer as a guide for the imprecision study.

4.6.2.2 Intra–Batch (Within–Run) Imprecision Assay

Each aliquot was measured several times in duplicates, and then %CV was calculated for each aliquot by dividing the standard deviation (SD) of the duplicate measurements by the mean of the duplicate measurements and multiplying the quotient by 100. The average of the individual CVs from the

several measurements was taken as the intra–batch CV for the particular patient group.

Intra–batch imprecision was determined through three methods:

(i) Pooled serum samples from IBD patients whose concentrations of calprotectin and S100A12 were highly elevated were used. They were analysed within the same assay run using three different kits for a minimum of five times in duplicate and this was dependent on the availability of wells on the ELISA microtitre plate (n = 10).

(ii) Pooled serum samples from IBD patients whose concentrations of calprotectin and S100A12 were moderately elevated were used. They were analysed within the same assay run using three different kits for a minimum of five times in duplicate and this was dependent on the availability of wells on the ELISA microtitre plate (n = 10).

(iii) Pooled serum samples from controls. They were analysed within the same assay run using three different kits for a minimum of five times in duplicate and this was dependent on the availability of wells on the ELISA microtitre plate (n = 10).

4.6.2.3 Inter–Batch (Between–Run) Imprecision Assay

An aliquot was measured once in duplicate during different assay runs and the %CV was calculated as for each run by dividing the standard deviation (SD) of the duplicate measurements by the mean of the duplicate measurements and multiplying the quotient by 100. The average of the individual CVs from the several measurements was taken as the inter–batch CV for the particular patient group.

Inter–batch imprecision was determined through three methods:

(i) Pooled serum samples from IBD patients whose concentrations of calprotectin and S100A12 were highly elevated were used. An aliquot was analysed once in duplicate over at least four different, consecutive assay runs using the three different ELISA kits (n = 8–12 for each method).

(ii) Pooled serum samples from IBD patients whose concentrations of calprotectin and S100A12 were moderately elevated were used. An aliquot was analysed once in duplicate over at least four different, consecutive assay runs using the three different ELISA kits (n = 8–12 for each method).

(iii) Two levels (low and high) of IQC materials supplied by each ELISA kit manufacturer were used. Each reconstituted level (as in Immunodiagnostik™) was analysed once in duplicate over four different, consecutive assay runs using the three ELISA kits (n = 8 for each method) according to the guideline provided by CLSI EP5 – A2 (CLSI, 2004b). The appropriate levels of individual control material of each ELISA kit are shown in table 4.2.

Assay Method	Concentration (ng/mL)					
	Level 1 (Low IQ Control)			Level 2 (High IQ Control)		
	Mean	SD	–1SD to 2SD	Mean	SD	–1SD to 2SD
BMN®-Cp	1400	200	1000 – 1800	5600	300	5000 – 6200
IDK®-Cp	15.4	4.0	7.4 – 23.4	49.0	9.0	31.0 – 67.0
IDK®-A12	0.54	0.17	0.37 – 0.88	6.5	1.5	5.0 – 9.5

Table 4.2 – Approximate concentrations of calprotectin and S100A12 in supplied levels of quality control materials from ELISA kit manufacturers. Both level 1 (low) and level 2 (high) of the control materials were measured neat in the assay process. IDK®-Cp and IDK®-A12 were supplied in nanogram per millilitre (ng/mL). However, BMN®-Cp was supplied in µg/mL but assay result had to be reported in ng/mL for ease of comparison with results from other kits (See Chapter 3 under section 3.3.5). IQ = Internal quality.

In all cases, a freshly reconstituted aliquot was used for a particular run. The Diamedix™ Dynex DS2™ Automated ELISA System was modified to run the control materials neat as additional controls without performing the normal pre–analytical dilution step necessary for the measurement of the analyte: as in 1 in 10 (S100A12) or 1 in 100 (calprotectin).

4.6.2.4 Result

4.6.2.5 Intra–Batch (Within–Run) Imprecision Assay

The results of the intra–batch (within–run) imprecision study varied between 1.0% and 9.2% as set out in table 4.3.

%CV for intra–assay variability for pooled highly elevated IBD samples and pooled moderately elevated IBD samples for IDK®-A12 assay were 9.2% and 4.8% respectively.

%CV for intra–assay variability for pooled highly elevated IBD samples, pooled moderately elevated IBD samples and pooled normal samples for IDK®-Cp assay were 1.0%, 3.8% and 3.9% respectively.

%CV for intra–assay variability for pooled highly elevated IBD samples, pooled moderately elevated IBD samples and pooled normal samples for BMN®-Cp assay were 2.8%, 1.8% and 4.6% respectively.

The observed mean intra–batch imprecision for serum S100A12 and serum calprotectin was < 10% for each assay: (BMN®-Cp: 3.1%; IDK®-A12: 7.0% and IDK®-Cp: 2.9%). IDK®-Cp assay posted the best overall performance with a demonstrable lowest mean %CV and narrowest %CV range.

ELISA Kit	Sample Type	n	Mean	SD	%CV
BMN®-Cp (ng/mL)	Pooled Highly Elevated IBD	10	9691	278.8	2.8
	Pooled Moderately Elevated IBD		4553	86.3	1.8
	Pooled Healthy Controls (Normal)		3940	179.4	4.6
	Mean				**3.1**
IDK®-A12 (ng/mL)	Pooled Highly Elevated IBD	10	1257	115.8	9.2
	Pooled Moderately Elevated IBD		535	25.8	4.8
	Mean				**7.0**
IDK®-Cp (ng/mL)	Pooled Highly Elevated IBD	10	15184	156.1	1.0
	Pooled Moderately Elevated IBD		3038	119.9	3.9
	Pooled Healthy Controls (Normal)		2880	111.3	3.8
	Mean				**2.9**

Table 4.3 – Imprecision study. Intra–batch (within–batch) imprecision data for BMN®-Cp, IDK®-A12 and IDK®-Cp assays using pooled IBD samples containing highly and moderately elevated concentrations of serum calprotectin/serum S100A12 and healthy controls. n = the number of replicates; SD = standard deviation; CV = coefficient of variation.

4.6.2.6 Inter–Batch (Between–Run) Imprecision Assay

The results of inter–batch (between–run) imprecision study varied between 0.1% and 7.6% as set out in table 4.4.

%CV for inter–assay variability for pooled highly elevated IBD samples, pooled moderately elevated IBD samples, low IQC and high IQC for IDK®-A12 assay were 2.3%, 1.2%, 4.3% and 7.6% respectively.

%CV for inter–assay variability for pooled highly elevated IBD samples, pooled moderately elevated IBD samples, low IQC and high IQC for IDK®-Cp assay were 6.5%, 4.2%, 4.1% and 3.9% respectively.

%CV for inter–assay variability for pooled highly elevated IBD samples, pooled moderately elevated IBD samples, low IQC and high IQC for BMN®-Cp assay were 5.2%, 6.8%, 0.1% and 0.8% respectively.

The observed mean inter–batch imprecision for serum S100A12 and serum calprotectin was < 10% for each assay: (BMN®-Cp: 3.2%; IDK®-A12: 3.8% and IDK®-Cp: 4.7%). BMN®-Cp assay posted the best overall performance with a demonstrable lowest mean %CV and narrowest %CV range.

ELISA Kit	Sample Type (Target Range in ng/mL)	n	Mean	SD	%CV
BMN®-Cp (ng/mL)	Pooled Highly Elevated	10	18748	1109.9	5.2
	Pooled Moderately Elevated		3615	245.9	6.8
	Low IQC (1000 – 1800)	8	1526	2.2	0.1
	High IQC (5000 – 6200)		5158	42.7	0.8
	Mean				**3.2**
IDK®-A12 (ng/mL)	Pooled Highly Elevated	12	723	16.6	2.3
	Pooled Moderately Elevated		522	6.1	1.2
	Low IQC (0.20 – 0.88)	8	0.7	0.02	4.3
	High IQC (3.5 – 9.5)		6.6	0.5	7.6
	Mean				**3.8**
IDK®-Cp (ng/mL)	Pooled Highly Elevated	10	13489	874.8	6.5
	Pooled Moderately Elevated		2901	122.4	4.2
	Low IQC (7.4 – 23.4)	8	7.8	0.3	4.1
	High IQC (31 – 67)		38.1	1.5	3.9
	Mean				**4.7**

Table 4.4 – Imprecision study. Inter–batch (between–batch) imprecision assay data for BMN®-Cp, IDK®-A12 and IDK®-Cp assays using pooled IBD samples containing highly and moderately elevated concentrations of serum calprotectin/serum S100A12 and quality control materials provided by the kit manufacturers. n = the number of replicates; SD = standard deviation; CV = coefficient of variation. IQC analytical ranges are expressed in parenthesis.

4.6.3 Recovery

4.6.3.1 Method

Spiking recovery was determined for each assay method by adding known concentrations of the analyte (i.e., calprotectin or S100A12) to aliquots of respective sample or incubation buffer provided by the kit manufacturer. The analyte was taken from the kit calibrator solution supplied by the manufacturer.

Four aliquots were prepared for each assay method by adding a different concentration of the analyte to the serum. The baseline concentration of the analyte in each buffer aliquot ranged from 0.18 ng/mL to 540 ng/mL.

Each of the four aliquots was measured in duplicate (n = 8) for each assay method. The sum of the baseline concentration of the analyte in the incubation or sample buffer and the analyte concentration in the calibrator before measurement gave the Expected (E) concentration of the analyte. The Measured (M) concentration of the analyte represented the result obtained after measurement.

The per cent recovery (%Recovery) was calculated by dividing Measured by Expected, and multiplying the quotient by 100 for single run for each aliquot. The %CV was calculated for each aliquot by dividing the standard deviation (SD) of the duplicate %Recovery by the mean of the duplicate %Recovery and multiplying the quotient by 100.

Similarly, the overall per cent recovery (%Recovery) for each method was taken as the mean of the individual result of %Recovery (n = 8) obtained from the four aliquots. The overall %CV of the assay method was calculated by dividing the

standard deviation (SD) of the individual result of %Recovery (n = 8) obtained from the four aliquots by the mean of the individual results of %Recovery (n = 8) obtained from the four aliquots and multiplying the quotient by 100.

4.6.3.2 Result

The results of spiking recovery for the 3 assays were expressed as the ratios of Measured to Expected concentration of the analyte in the sample matrix. These are set out in tables 4.5 – 4.7.

Measured to Expected ratios for spiking recovery for IDK®-A12 assay mean was: 126.5% (range: 107.7 to 155.9%), SD was 18.4% and %CV was 14.5% (table 4.5); for IDK®-Cp assay, mean was: 89.5% (range: 84.7 to 93.8%), SD was 3.2% and %CV was 3.6% (table 4.6); and for BMN®-Cp assay, mean was: 82.1% (range: 65.5 to 95.5%), SD was 10.7% and %CV was 13.0% (table 4.7);

Overall, the 3 assays exhibited acceptable individual analytical performance judged by the mean %Recovery of 104 ± 22%. However, BMN®-Cp assay demonstrated 65.5 – 68.9% under recovery at the lowest spiked concentration of 1200 ng/mL calprotectin in serum (table 4.7).

	Immunodiagnostik™ S100A12 ELISA K 6938 (IDK®-A12)				
Buffer Aliquot	**Unspiked Buffer Aliquot (ng/mL)**	**S100A12 Added (ng/mL)**	**Expected (E) (ng/mL)**	**Measured (M) (ng/mL)**	**%Recovery = Measured / Expected**
#1	0.18	54.00	27.09	29.18	107.7
	0.18	54.00	27.09	29.43	108.6
#2	0.18	18.00	9.09	10.11	111.2
	0.18	18.00	9.09	10.38	114.2
#3	0.18	6.00	3.09	4.12	133.3
	0.18	6.00	3.09	4.27	138.2
#4	0.18	2.00	1.09	1.56	143.1
	0.18	2.00	1.09	1.70	155.9
	% Recovery				**126.5**
	SD				**18.4**
	%CV				**14.5**

Table 4.5 – Spiking recovery data for serum IDK®-A12 assay

	Immunodiagnostik™ K 6935 Calprotectin (IDK®-Cp)				
Buffer Aliquot	**Unspiked Buffer Aliquot (ng/mL)**	**Calpro. Added (ng/mL)**	**Expected (E) (ng/mL)**	**Measured (M) (ng/mL)**	**%Recovery = Measured / Expected**
#1	0.54	250.00	125.27	112.16	89.5
	0.54	250.00	125.27	108.99	87.0
#2	0.54	62.50	31.52	29.43	93.4
	0.54	62.50	31.52	28.74	91.2
#3	0.54	15.60	8.07	7.06	87.5
	0.54	15.60	8.07	7.57	93.8
#4	0.54	3.90	2.22	1.98	89.2
	0.54	3.90	2.22	1.88	84.7
	% Recovery				**89.5**
	SD				**3.2**
	%CV				**3.6**

Table 4.6 – Spiking recovery data for serum IDK®-Cp assay

	Bühlmann MRP8/14 S100A8/A9 (BMN®-Cp)				
Buffer Aliquot	**Unspiked Buffer Aliquot (ng/mL)**	**Calpro. Added (ng/mL)**	**Expected (E) (ng/mL)**	**Measured (M) (ng/mL)**	**%Recovery = Measured / Expected**
#1	540	24000	12270	11720	95.5
	540	24000	12270	11260	91.8
#2	540	12000	6270	5390	85.9
	540	12000	6270	5410	86.3
#3	540	4000	2270	1740	76.7
	540	4000	2270	1950	85.9
#4	540	1200	870	570	65.5
	540	1200	870	600	68.9
	% Recovery				**82.1**
	SD				**10.7**
	%CV				**13.0**

Table 4.7 – Spiking recovery data for serum BMN®-Cp assay

4.6.4 Linearity of Dilution (Parallelism)

4.6.4.1 Method

Linearity of dilution was determined by using pooled serum samples containing elevated concentration of the analyte as shown in table 4.8. Except in the Bühlmann calprotectin assay where normal saline was used in place of incubation buffer as the diluent because of the bluish colour background that could influence the optical density, a series of seven serial doubling dilutions (i.e., 1 in 2, 1 in 4, 1 in 8, 1 in 16, 1 in 32, 1 in 64 and 1 in 128) were prepared from each serum sample using the sample matrix (pooled control serum samples diluted 1 in 2 with the respective assay sample buffer) as the diluent and subsequently assayed in duplicate in the same batch run.

Assay Method	Concentration of Calprotectin / S100A12 in pooled serum samples of control patients
BMN®-Cp (ng/mL)	15380
IDK®-Cp (ng/mL)	8560
IDK®-A12 (ng/mL)	840

Table 4.8 – Concentrations of calprotectin and S100A12 in serum samples used for the determination of linearity of dilution (parallelism). Linearity of dilution (parallelism) was determined by evaluating the serum sample at its initial strength (undiluted) and at dilutions of 1 in 2; 1 in 4; 1 in 8; 1 in 16; 1 in 32; 1 in 64 and 1 in 128.

The concentration of the analyte at each dilution prior to measurement represented the Expected (E) concentration of the analyte. The Measured (M) concentration of the analyte was taken as the result obtained after measurement. The per cent linearity of dilution (%linearity of dilution) at a particular dilution (e.g., 1 in 2) of the sample was calculated by dividing Measured by Expected and multiplying the quotient by 100. The overall per cent linearity of dilution (%linearity of dilution) for each method was calculated as the mean of the individual result of %linearity of dilution obtained at different dilutions of the sample.

4.6.4.2 Result

The results from the serially diluted samples containing elevated concentration of serum calprotectin or serum S100A12 for each assay are shown in tables 4.9 – 4.11.

The mean (range) Measured to Expected ratios for dilutional parallelism (n = 7) for BMN®-Cp assay was 100.8% (range: from 92.7 to 108.3%, table 4.9); for

IDK®-A12 assay, 103.7% (range: from 71.4 to 148.8%, table 4.10) and for IDK®-Cp assay was 98.4% (range: from 89.2 to 112.3%, table 4.11).

Linear regression was also carried out on the plot of the Expected and Measured results of the dilutions (n = 7) for each assay as shown in figures 4.1 – 4.3.

The slope, intercept and square of regression coefficient (r^2) were: 0.939, +73.061, 0.9994 (for BMN®-Cp assay, figure 4.1); 1.5107, –20.998, 0.9965 (for IDK®-A12 assay, figure 4.2) and 1.1103, –22.615, 0.9997 (for IDK®-Cp assay, figure 4.3) respectively.

In each case, the dilutions were linear over the range tested and the results met the > 80% of target acceptance criterion. A summary of the linear regression fits characteristics of each of the 3 assays are shown in table 4.12.

Linearity of dilution (parallelism) data for serum BMN®-Cp assay				
ELISA Kit	**Dilution Factor (DF)**	**Measured (M) (ng/mL)**	**Expected (E) (ng/mL)**	**Measured / Expected (%)**
BMN®-Cp (ng/mL)	1 in 1	15380	–	–
	1 in 2	7250	7690	94.3
	1 in 4	3720	3845	96.6
	1 in 8	1990	1923	103.1
	1 in 16	1020	962	106.3
	1 in 32	500	481	104.2
	1 in 64	260	241	108.3
	1 in 128	110	121	92.7
	Mean			**100.8**

Table 4.9 – Linearity of dilution (parallelism) data for BMN®-Cp assay using elevated concentration of serum calprotectin at serial dilutions of 1:2; 1:4; 1:8; 1:16; 1:32; 1:64 and 1:128. The Measured to Expected ratios are given in %.

Linearity of Dilution (Parallelism) Data for Serum IDK®-A12 Assay				
ELISA Kit	**Dilution Factor (DF)**	**Measured (M) (ng/mL)**	**Expected (E) (ng/mL)**	**Measured / Expected (%)**
IDK®-A12 (ng/mL)	1 in 1	840	–	–
	1 in 2	625	420	148.8
	1 in 4	283	210	134.8
	1 in 8	120	105	114.3
	1 in 16	49	53	92.5
	1 in 32	23	27	85.2
	1 in 64	11	14	78.6
	1 in 128	5	7	71.4
	Mean			**103.7**

Table 4.10 – Linearity of dilution (parallelism) data for IDK®-A12 assay using elevated concentration of serum S100A12 at serial dilutions of 1:2; 1:4; 1:8; 1:16; 1:32; 1:64 and 1:128. The Measured to Expected ratios are given in %.

Linearity of Dilution (Parallelism) Data for Serum IDK®-Cp Assay				
ELISA Kit	**Dilution Factor (DF)**	**Measured (M) (ng/mL)**	**Expected (E) (ng/mL)**	**Measured / Expected (%)**
IDK®-Cp (ng/mL)	1 in 1	8556	–	–
	1 in 2	3892	4278	90.9
	1 in 4	1909	2139	89.2
	1 in 8	970	1070	90.7
	1 in 16	507	535	94.8
	1 in 32	301	268	112.3
	1 in 64	139	134	103.7
	1 in 128	72	67	107.5
	Mean			**98.4**

Table 4.11 – Linearity of dilution (parallelism) data for IDK®-Cp assay using elevated concentration of serum calprotectin at serial dilutions of 1:2; 1:4; 1:8; 1:16; 1:32; 1:64 and 1:128. The Measured to Expected ratios are given in %.

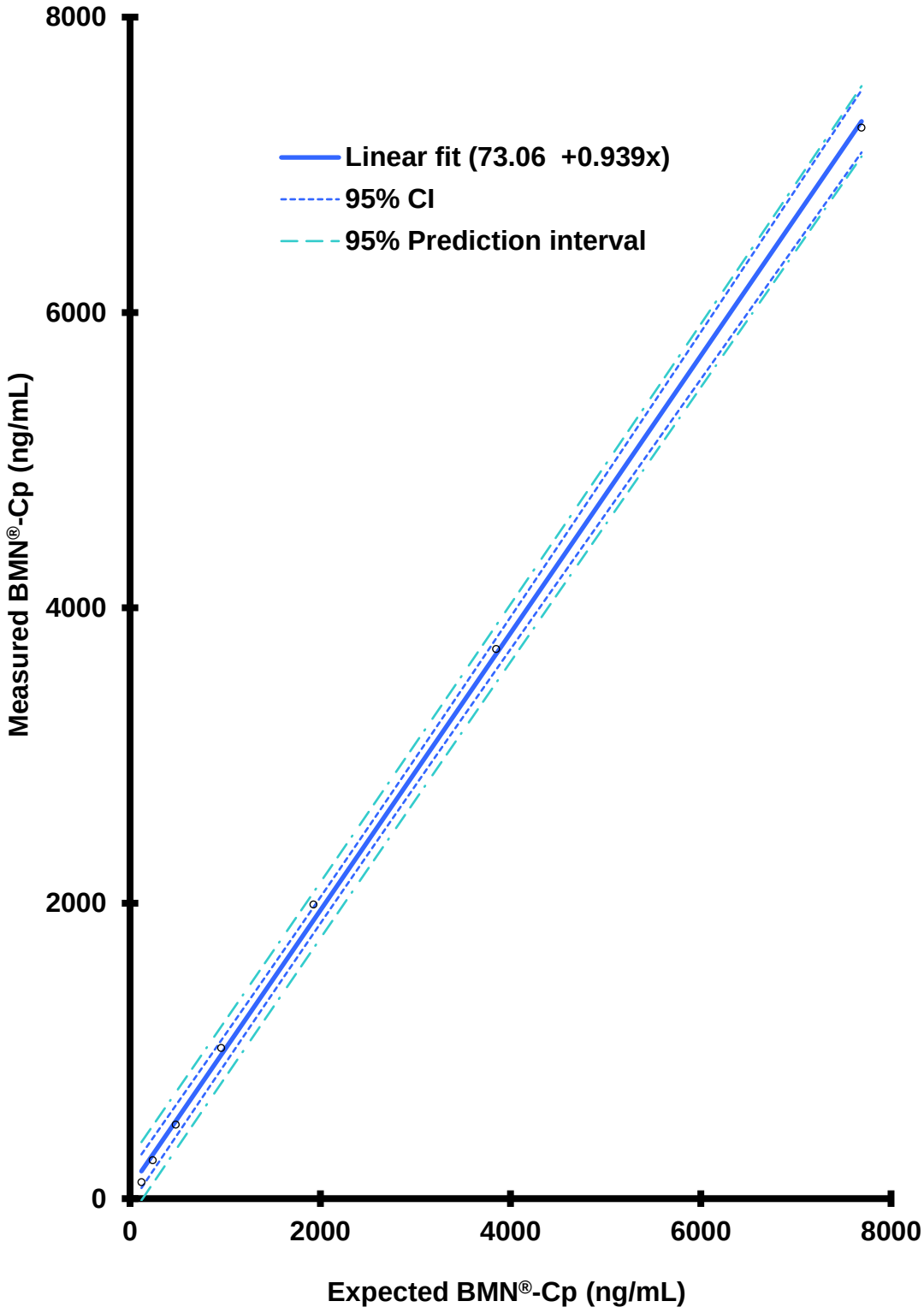

Figure 4.1 – Scatter plot with fit for serum BMN®-Cp assay. Measured and Expected results for the elevated sample dilutions demonstrate reliability of BMN®-Cp Kit. 95% CI = –15.02 to 161.15 (Intercept), 0.913 to 0.965 (Slope) and t–statistic = 2.13 (Intercept), 92.00 (Slope).

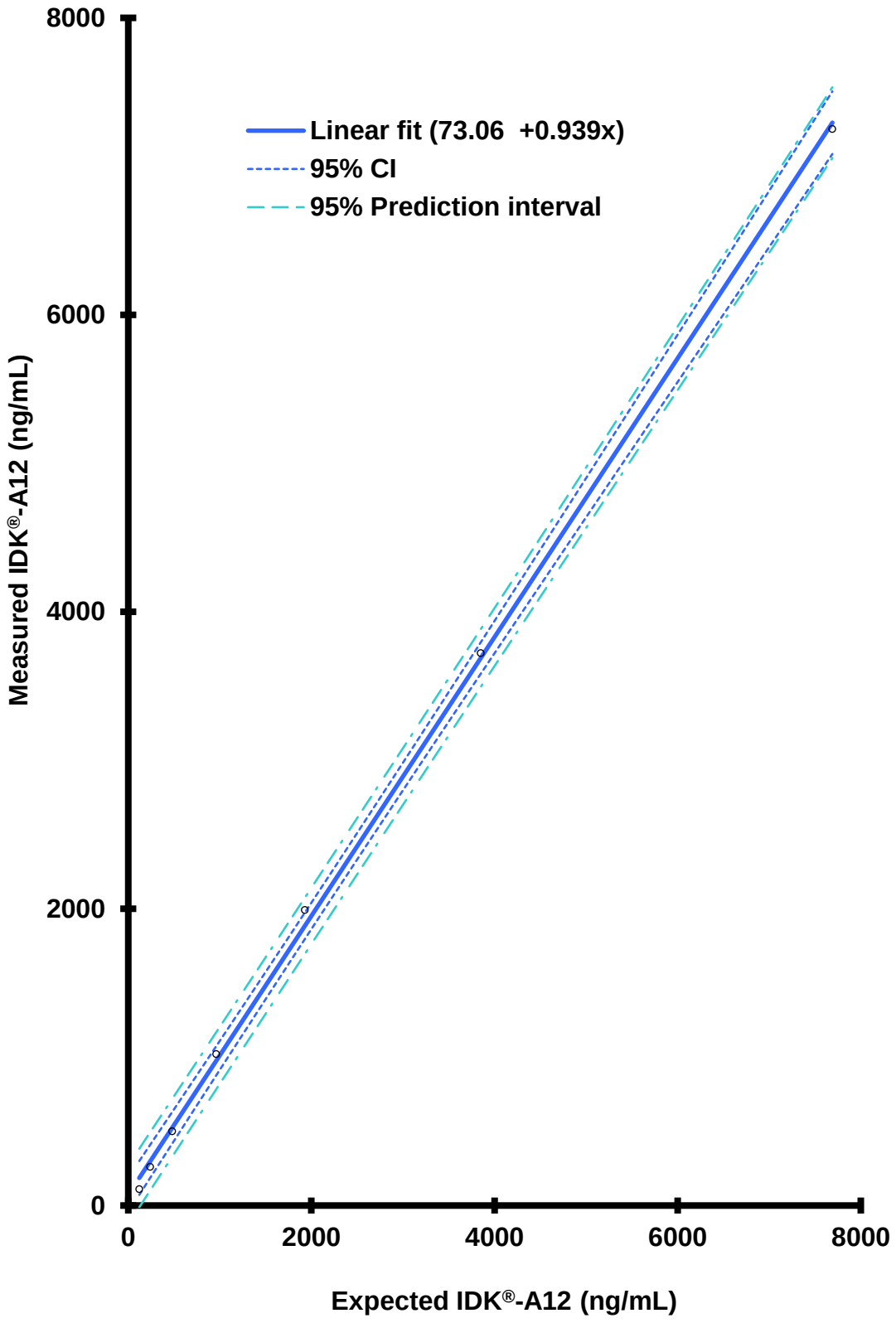

Figure 4.2 – Scatter plot with fit for serum IDK®-A12 assay. Measured and Expected results for the elevated sample dilutions demonstrate reliability of the IDK®-A12 Kit. 95% CI = –40 to –2 (Intercept), 1.408 to 1.614 (Slope) and t–statistic = –2.86 (Intercept), 37.73 (Slope).

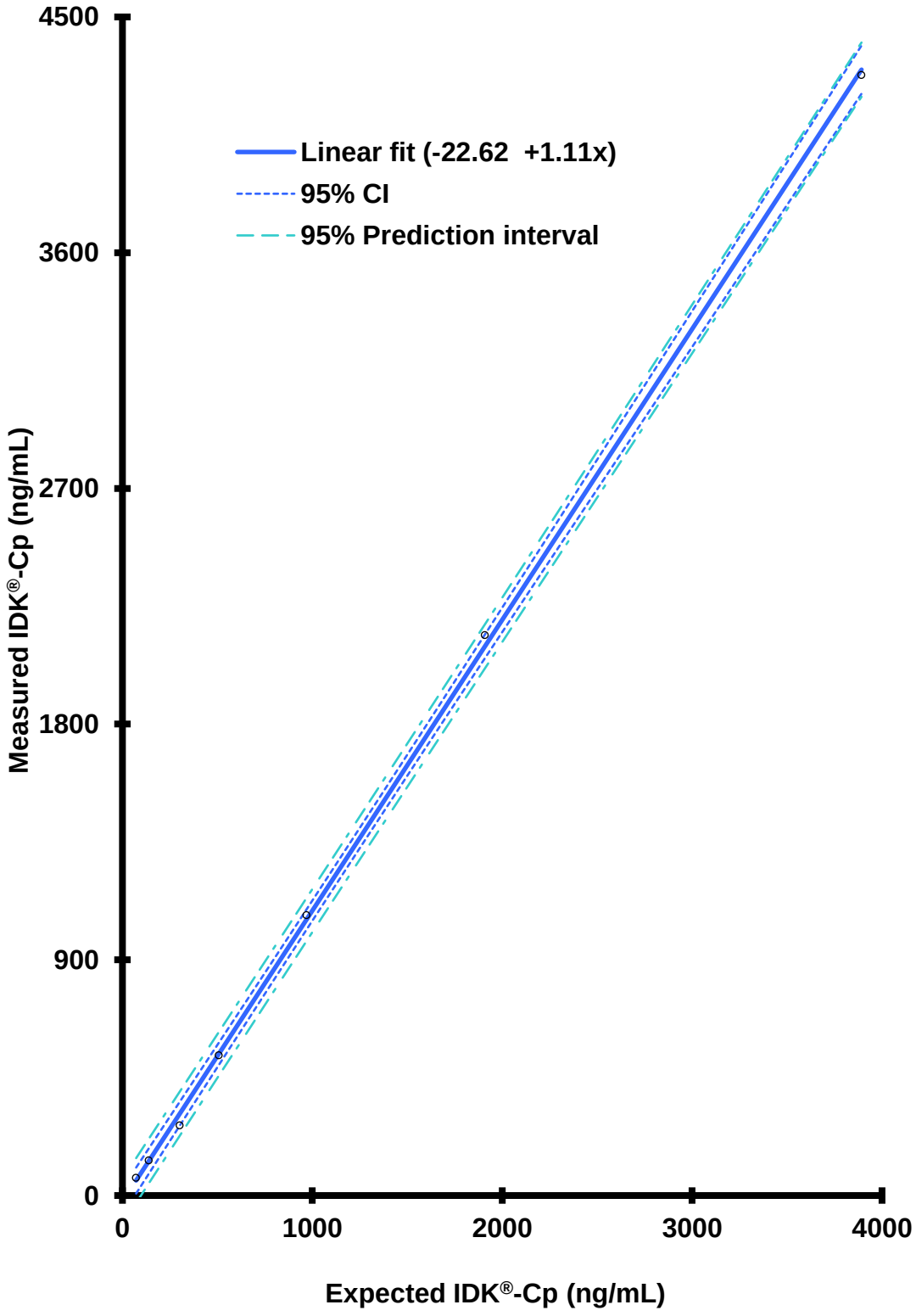

Figure 4.3 – Scatter plot with fit for serum IDK®-Cp assay. Measured and Expected results for the elevated sample dilutions demonstrate reliability of the IDK®-Cp Kit. 95% CI = –61.12 to 15.89 (Intercept), 1.09 to 1.13 (Slope) and t–statistic = –1.51 (Intercept), 125.63 (Slope).

ELISA Kit	Slope	Intercept	Linear Fit	Relationship of Measured (M) to Expected (E)	Line Equation¶ (y = mx + c)	*r*	r^2	*p*	SEM
BMN®-Cp (ng/mL)	0.9390	73.061	(73.06 + 0.939*x*)	M = 73.06 + 0.939E	y = 0.939*x* + 73.061	0.99	0.98	0.0001	68.9
IDK®-A12 (ng/mL)	1.5107	–20.998	(–21 + 1.511*x*)	M = –21 + 1.511E	y = 1.5107*x* – 20.998	0.99	0.98	0.0001	14.7
IDK®-Cp (ng/mL)	1.1103	22.615	(–22.62 + 1.11*x*)	M = –22.62 + 1.11E	y = 1.1103*x* – 22.615	0.99	0.98	0.0001	29.9

Table 4.12 – A summary of the linear regression fits analysis (figures 4.1 – 4.3) of the results of Measured and Expected concentrations of serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 from serial dilutions (n = 7) of the analyte for the respective ELISA kits. ¶The relationship between the Measured and Expected concentration of the analyte is expressed in the form of the equation: *y* = m*x* + c where *x* and *y* represents the Expected and Measured result respectively. SEM = Standard error of the mean.

4.6.5 Analytical Sensitivity: Limit of the Blank and Lower limit of Detection

For the purpose of developing and validating Bühlmann Laboratories AG and Immunodiagnostik™ AG ELISA assays, the respective dilution buffer or sample buffer supplied by the assay manufacturer for each of the analyte (calprotectin and S100A12) was used. Five (5) aliquots of each dilution buffer was prepared and measured in duplicates. The mean absorbance was used to calculate the limit of the blank (LoB) for each analyte.

4.6.5.1 Method

4.6.5.2 Limit of the Blank (LoB)

Limit of the blank (LoB) is the highest analyte concentration expected to be found when replicates of a sample containing no analyte are tested. LoB was derived by measuring replicates of a blank sample or dilution buffer (zero concentration of analyte) and calculating the mean result and the standard deviation.

Five aliquots of sample/incubation buffer containing zero concentration of analyte (blank solution) was each measured in duplicates (n = 10) on the same ELISA microtitre plate according to the assay protocol. The average concentration of the analyte (calprotectin or S100A12) and standard deviation (SD) was calculated and used to calculate the LoB according to the following formula:

$$\mathbf{LoB = Mean_{(BLANK)} + 2(SD_{BLANK})}$$

4.6.5.3 Lower limit of Detection (LLoD)

Lower limit of detection (LLoD) was determined by using the calculated LoB and test replicates of a sample known to contain a low concentration of the analyte under consideration. LLoD is estimated as the sum of the LoB and 2SD of low concentration of sample.

Five aliquots of sample/incubation buffer containing zero concentration of analyte (blank solution) were spiked with a small known concentration of calprotectin or S100A12 using the manufacturer's supplied calibrator solutions. Concentrations of calprotectin and S100A12 in the spiked samples were chosen based upon the manufacturer's claimed analytical sensitivity. These were 400 ng/mL and 3.9 ng/mL for Bühlmann calprotectin assay and Immunodiagnostik™ calprotectin assay respectively, and 0.66 ng/mL for Immunodiagnostik™ S100A12 assay. The spiked samples were analysed five consecutive occasions in duplicates (n = 10) on the same ELISA microtitre plate as per the assay protocol used for LoB samples. Subsequently, the mean concentration of the analyte and SD were calculated as described as per the assay protocol used for LoB.

The LLoD was calculated using the following formula:

$$\mathbf{LLoD = LoB + 2(SD_{LOW\ CONCENTRATION\ OF\ SPIKED\ SAMPLE})}$$

4.6.5.4 Result

The calculated results of the limit of the blank (LoB) and the lower limit of detection (LLoD) for the 3 ELISA assays are set out in table 4.13. Unlike the results of IDK®-Cp and IDK®-A12 assays, the LoB and LLoD values for BMN®-

Cp assay were deliberately reported in µg/mL as against ng/mL for easy and quick comparison with the decision threshold provided by assay manufacturer.

4.6.5.5 Limit of the Blank

I. IDK®-A12 Kit

Limit of the blank (LoB) = $Mean_{BLANK} + 2(SD_{BLANK})$
= 0.319 + 2(0.177)
= 0.319 + 0.354
= 0.673 ng/mL

II. IDK®-Cp Kit

Limit of the blank (LoB) = $Mean_{BLANK} + 2(SD_{BLANK})$
= 0.539 + 2(0.303)
= 0.539 + 0.606
= 1.145 ng/mL

III. BMN®-Cp Kit

Limit of the blank (LoB) = $Mean_{BLANK} + 2(SD_{BLANK})$
= 0.545 + 2(0.016)
= 0.545 + 0.032
= 0.577 µg/mL

4.6.5.6 Lower Limit of Detection

I. IDK®-A12 Kit

Lower limit of detection (LLoD) = $LoB + 2(SD_{LOW\ CONCENTRATION\ OF\ SPIKED\ SAMPLE})$
= 0.673 + 2(0.223)
= 0.673 + 0.446
= 1.119 ng/mL

II. IDK®-Cp Kit

Lower limit of detection (LLoD) = LoB + 2($SD_{LOW\ CONCENTRATION\ OF\ SPIKED\ SAMPLE}$)
= 1.145 + 2(0.244)
= 1.145 + 0.488
= 1.633 ng/mL

III. BMN®-Cp Kit

Lower limit of detection (LLoD) = LoB + 2($SD_{LOW\ CONCENTRATION\ OF\ SPIKED\ SAMPLE}$)
= 0.577 + 2(0.010)
= 0.577 + 0.020
= 0.597 µg/mL

4.6.5.7 Limit of Quantitation (LoQ) or Functional Sensitivity

4.6.5.8 Method

The limit of quantitation (LoQ) or functional sensitivity was determined as part of the imprecision experiments in section 4.6.2 above by evaluating the %CV of the within–batch (Intra–assay) and between–batch (Inter–assay) imprecision experiments conducted with pooled control (normal) serum samples and pooled serum samples from IBD patients whose concentrations of calprotectin and S100A12 were moderately and highly elevated respectively, to identify the lowest concentration of calprotectin or S100A12 at which the %CV was < 20%.

4.6.5.9 Result

The intra–batch and inter–batch imprecision results for the moderately and highly elevated fractions for BMN®-Cp, IDK®-A12 and IDK®-Cp assays (tables 4.3 and 4.4) showed %CVs of < 20%. The LoQ derived from the moderately

elevated pooled concentrations were found to be 3615 and 522 ng/mL for BMN®-Cp assay and IDK®-A12 assay respectively, and from pooled control (normal) serum samples to be 2880 ng/mL for IDK®-Cp assay. All imprecision was at an acceptable %CV criterion of < 20% and the LoQ > LLoD (Armbruster and Pry, 2008; CLSI, 2004a).

ELISA Kit	n	Mean BLANK (A12/CALP.)	SD BLANK	Cal. LoB	Mean (LOW CONC. OF SPIKED SAMPLE)	SD (LOW CONC. OF SPIKED SAMPLE)	Cal. LLoD
IDK®-A12 (ng/mL)	10	0.319	0.177	0.673	0.517	0.223	1.119
IDK®-Cp (ng/mL)	10	0.539	0.303	1.145	0.436	0.244	1.633
BMN®-Cp (µg/mL)	10	0.545	0.016	0.577	0.533	0.010	0.597

Table 4.13 – Analytical sensitivity data. Calculated values for limit of the blank (LoB) and the lower limit of detection (LLoD); n = the number of replicates.

4.6.6 Assay carry over

4.6.6.1 Method

The concentration of serum samples used to evaluate carry over assay were 393 ng/mL (for IDK®-A12 assay), 3903 ng/mL (for IDK®-Cp assay) and 14806 ng/mL (for BMN®-Cp assay). In order to determine the concentration of calprotectin or S100A12 carried over into the blank sample (incubation or sample buffer), each of the serum samples were individually placed in the microtitre well preceding the two neighbouring wells that contained the sample/incubation buffer blank in the following sequence: HBB, where H is the

serum sample with high calprotectin or high S100A12 concentration and B is the blank according to a protocol devised by Haeckel (1988).

Measurements were repeated in duplicate (n = 4). Consequently, any significant cross–contamination (carry over) of calprotectin or S100A12 between the wells during the ELISA washer purge step and plate–wash cycle could be detected in the blank (sample or incubation buffer) solution. Any cross contamination was regarded as being significant when the mean calprotectin or S100A12 concentration measured in the two blank replicates (i.e., BB) was greater than the previously calculated LoB for the assay in section 4.6.5.5.

4.6.6.2 Result

A significant level of assay carry over from one microtitre well to a neighbouring one is confirmed if the mean serum calprotectin or serum S100A12 levels in the blank (sample or incubation buffer) solution expressed as LoB of carry over assay (LoB^{c}) are greater than the LoB of sample or incubation buffer ($LoB^{§}$) for the particular assay previously determined in the above section 4.6.5.5.

As shown in the dataset of table 4.14, no concentration of serum calprotectin or serum S100A12 was detected at or above the respective $LoB^{§}$ in the neighbouring wells that followed the elevated samples. This confirms that no significant carry over was detected ($LoB^{c} < LoB^{§}$) in all the three methods.

ELISA Kit	n	Mean Concentration		SD^C	Calculated Value		Result
		LOW CONC. OF SPIKED SAMPLE	CARRY OVER BLANK (S100A12/CALPRO.)		LoB^C	$LoB^§$	
IDK®-A12 (ng/mL)	4	0.517	0.251	0.086	**0.424**	**0.673**	**LoB^C < $LoB^§$**
IDK®-Cp (ng/mL)	4	0.436	0.778	0.095	**0.968**	**1.145**	**LoB^C < $LoB^§$**
BMN®-Cp (µg/mL)	4	0.533	0.517	0.026	**0.569**	**0.577**	**LoB^C < $LoB^§$**

Table 4.14 – Limit of the blank (calculated from sample/incubation buffer) versus limit of the blank (determined from the carry over assay). The mean concentration of serum calprotectin and serum S100A12 of low spiked sample and 'carry over blank', SD of 'carry over blank' (SD^C), their respective calculated LoB from section 4.6.5.5 and results. LoB^C = Limit of the Blank of carry over assay; $LoB^§$ = Limit of the Blank of sample/incubation buffer as was previously determined in section 4.6.5.2 above for the particular assay protocol; n = the number of replicates.

4.6.7 Assay Drift

4.6.7.1 Method

Good laboratory practice (GLP) requires that assay development and validation, includes evaluation for possible drift before its introduction into routine clinical laboratory use.

Specifically, and as part of routine practical efforts to uncover assay drift, the quality control materials (supplied by ELISA kits manufacturers) used in this study were allocated to different positions at the beginning and towards the end of the microtitre plate for each particular run of calprotectin and S100A12. Level 1 (low) internal quality control material was placed at positions C2 and

G12 while level 2 (high) internal quality control material was placed at positions D2 and H12 on the 96–well microtitre plate that was used for ELISA template.

4.6.7.2 Result

There was no drift in the results of both levels (low and high) of internal quality control materials recorded after a particular run for calprotectin and S100A12.

4.7 Discussion

4.7.1 Serum calprotectin and serum S100A12

BMN®-Cp manufactured by Bühlmann Laboratories AG, Baselstrasse 55, CH – 4124 Schönenbuch, Switzerland, IDK®-Cp and IDK®-A12 manufactured by Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany assays demonstrated good analytical performance on validation.

Serum BMN®-Cp results were 1.7–fold higher than serum IDK®-Cp consistent with other studies reporting between–assay variability of faecal calprotectin ELISA assay kits (Whitehead et al., 2013). Reasons for this include a possible difference in assay antibodies and assay format. The BMN®-Cp and IDK®-Cp assays use a similar type of detection antibody (monoclonal vs. monoclonal) but were of different origins (horse vs human respectively) and different assay format (sandwich vs. two–site sandwich respectively). The lack of agreement in the results of serum calprotectin determined by both methods (i.e., BMN®-Cp and IDK®-Cp assays) indicate that absolute results of serum calprotectin are not interchangeable between methods. The upper reference ranges of BMN®-Cp (>3900 ng/mL) and serum IDK®-Cp (>3000 ng/mL), however, reflect

this 1.7–fold difference and therefore calprotectin results from both assays relative to their reference ranges may be usefully compared.

In the present study, measurement of S100A12 in serum was adapted from the S100A12 ELISA kit for the *in–vitro* determination of S100A12 in stool. Quantification of serum S100A12, in the present study, was not chosen for further optimisation and application in clinical studies of IBD since there were no reference ranges provided by Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany. We did not determine reference ranges for serum S100A12 as this was beyond the scope of the present study. The performance characteristics of Immunodiagnostik™ S100A12 ELISA K 6938 assay was, however, validated against characteristics criteria provided by the Immunodiagnostik™ AG as the assay manufacturer.

4.7.2 Method validation for serum calprotectin and serum S100A12

A limiting factor in the evaluation of performance characteristics of the three ELISA assays: BMN®-Cp, IDK®-Cp and IDK®-A12 was the smaller than desired number of samples available for measuring serum calprotectin and serum S100A12 and high ELISA kit costs. All assays, however, were fully validated for the quantitation of serum calprotectin and serum S100A12 and showed good performance that compared favourably with assay parameters supplied by their respective kit manufacturers. Linearity (parallelism), recovery and imprecision studies indicate assays to be linear, accurate and reproducible. Due to unavailability of calprotectin and S100A12 analogues, analytical specificity of the ELISA kits for calprotectin and S100A12 assays could only be demonstrated by dilution linearity (parallelism) and spiking recovery of calprotectin and S100A12. However, as more S100 protein family and/or

analogues may become available in future, analytical specificity of calprotectin and S100A12 ELISA assays may need to be further evaluated.

4.7.3 Analytical sensitivity

Analytical sensitivity or LLoD for IDK®-A12 (1.119 ng/mL), IDK®-Cp (1.633 ng/mL) and BMN®-Cp (597 ng/mL) were calculated with a working range from 6.73 to 540 ng/mL for IDK®-A12, 114.5 to 25000 ng/mL for IDK®-Cp and 57700 to 2400000 ng/mL for BMN®-Cp. The lowest standard of 0 ng/mL, however, was not consistently detectable by IDK®-Cp and IDK®-A12 serum assays. The next higher standard for IDK®-Cp (3.90ngm/L) and IDK®-A12 (0.66 ng/mL) were consistently measurable. Thus, the practical sensitivities of the assays were set at 4 ng/mL for IDK®-Cp and 1 ng/mL for IDK®-A12, and the working ranges were defined as 4 to 250 and 1 to 54 ng/mL for IDK®-Cp and IDK®-A12 respectively.

Taking into account the dilution of serum samples to a factor of 1 in 100 for IDK®-Cp and 1 in 10 for IDK®-A12, this translates into a practical working range of 400 to 25000 ng/mL (IDK®-Cp) and 10 to 540 ng/mL (IDK®-A12) for serum samples. Similarly, the lowest standard of 4 ng/mL was not consistently detectable by the BMN®-Cp assay, while the next higher standard of 12 ng/mL was consistently measurable and therefore the practical sensitivity of BMN®-Cp assay was set at 12 ng/mL and the working range was defined as 12 to 240 ng/mL for serum samples. Considering that the serum samples were diluted to a factor of 1 in 100, this translates into a practical working range of 1200 to 24000 ng/mL for serum samples.

These adjusted or practical working ranges for the IDK®-Cp, IDK®-A12 and BMN®-Cp assays shows a wide range suitable for routine clinical laboratory

practice. The LLoD for serum IDK®-Cp and BMN®-Cp are adequate when considered against their respective upper limit of the manufacturer provide reference intervals (>3000 ng/mL for IDK®-Cp and >3900 ng/mL for BMN®-Cp). As previously stated, the reference interval for IDK®-A12 assay was neither supplied by the kit manufacturer nor determined as part of the analytical validation process for the ELISA kits in the present study.

The standards (calibrators) used in the present study include the same lyophilised materials in five ampoules of varying concentrations of calprotectin (i.e. 0, 3.9, 15.6, 62.5 and 250.0 ng/mL for IDK®-Cp assay; 0.4, 1.2, 4.0, 12.0 and 24.0 µg/mL for BMN®-Cp assay) that had been used to calibrate the BMN®-Cp and IDK®-Cp assays, and the practical working range in the present study is comparable to that reported by their respective manufacturers, making the data comparable, thus facilitating data analysis and interpretation.

4.7.4 Functional sensitivity

Functional sensitivity or LoQ is the lowest concentration of an analyte that may be discriminated from zero with a high degree of confidence and it is reported as the lowest analyte value whose %CV is < 20% (Shrivastava and Gupta, 2011). In all cases and at an acceptable criterion of < 20 %CV, the LoQ is greater than LLoD (LoQ > LLoD). In the present study, the LoQ for the three ELISA Kits was reported as 3615 ng/mL (BMN®-Cp assay), 522 ng/mL (IDK®-A12 assay) and 2880 ng/mL (IDK®-Cp assay) and this satisfied the 'fit for purpose' criteria of analytical methods as the corresponding values for LLoD were 597 ng/mL (BMN®-Cp assay), 1.119 ng/mL (IDK®-A12 assay) and 1.633 ng/mL (IDK®-Cp assay).

4.7.5 Linearity of dilution (Parallelism)

All three assays were linear between 840 and 15380 ng/mL, which will provide robust comparison within the analytical range of between 10 and 25000 ng/mL that cut across the linearity bracket provided by the commercially available ELISA kit manufacturers.

In the present study, a mean value of 103.7%, 98.4% and 100.8% for Measured to Expected ratios for dilution linearity (parallelism) for IDK®-A12, IDK®-Cp and BMN®-Cp assays respectively, compared favourably with the kit manufacturers' provided mean data of 91.8%, 95.8% and 97.8% for IDK®-A12, IDK®-Cp and BMN®-Cp assays respectively, and these values are within the targeted Measured to Expected ratios of between 80% to 120% acceptance criteria for immunoassays.

Measured to Expected ratios for dilution linearity (parallelism) ranged between 71.4% and 148.8% for the three assays. Some values were outside the range of 80% to 120% acceptance criteria for immunoassays. The two highest values of 148.8% and 134.8% were observed for the sample with the lowest S100A12 concentration of 840 ng/mL (i.e., 1 in 1 or neat dilution), suggesting that the S100A12 assay has a limited linearity in the lower limit of the working range (i.e., 10 to 540 ng/mL). Nevertheless, for the assessment of IBD, it would be expected that serum S100A12 values would be in the upper rather than the lower area of the working range and would not affect the clinical usefulness of serum S100A12. In conclusion, the assays are linear within the analytical range for clinical application, with decreased linearity for extremely low and extremely high serum concentrations of calprotectin and S100A12.

4.7.6 Recovery

Recovery for IDK®-Cp assay (89.5%) and BMN®-Cp assay (82.1%) were within accepted target criteria of 80% to 120% for immunoassays (Tables 4.6 and 4.7). Recovery for IDK®-A12 assay (126.5%) was acceptable even though just outside the target criteria (Table 4.5). These recoveries compare favourably to those provided by the assay manufacturers, i.e., 95.8%, 98.7% and 94.4% for IDK®-Cp, BMN®-Cp and IDK®-A12 assays respectively.

Recovery of calprotectin with the IDK®-Cp assay was adequate at all concentrations studied. Recovery at low concentrations of calprotectin with the BMN®-Cp assay was just outside the target criteria but acceptable because it would not affect the clinical utility of the assay in detecting inflammation due to IBD. Similarly, over–recovery of S100A12 at low concentrations with IDK®-A12 assay would not affect the clinical utility of the assay in detecting inflammation due to IBD.

4.7.7 Imprecision

It is generally recognized that intra–assay (within–batch) and inter–assay (between–batch) variability for immunoassay of less than 10% to 20% are acceptable. In the present study, intra–assay and inter–assay imprecision were all less than 10% and compared favourably to manufacturers' supplied imprecision values. This reinforces the accuracy of the three assays for clinical usefulness.

4.7.8 Analytical interference and Stability

Serum samples may be haemolysed, icteric or lipaemic. The potential effects of these factors were not investigated but icteric, lipaemic and haemolysed

samples were excluded. The assay protocols, however, require dilution of samples which may provide protection from these interferences.

The shelf–life of the ELISA kit components used in the present study was over 12 months of refrigerated storage. The fact that the values of the calibrator for calprotectin and S100A12 remained constant during the course of the over 12–month shelf–life of the assays indicated that the reagent and assays were stable.

The effect of sample stability and repeated freeze–thaw cycles on calprotectin and S100A12 assays was not investigated in the present study. Previous studies have been reported that calprotectin and S100A12 are stable in serum samples when stored frozen at –20^{o}C for at least 6 months (Larsen et al., 2007a). Larsen et al (2007a), however, reported that storage at ambient temperature may give a 6–fold or greater increase in calprotectin and S100A12 concentrations. Caution must, therefore, be exercised in analysing old samples not appropriately stored. Larsen et al (2007a) also reported that repeated freeze–thaw cycles did not alter the analytes concentrations in blood. This was supported from good inter–assay (between–batch) imprecision reported in this study.

Data by Larsen et al (2007a) that evaluated the pre–analytical handling of calprotectin and S100A12 in serum assays is relatively old. It is, therefore, imperative that future clinical studies on calprotectin and S100A12 in serum using current immunoassay kits should evaluate the influence of storage conditions and stability on these assays at different temperatures: 18 – 25°C (ambient), 4°C (in the refrigerator), –20°C (in the freezer), –80°C (in the ultra low temperature (ULT) freezer)), –150°C and lower (in a liquid nitrogen

cryogenic tank or freezer) and for varying periods (Hedayati et al., 2020; Kachhawa et al., 2017; Cuhadar et al., 2013).

4.8 Conclusion

The present study has shown that serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 assays are suitable assays for routine application in a clinical laboratory. The performance characteristics were robust and sensitive from limit of the blank (LoB), lower limit of detection (LLoD) or analytical sensitivity, limit of quantitation (LoQ) or functional sensitivity and carry over studies. The three assays are reliable from the results of imprecision, reproducibility, spiking recovery and linearity of dilution or parallelism experiments, and compared favourably to manufacturer provided performance characteristics. The large difference in numerical values between serum BMN®-Cp and serum IDK®-Cp concentrations indicate that the results and any derived cut–offs between the assays are not directly inter–changeable.

CHAPTER 5

CLINICAL STUDY I: DIAGNOSIS OF INFLAMMATORY BOWEL DISEASE

5.1 Introduction

The distinction between IBD and IBS is made using faecal and recently but to a lesser extent serum laboratory biomarkers of inflammation to reduce the need for more invasive and expensive endoscopies (Cury et al., 2013). Current diagnostic procedures of IBD rely heavily on faecal biomarkers as heterogeneous groups of biomolecules that either drip from or are actively released by inflamed mucosal cells, activated neutrophils or fast–separating cells following divergent episodes of gastrointestinal tract inflammation (Lehmann et al., 2015; Vermeire et al., 2006; Lewis, 2011; Diamanti et al., 2010; WGO, 2009).

Calprotectin and S100A12 are conventional biomarkers of IBD. Both are members of the calcium–binding, low molecular weight S100 protein family of Calgranulins that are soluble in a 100%–saturated solution of ammonium sulphate at neutral pH (Cury et al., 2013; Meijer B et al., 2012; Hare et al., 2013). As proinflammatory proteins released by neutrophils in the gut of patients with CD and UC (Foell et al., 2003b; Däbritz et al., 2013), both calprotectin and S100A12 trigger important extracellular activities that contribute towards the immune responses (Manolakis et al., 2010).

Faecal calprotectin has been used in most IBD studies as the 'gold standard' against which faecal biomarkers are benchmarked (Lehmann et al., 2015). Faecal calprotectin has higher sensitivity and specificity compared to blood

parameters like CRP and ESR, and is a better diagnostic biomarker of IBD (Tibble et al., 2000b; Schoepfer et al., 2008; Henderson et al., 2014).

Faecal calprotectin, like most biomarkers, has limitations. These include a lack of specificity, intra–individual and inter–individual variation, lack of standardised cut–offs, an overlap between disease and non–disease cohort, between method bias and patient inconvenience. Increased concentrations of faecal calprotectin occur not only in IBD, but also in other inflammatory and gastrointestinal pathological conditions including infective colitis, microscopic colitis, eosinophilic colitis, adenomas and colorectal cancer (Khaki–Khatibi et al., 2020; Ricciuto and Griffiths, 2019; Laserna–Mendieta and Lucendo, 2019; Caviglia et al., 2018; Manceau et al., 2017; Manz et al., 2012; Ayling and Kok, 2018; Sipponen and Kolho, 2015; Johnson et al., 2008). In addition, there are several non–pathological conditions that can lead to altered faecal calprotectin values (Caviglia et al., 2018; Waugh et al., 2013). Faecal calprotectin levels are dependent on age and clinical comorbidities which vary considerably every 24 hours (Røseth et al., 1992).

A substantial day–to–day variation of levels of calprotectin in the stool of patients presenting with IBD (Husebye et al., 2001), suggests that marked intra–individual variability of faecal calprotectin concentration may be influenced by factors other than disease condition (Naismith et al., 2013; Moum et al., 2010; Lasson et al., 2014). Inter–individual biological variation has been reported to be high for faecal calprotectin, and spot variability during multiple sampling from the same faecal collection (Husebye et al., 2001). Others include a lack of standardised faecal calprotectin optimal cut–offs to distinguish IBD from IBS, characterize disease activity, predict clinical

remission, and assess response to treatment (Burri and Beglinger, 2012; D'Inca et al., 2007; Tibble et al., 2000b).

There exists a significant overlap in faecal calprotectin levels between 50 – 150 µg/g stool in patients with IBD and IBS (Lehmann et al., 2015). Quantitative measurement of faecal calprotectin by ELISA is well–established, and commercially prepared ELISA kits are available for routine clinical laboratory use. Variations in kit performance characteristics, however, have been reported (Burri et al., 2013). Assays employing monoclonal antibodies compared to those using polyclonal antibodies have superior assay performance including accuracy, imprecision, functional sensitivity, recovery, linearity of dilution, assay drift, stability, and interference.

ELISA kit manufacturers may also select diseased and non–diseased groups to demonstrate maximal assay performance and to occasionally justify commercial interest (Delefortrie et al., 2016; Oyaert et al., 2017; Oyaert et al., 2014; Jones and Payne,1997), and their evaluation consequently may not necessarily reflect the performance in routine use (Nilsen et al., 2016; Labaere et al., 2014; Jones and Payne,1997). Finally, the reluctance of patients to provide stool samples makes is another potential limitation (Vermeire et al., 2006; Fengming and Jianbing, 2014; Judd et al., 2011; Lehmann et al., 2014).

These issues may be overcome by using serum samples for the measurement of calprotectin and S100A12 (Baran and Karaca, 2013; Lehmann et al., 2015; Sunkara et al., 2011; Xavier and Podolsky, 2007). This chapter reports on an evaluation of serum calprotectin (using BMN®-Cp and IDK®-Cp assays) and serum S100A12 (using IDK®-A12 assay) in the diagnosis of IBD.

5.1.1 Assessing assay diagnostic performance

The overlap of biomarker concentrations, such as calprotectin, between healthy populations and diseased populations such as patients with IBD and without IBD, explains the sensitivity and specificity of a test being less than 100% (Jones and Payne, 1997). To improve diagnostic performance, it has been suggested that inclusion of clinical data in the calculation of test results should be considered to reduce the basis for the selection of a diagnostic method in clinical studies (Bossuyt, 2009; Garcia–Romero et al., 1996).

The predictive power of a diagnostic test predicts the probability that the patient does or does not have the disease (e.g., IBD) in question. Whilst the positive predictive value (PPV) is the index that expresses the proportion of patients with a positive test result who are correctly diagnosed as disease positive, the negative predictive value (NPV) is the index that expresses the proportion of patients with a negative test result who are correctly diagnosed as being disease free (Jones and Payne, 1997) but this is related to disease prevalence (WGO, 2009; Silicia and Saro, 2008; Soon et al., 2012). Therefore, a measure of the efficiency of a diagnostic test procedure lies in its ability to correctly categorise patients into those with the disease (e.g., IBD) by using the index referred to as the positive likelihood ratio (PLR) and those without the disease (e.g., non–IBD) as the negative likelihood ratio (NLR).

5.1.2 Aim of this chapter

The aim of the study presented in this chapter was to investigate the diagnostic potential of serum calprotectin and serum S100A12 as biomarkers to replace or supplement the utility of faecal calprotectin in the assessment of IBD.

5.2 Patients and Methods

5.2.1 Comparison of results of faecal calprotectin, serum calprotectin and serum S100A12 assays in 40 patients with IBD

5.2.2 Patients Recruitment and Study Design

Two groups of patients were included in the clinical study. The first group were patients with IBD whose stool samples were received as part of routine sample delivery and processing in the clinical chemistry laboratory at New Cross Hospital Wolverhampton. The second group were healthy control patients. Details about their sampling characteristics and profile are supplied in section 5.2.2.2 below.

5.2.2.1 Patients with inflammatory bowel disease

Forty patients were recruited under this group. Of these, 21 were males and 19 were females. These participants were aged between 18 and 85 years, with an average age of 43 years and a standard deviation (SD) of 18.0. The rest of the statistics including frequency of participants' demographics and percentages are shown in table 5.1.

Patients presenting at the twice–weekly IBD Clinic of the Department of Gastroenterology, New Cross Hospital Wolverhampton scheduled for faecal calprotectin assay were included. Inclusion criteria were based on symptoms contained in the guidelines set out in the Department of Clinical Chemistry’s standard operating procedure (SOP) for requesting faecal calprotectin measurement to exclude IBD. These are symptoms associated with chronic diarrhoea (> 6 weeks duration). Data were also collected on rectal bleeding, patients > 45 years old, unplanned weight loss, presence of abdominal or rectal mass and anaemia. In these patients, endoscopy is the recommended first line

investigation after exclusion of infection. Another exclusion criterion was current therapy with non–steroidal anti–inflammatory drugs (NSAIDs).

5.2.2.2 Control Patients

Five patients were recruited under this group. Of these, 2 were males and 3 were females. These participants were aged between 13 and 83 years, with an average age of 37 years and a standard deviation (SD) of 26.8. The rest of the statistics including frequency of participants' demographics and percentages are shown in table 5.1.

Healthy controls were defined as those without any criteria listed in IBD and acute phase response (APR) patients in whom there were no chronic diseases, and who were known or postulated to have increased systemic concentrations of calprotectin or S100A12. The healthy controls were selected from among those requests from primary care practices scheduled for routine annual general check–up that met the exclusion criteria of both IBD and APR patients. In addition, their CRP results were less than or equal to five milligram per litre (≤ 5 mg/L).

Patient Group	N	Frequency		Percent (%)		Age (years)			
		M	F	M	F	Min.	Max.	Mean	SD
IBD	40	21	19	54	46	18	85	43	18.0
Controls	5	2	3	40	60	13	83	37	26.8
Total	45	23	22						

Table 5.1 – Demographics of study participants. The Age and gender: male and female distribution of 40 IBD patients and 5 healthy control participants that were recruited for the analytical validation of serum calprotectin and serum S100A12 using Bühlmann Laboratories AG and Immunodiagnostik™ AG ELISA Kits.

5.3 Sample Collection and General Serum Preparation

Five millilitres (mL) of blood were collected into plain tubes: BD Vacutainer SST Advance Tubes (Medisave UK Limited, Weymouth, Dorset, UK), with great care taken to avoid haemolysis. This was mixed severally by gentle inversion of the sample tube and then left on the laboratory bench at ambient temperature (18–25°C) for not less than 60 minutes to clot while being protected from light. Thereafter it was centrifuged at a speed of 1800 x g for 15 minutes at ambient temperature (18–25°C). The supernatant (serum) was collected and stored at –80ºC. The whole process of separating the serum from centrifugation, aliquoting and up to storage occurred within 2 hours after the phlebotomy exercise.

Although variations in ambient temperature in the clinical chemistry laboratory did not occur throughout the duration of this study, it was still important to define ambient temperature as 18–25ºC. All serum samples used in this study met the above stated criteria.

5.3.1 IBD Samples

Serum samples used in this study were obtained from patients who were confirmed as positive IBD cases based on the result of their faecal calprotectin assay. The department of clinical chemistry at New Cross Hospital Wolverhampton uses Bühlmann fCAL™ ELISA Calprotectin method for the in–house measurement of faecal calprotectin. This method reports a faecal calprotectin result of greater than two hundred microgram per gram stool (> 200 µg/g stool) for positive IBD case.

Five mL of serum were collected from both patient groups for this study. Unless indicated otherwise, all serum samples were stored at –80ºC until analyses. The serum samples were however allowed to thaw slowly and equilibrate at ambient

temperature (18–25°C) for at least two hours prior to being assayed for calprotectin or S100A12 on the DS2 by the appropriate method.

Part of the pre–analytic handling of sample collection and preparation include taking extreme care to collect only serum samples that were used for this study. This was to avoid the inclusion of plasma samples based on two key reasons: (a) plasma calprotectin levels are 10–fold lower compared to serum levels. With serum levels, there is the presence of granulocyte–activating biomarkers that are released when granulocytes are activated during serum clotting and (b) there is a variation in plasma calprotectin levels due to variation in the time between sampling and analysis, and the cumulative effect of the number of freezes–thaw cycles while the assay procedure lasted. Since the pre–analytical conditions that relate to the use of plasma samples cannot be absolutely guaranteed to be held constant, it is most advisable to exclude plasma samples as part of the efforts to maintain good pre–analytical handling requirements (Larsen et al., 2007a). This is a general requirement to avoid significant differences in the levels of calprotectin that can be observed due to different sample preparation procedures, and this is independent of the assay procedure used (Lönnkvist et al., 2011; Börekçi et al., 2009; Larsen et al., 2007a).

However, the choice of serum samples was best explained by the fact that the time between serum collection and analysis as well as repeated freeze–thaw cycles do not cause any variation in the observed calprotectin levels regardless of the test method employed (Larsen et al., 2007a). Both Bühlmann (Bühlmann Laboratories AG, Baselstrasse 55, CH – 4124 Schönenbuch, Switzerland) and Immunodiagnostik™ (Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) recommended the use of serum samples for calprotectin determinations.

As a general rule, both lipaemic and haemolysed samples were not included in this study because of their tendency to give erroneous results when analysed.

5.3.2 Control Samples

Five mL of serum sample was collected from individual patient. These were then pooled, mixed properly by placing them for not less than 120 minutes at ambient temperature (18–25°C) on Stuart SRT9 tube roller mixer, with 9 roller and fixed speed of 33 revolutions per minute (rpm) measuring 340 mm in length and 30 mm in diameter, connected to a 230V 50 Hz a.c. They were then divided into aliquots and stored away at –80C until analysis.

5.4 Method

The experimental procedures that provided details of principles of serum calprotectin and serum S100A12 assays, test procedures for both IBD and control samples, and assay working range were the same as those set out in Chapter 4 under section 4.5. Serum calprotectin results above the measuring range of the assay were diluted with Incubation Buffer (Code: B–MRP8/14–IB, for Bühlmann BMN®-Cp assay) or Sample Buffer (Cat. No. K 6935; Label: SAMPLEBUF, for Immunodiagnostik™ IDK®-Cp assay) as appropriate, and re–assayed to enable quantitation. Serum S100A12 results above the measuring range of the S100A12 assay were diluted with Sample Buffer (Cat. No. K 6938; Label: SAMPLEBUF, for Immunodiagnostik™ IDK®-A12 assay) and re–assayed to enable quantitation.

5.4.1 Statistical Analysis

Data processing and statistical analysis were performed as described in Chapter 4.

The faecal calprotectin assay was linear between 20 and 1932 µg/g and results <20 and >1932 µg/g were arbitrarily assigned a value of 20 and 1932 µg/g respectively, for statistical purposes. Since the data were non–parametric (Kolmogorov–Smirnov and Shapiro–Wilk tests), data are expressed as medians with inter–quartile ranges (IQR). Spearman rank–order correlation was used to measure the degree of association between variables. Spearman's rank–order values (r) between 0.5 and 1.0 indicate a good correlation. A p–value of < 0.05 was considered statistically significant. Linearity was assessed by linear regression analysis or Passing–Bablok (Passing and Bablok, 1983).

The receiver operating characteristic (ROC) curve and the area under the curve (AUC) analysis was used to evaluate the diagnostic performance of serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 compared to faecal calprotectin, the in–house reference method for the diagnosis of IBD. An assay with an AUC value of ≥ 0.7 was considered relevant for the diagnosis of IBD (DeLong et al., 1988),

Agreement between interpretative results of faecal calprotectin and serum calprotectin was evaluated using concordance tables. The kappa statistic (k_w) (Sim and Wright, 2005) was applied to determine the diagnostic agreement between faecal calprotectin results and the results of serum BMN®-Cp and serum IDK®-Cp assays. The k_w values between 0.00 – 0.20, 0.21 – 0.40, 0.41 – 0.60, 0.61 – 0.80 and 0.81 – 1.00 were classified as poor, fair, moderate, good, and very good respectively.

5.4.2 Results

5.4.2.1 Descriptive statistical representation of concentrations of faecal calprotectin, serum calprotectin and serum S100A12 in 40 patients with IBD

The results of the cross–kit comparison for faecal calprotectin, serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 assays in 40 patients with IBD to evaluate the diagnostic performance of the serum assays in the assessment of IBD are presented in table 5.2. Median faecal calprotectin was 297 µg/g (IQR: 102 – 1454 µg/g). Median serum concentration of BMN®-Cp, 5428 ng/mL (IQR: 3728 – 8603 ng/mL) was higher (p = 0.0001) than median serum concentration of IDK®-Cp, 3254 ng/mL (IQR: 2085 – 4606 ng/mL). Median serum concentration of S100A12 was 412 ng/mL (IQR: 321 – 565ng/mL).

Parameters	fCAL™ (µg/g)	BMN®-Cp (ng/mL)	IDK®-Cp (ng/mL)	IDK®-A12 (ng/mL)
N	40	40	40	40
Median	297	5428	3254	412
Minimum	47	1290	675	202
Maximum	1932	22743	12808	1285
IQR	102–1454	3728–8603	2085–4606	321–565
Reference range	**> 200[a]**	**400 – 3900[b]**	**< 3000[b]**	**35 – 1570[c]**

Table 5.2 – Faecal calprotectin, serum calprotectin and serum S100A12 concentrations for 40 IBD patients. [a]In–house reference method. [b]Reference range supplied by kit manufacturer. [c]Reference range quoted in a published study by Larsen et al (2007a). IQR = Interquartile range.

5.5 Method Comparison: Evaluation of the diagnostic performance of serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 assays using Receiver Operating Characteristic curves and Area Under the Curve analyses

The diagnostic performances of serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 assays were compared against faecal calprotectin using ROC curves (figure 5.1) and AUC analyses (table 5.3). ROC AUC analysis indicated that all the three serum assays performed acceptably well (table 5.3). Serum IDK®-Cp performed best followed by serum BMN®-Cp and then serum IDK®-A12.

At the kit manufacturer's (Bühlmann Laboratories AG) recommended diagnostic reference cut–off value of >3900 ng/mL, serum BMN®-Cp assay had a sensitivity of 85% and a specificity of 50% for IBD diagnosis (table 5.4). Similarly, at the kit manufacturer's (Immunodiagnostik™ AG) recommended diagnostic reference cut–off value of >3000 ng/mL, serum IDK®-Cp assay had a sensitivity of 75% and a specificity of 79% for IBD diagnosis.

The analytical performance and diagnostic efficiency of the serum IDK®-Cp assay at an optimum cut–off value of >2500 ng/mL with a sensitivity of 80% and specificity of 65% was comparatively more vigorous to the serum BMN®-Cp assay at an optimum cut–off value of >4200 ng/mL with a sensitivity of 80% and specificity of 58% for the diagnosis of IBD (table 5.4).

The results of the coordinates of the ROC curve analyses and cut–off values with the highest sensitivity and specificity for the Bühlmann MRP8/MRP14 Calprotectin S100A8/S100A9 assay (serum BMN®-Cp), Immunodiagnostik™ Calprotectin ELISA K 6935 assay (serum IDK®-Cp) and Immunodiagnostik™ S100A12 ELISA K 6938 assay (serum IDK®-A12) are contained in Appendix G.

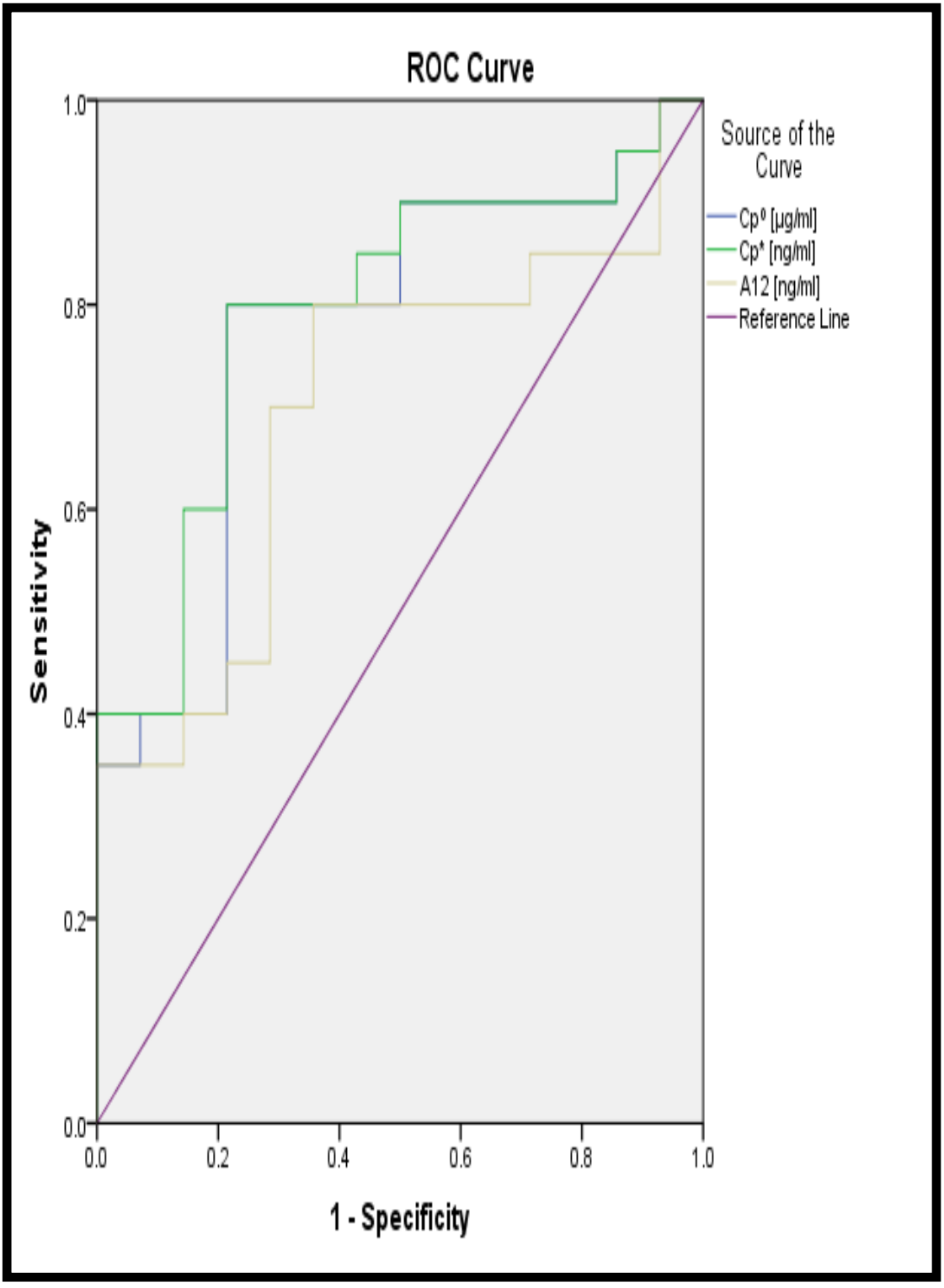

Figure 5.1 – Receiver operating characteristic (ROC) curves of serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 assays for predicting the diagnosis of IBD in 40 patients. Diagnostic performance of the serum calprotectin and serum S100A12 assays: BMN®-Cp (blue line), IDK®-Cp (green line) and IDK®-A12 (yellow line) assays for a common set of serum samples. The reference line is purple.

Test Result Variables	Area Under the Curve (AUC)	Standard Error of the Mean[a]	95 Percent Confidence Interval (95%CI)	
			Lower Bound	Upper Bound
BMN®-Cp (ng/mL)	0.771	0.083	0.608	0.935
IDK®-Cp (ng/mL)	0.793	0.079	0.638	0.947
IDK®-A12 (ng/mL)	0.700	0.092	0.521	0.879

Table 5.3 – Diagnostic utility of serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 in IBD. [a]Under the nonparametric assumption.

BÜHLMANN MRP8/MRP14 CALPROTECTIN S100A8/S100A9 ELISA (FOR SERUM BMN®-Cp ASSAY)			IMMUNODIAGNOSTIK™ CALPROTECTIN ELISA K 6935 (FOR SERUM IDK®-Cp ASSAY)		
Cut–off value (ng/mL)	**Sensitivity (%)**	**Specificity (%)**	**Cut–off value (ng/mL)**	**Sensitivity (%)**	**Specificity (%)**
> 3000	95	15	> 2000	90	43
> 3500	90	36	> 2200	85	58
> 3900	**85**	**50**	**> 2500**	**80**	**65**
> 4200	**80**	**58**	**> 3000**	**75**	**79**
> 4700	80	71	> 3300	65	79
> 5200	75	79	> 3600	60	86

Table 5.4 – The cut–off values, sensitivity, and specificity of the serum BMN®-Cp and serum IDK®-Cp assays for the measurement of serum calprotectin in IBD diagnosis. Kit manufacturer's recommended cut–off value for each assay is highlighted.

5.6 Correlations

Correlations between faecal calprotectin, serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 are shown in table 5.5 and figures 5.2 – 5.7

Faecal calprotectin correlated moderately well with serum BMN®-Cp (*r* = 0.65) and serum IDK®-Cp (*r* = 0.69) but not with serum S100A12 (*r* = 0.44), although all were statistically significant.

Serum IDK®-A12 correlated strongly positive with serum BMN®-Cp (*r* = 0.81) and serum IDK®-Cp assay (*r* = 0.80). Serum BMN®-Cp assay correlated very strongly positive with serum IDK®-Cp assay (*r* = 0.99).

Assay			***r***	***r²***	***p***	***y = mx + c***
fCAL™ (µg/g)	**vs.**	**BMN®-Cp (ng/mL)**	0.65	0.42	0.0001	0.1059*x* + 46.098
fCAL™ (µg/g)	**vs.**	**IDK®-Cp (ng/mL)**	0.69	0.48	0.0001	0.1929*x* – 78.441
fCAL™ (µg/g)	**vs.**	**IDK®-A12 (ng/mL)**	0.44	0.19	0.0001	1.3309*x* + 66.066
BMN®-Cp (ng/mL)	**vs.**	**IDK®-Cp (ng/mL)**	0.99	0.98	0.0004	0.5835*x* – 55.507
BMN®-Cp (ng/mL)	**vs.**	**IDK®-A12 (ng/mL)**	0.81	0.65	0.0001	0.0428*x* + 194.71
IDK®-Cp (ng/mL)	**vs.**	**IDK®-A12 (ng/mL)**	0.80	0.64	0.0001	0.0727*x* + 201.07

Table 5.5 – Spearman's rank–order correlations between fCAL™, serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 assays. Correlation (*r*) was significant at the 0.01 level (2–tailed).

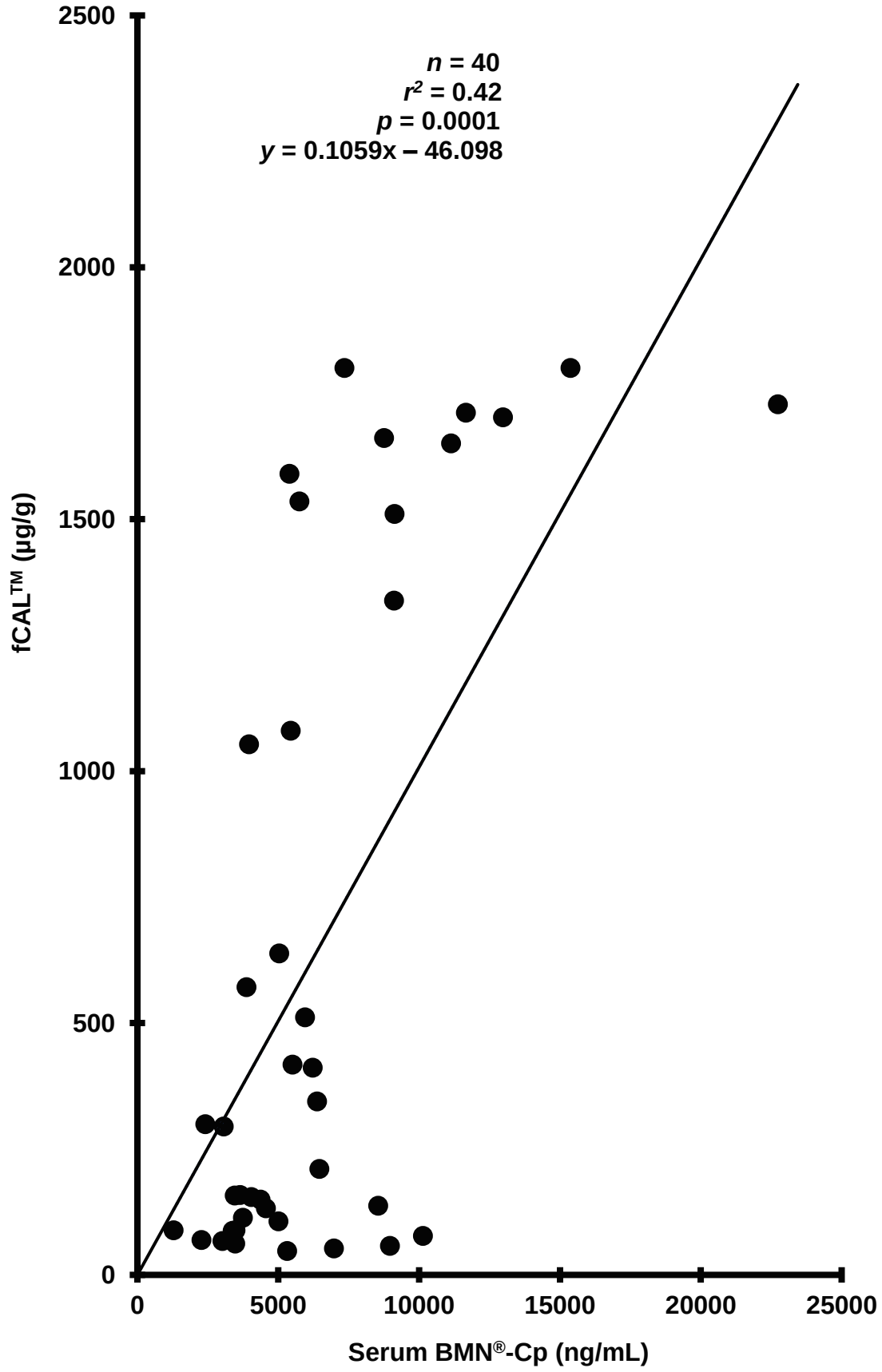

Figure 5.2 – fCAL™ versus serum BMN®-Cp in the diagnosis of IBD.

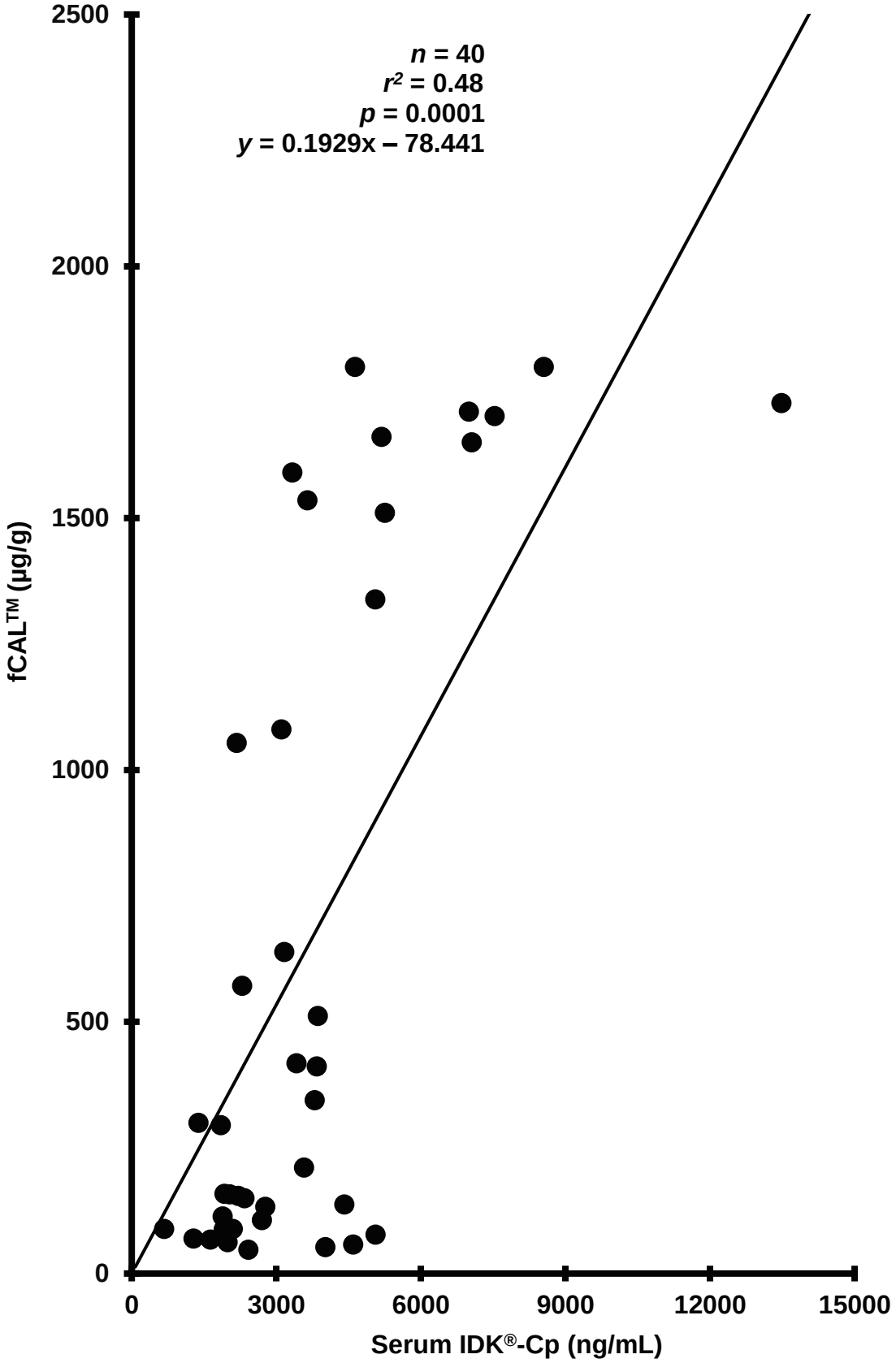

Figure 5.3 – fCAL™ versus serum IDK®-Cp in the diagnosis of IBD.

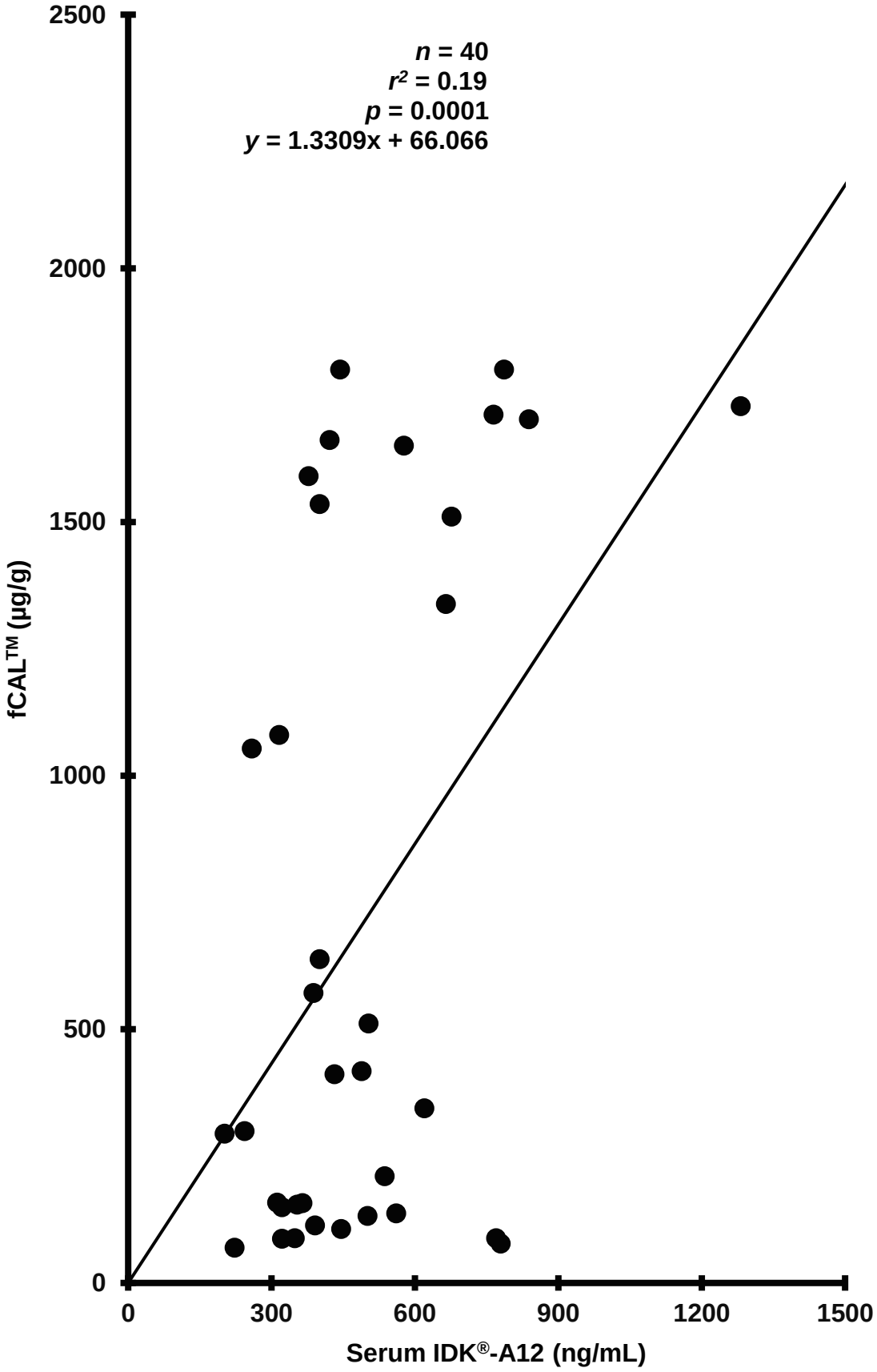

Figure 5.4 – fCAL™ versus serum IDK®-A12 in the diagnosis of IBD.

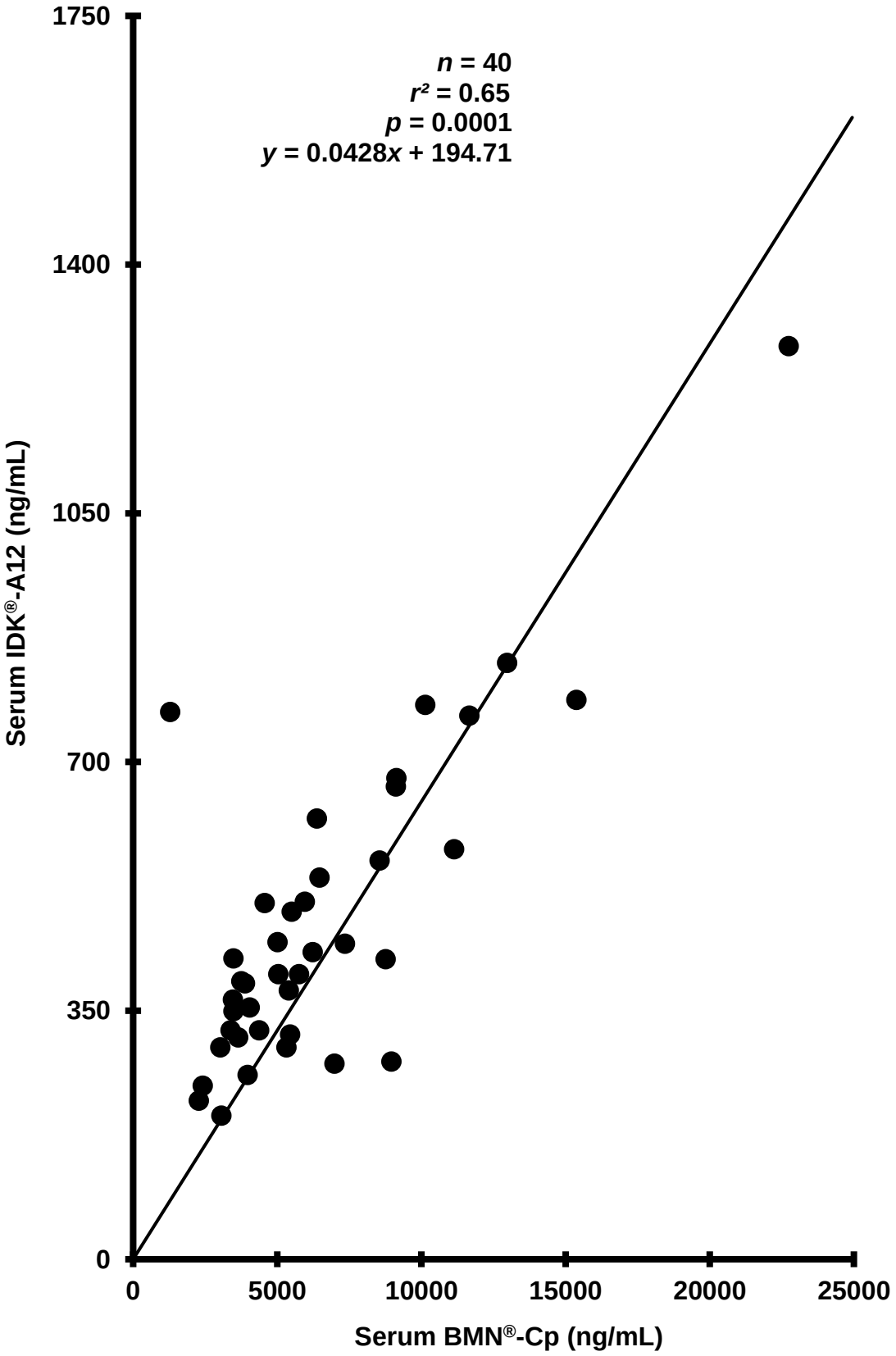

Figure 5.5 – Serum BMN®-Cp versus Serum IDK®-A12 in the diagnosis of IBD.

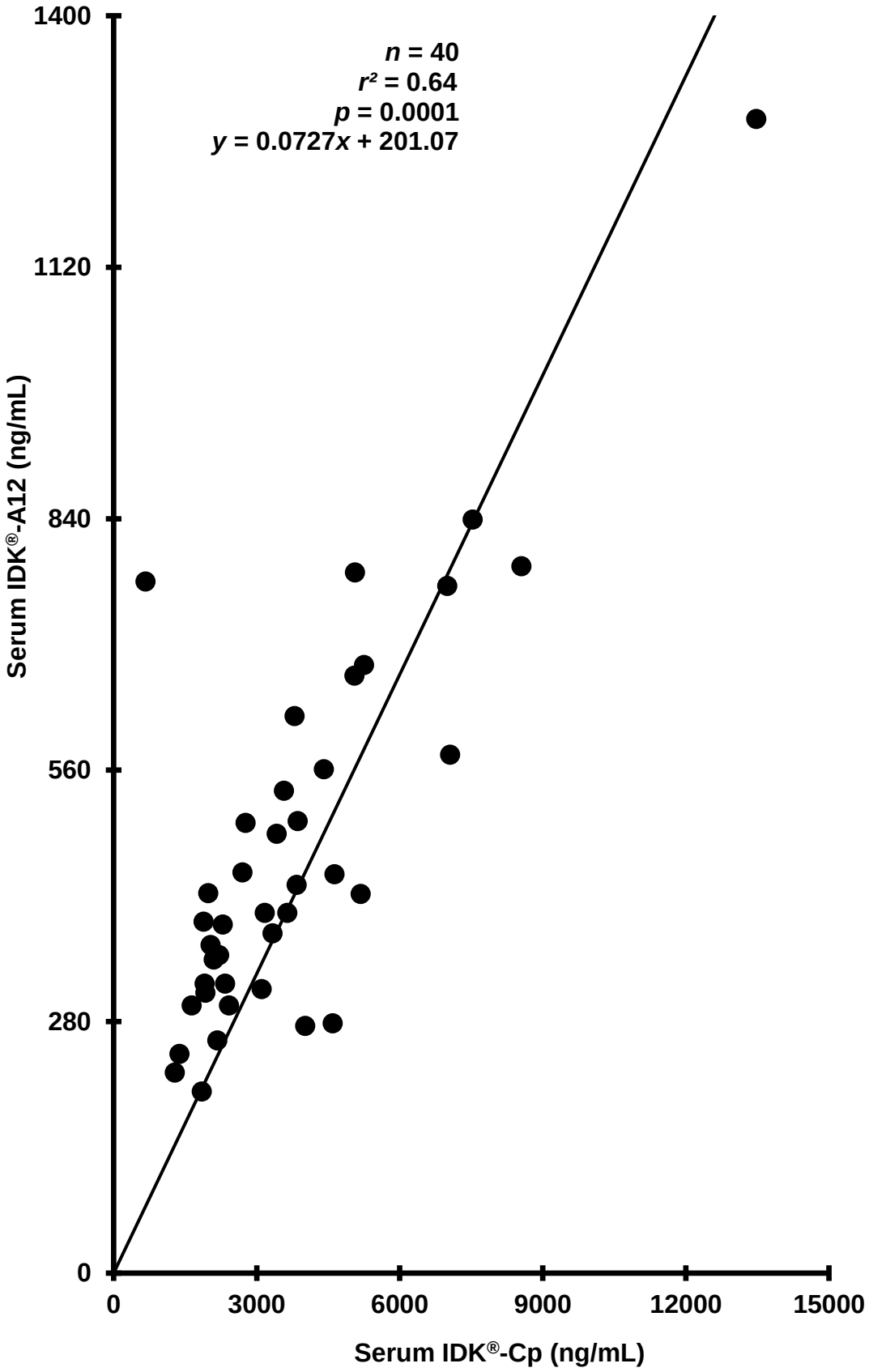

Figure 5.6 – Serum IDK®-Cp versus serum IDK®-A12 in the diagnosis of IBD.

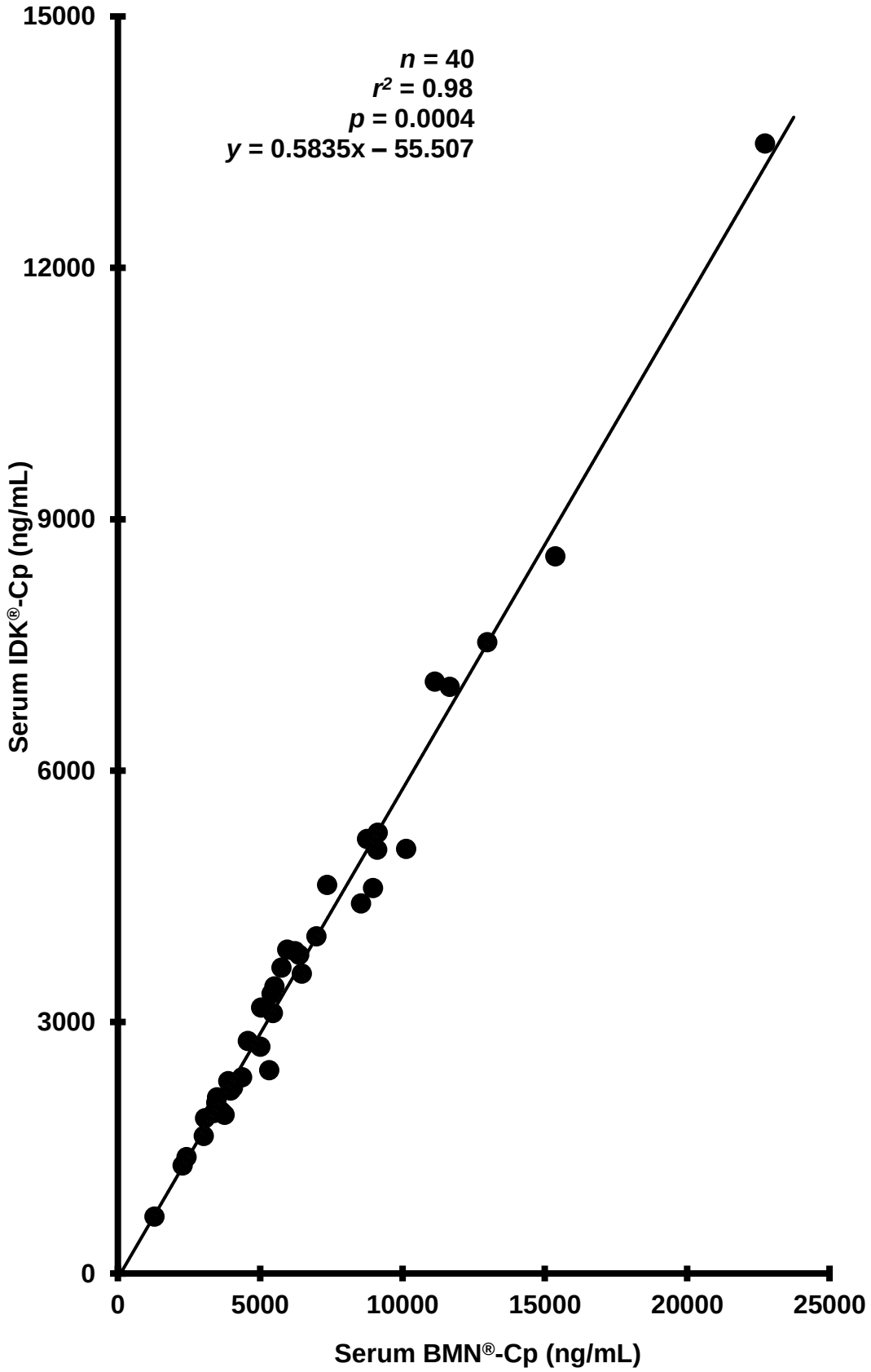

Figure 5.7 – Serum BMN®-Cp versus serum IDK®-Cp in the diagnosis of IBD.

5.7 Concordance between faecal calprotectin and serum calprotectin concentrations analysed by the serum BMN®-Cp and serum IDK®-Cp assays in 40 patients with IBD

The diagnostic concordance between faecal calprotectin (fCAL™), serum BMN®-Cp and serum IDK®-Cp in 40 patients is shown tables 5.6 – 5.8. Diagnostic concordance between faecal calprotectin and serum IDK®-A12 were not studied because diagnostic cut–offs between disease and non–disease cohorts was not provided by the manufacturer. Faecal calprotectin results >200 µg/g stool, 50 – 200 µg/g stool and < 50 µg/g stool were classified as positive, indeterminate, and negative results respectively. Serum BMN®-Cp assay (Bühlmann MRP8/MRP14 Calprotectin S100A8/S100A9 ELISA) and serum IDK®-Cp assay (Immunodiagnostik™ Calprotectin ELISA K 6935) results were classified as positive or negative based on the assay manufacturer's recommended cut–offs of > 3900 ng/mL and > 3000 ng/mL respectively.

The two ELISA kits are formulated by two different manufacturers: Bühlmann Laboratories AG and Immunodiagnostik™ AG. Therefore, both assays (BMN®-Cp and IDK®-Cp) are composed of different concentrations of macromolecules of several parameters that include primary antibody, secondary antibody, calibrators, quality control materials, incubation buffers, wash buffers, TMB substrate and horseradish–peroxidase that accounted for dissimilarities in their analytical performance in respect of orbital shaking at low to medium speed during incubation periods, wash cycles, and measurement protocols to facilitate the push for antigen molecules from a favourable position for antibody binding prior to establishing a bond.

In summary, the Kappa (k_w) statistic of 0.32, indicated a fair agreement between the fCAL™ assay and serum BMN®-Cp assay for the diagnosis of IBD. Similarly,

the k_w statistic of 0.33, indicated fair agreement between the fCAL™ assay and serum IDK®-Cp assay for the diagnosis of IBD. The k_w statistic of 0.70 indicated a good diagnostic concordance agreement between serum BMN®-Cp and serum IDK®-Cp assays. It is worth noting that the k_w value makes no judgement about right or wrong, or of the implications of the comparative analysis of both methods.

		Faecal Calprotectin (fCAL™), µg/g			
	Kappa Statistic (k_w) = 0.32	**Positive**	**Indeterminate**	**Negative**	**Total (%)**
Serum BMN®-Cp (ng/mL)	**Positive**	**18**	**5**	**1**	**24 (60.0)**
	Indeterminate	**0**	**5**	**0**	**5 (12.5)**
	Negative	**3**	**4**	**4**	**11 (27.5)**
	Total (%)	**21 (52.5)**	**14 (35.0)**	**5 (12.5)**	**40 (100)**

Table 5.6 – Concordance table for fCAL™ and serum BMN®-Cp in 40 patients with IBD. The Kappa statistic (k_w) was calculated to be 0.32.

		Faecal Calprotectin (fCAL™), µg/g			
	Kappa Statistic (k_w) = 0.33	Positive	Indeterminate	Negative	Total (%)
Serum IDK®-Cp (ng/mL)	Positive	17	2	1	20 (50.0)
	Indeterminate	0	5	0	5 (12.5)
	Negative	4	7	4	15 (37.5)
	Total (%)	21 (52.5)	14 (35.0)	5 (12.5)	40 (100)

Table 5.7 – Concordance table for fCAL™ and serum IDK®-Cp in 40 patients with IBD. The Kappa statistic (k_w) was calculated to be 0.33.

		Serum BMN®-Cp (ng/mL)			
	Kappa Statistic (k_w) = 0.70	Positive	Indeterminate	Negative	Total (%)
Serum IDK®-Cp (ng/mL)	Positive	22	0	0	22 (55.0)
	Indeterminate	0	0	0	0 (0.0)
	Negative	5	0	13	18 (45.0)
	Total (%)	27 (67.5)	0 (0.0)	13 (32.5)	40 (100)

Table 5.8 – Concordance table for serum BMN®-Cp and serum IDK®-Cp in 40 patients with IBD. The Kappa statistic (k_w) was calculated to be 0.70

5.8 Discussion

The results presented in this study investigated the potential value of serum calprotectin and serum S100A12 relative to faecal calprotectin, the study 'gold standard' biomarker, in the diagnosis of IBD.

Serum BMN®-Cp and serum IDK®-Cp assays had an excellent correlation. Although serum BMN®-Cp was significantly higher than serum IDK®-Cp, this was just due to matched results for serum BMN®-Cp being 1.7–fold higher than serum IDK®-Cp and accounted for in the manufacturer provided cut–off values of > 3900 ng/mL and > 3000 ng/mL respectively.

Even though serum biomarkers correlated strongly with each other, their correlation with faecal calprotectin was not as strong. We suggest that this is a reflection of the different sources of inflammatory markers in stool and blood. Levels of calprotectin in stool are largely reflective of the extent of gastrointestinal mucosal inflammation unlike calprotectin levels in serum which indicate the magnitude of systemic inflammation. Nevertheless, faecal calprotectin correlated best with serum IDK®-Cp and least with serum IDK®-A12.

Analyses of AUC from ROC curves indicated that compared to 'gold standard' faecal calprotectin, serum IDK®-Cp assay (AUC = 0.793) performed better than serum BMN®-Cp assay (AUC = 0.771) and both performed better than serum S100A12 assay (AUC = 0.700). This characterisation of the IDK®-Cp assay is consistent with the best diagnostic efficiency at an optimum cut–off value of > 2500 ng/mL which had a sensitivity of 80% and specificity of 65%. This was also supported by the concordance tables in which concordance with faecal calprotectin measurement was greater for serum IDK®-Cp assay (k_w = 0.33)

than serum BMN®-Cp assay (k_w = 0.32). Our data indicate that serum biomarkers, particularly serum IDK®-A12, are unlikely to replace faecal calprotectin. A raised serum calprotectin, however, may have a role identifying which patients require endoscopy without recourse to faecal studies but a normal serum calprotectin does not exclude IBD.

This study has several limitations. The small number of study subjects, and particularly those who were IBD negative, increases the risk of type I and II statistical errors. We did not have clinical data on those with raised faecal calprotectin, such as UC or CD and these may have different effects on faecal and circulating biomarkers. Ideally, faecal and circulating biomarkers should have been evaluated against the widely accepted gold standard of endoscopic biopsy supported by imaging or disease activity scoring systems but these are not practical in routine practice.

The stability of calprotectin and S100A12 over time, in this study, was not evaluated. Previous studies reported calprotectin to be stable in blood before separation during pre–analytic handling and during storage as serum calprotectin (Røseth et al., 1992; Tøn et al., 2000). Conversely, levels of S100A12 in blood may increase over time (Larsen et al., 2007b) but this is unclear (BioVendor, 2016). This may have been a limiting factor in this study and has the potential to limit serum S100A12 application in the diagnosis of IBD as there could be a dramatic change from low to normal or high S100A12 concentration in blood. Variations and inconsistencies of S100A12 levels in blood between different ELISA kits have also been reported (Brinar et al., 2010).

The present study demonstrated difficulties in evaluating the utility of serum S100A12 in IBD diagnosis. No cut–off values were supplied by the kit (i.e.,

Immunodiagnostik™ S100A12 ELISA K 6938) manufacturer for the diagnosis of IBD. Moreover, using an adapted faecal S100A12 assay protocol with associated quality assurance issues for the measurement of S100A12 in serum proved practically redundant and thus both methods are not interchangeable.

5.9 Conclusion

Although the serum IDK®-Cp assay had marginally the best diagnostic performance, serum calprotectin analysed by both the IDK®-Cp and BMN®-Cp assays have good analytical performance and diagnostic accuracy. Serum calprotectin is unlikely to replace faecal calprotectin but may have a role supplementing faecal calprotectin in the diagnosis of IBD. An elevated serum calprotectin in patients with chronic diarrhoea would be an indication for endoscopy since it has a low false positive rate, but a normal serum calprotectin level does not exclude IBD. The diagnostic performance of serum S100A12, in this study, was poor as a biomarker for IBD assessment. Studies in larger stringently defined population cohorts are necessary to evaluate serum calprotectin and serum S1200A12 compared to faecal calprotectin using endoscopic biopsy with or without clinical activity scoring systems as gold standard in the assessment of IBD in patients presenting with chronic diarrhoea.

CHAPTER 6

CLINICAL STUDY II: EVALUATION OF UTILITY OF SERUM CALPROTECTIN TO DISCRIMINATE BETWEEN ACTIVE AND INACTIVE INFLAMMATORY BOWEL DISEASE

6.1 Introduction

CD and UC are chronic inflammatory gastrointestinal disorders that affect both adult and paediatric populations (Strober et al., 2007; Ford et al., 2013; Kalla et al., 2014). CD and UC are characterised by episodes of symptomatic relapse and remission. Preventing long–term complications and reducing irreversible luminal damage is a key goal. It is, therefore, important to select IBD patients who will benefit from early use of biological therapies, immunomodulators and/or surgery, given the increasing availability of treatment repertoire (Kalla et al., 2016).

Mucosal healing is regarded as the therapeutic objective for IBD assessment because it is associated with clinical remission, reduced hospitalisation and surgery, and lower prevalence of CRC (Frøslie et al., 2007; Pineton de Chambrun et al., 2010; Baert et al., 2010). Monitoring is, therefore, needed to assess disease activity and efficacy of treatment (Foell et al., 2009). Whilst endoscopy is the ‘gold standard’ for assessing intestinal inflammation and mucosal healing, it carries clinical risk. Recurrent endoscopy is invasive, unpleasant for the patient, labour intensive, expensive and requires a skilled operator (Blotière et al., 2014). A robust non–invasive biomarker of intestinal inflammation to reduce or replace endoscopy would carry many advantages.

Traditionally, monitoring disease activity, predicting disease course, establishing disease site, and predicting both risk of relapse and response to therapy in IBD is a clinical decision based on disease activity scores such as

CDAI, Harvey–Bradshaw Index (HBI), Rachmilewitz Index (RI), conventional blood–based biomarkers, radiology, and intermittent endoscopy (Scaioli et al., 2015; Lehmann et al., 2015). Disease activity scores are limited by the reporting bias of patients, signifying more about the patients' wellbeing rather than the degree of mucosal inflammation as well as the intrinsic difficulty involved in data collection (Lehmann et al., 2015). The goal of disease biomarkers and clinical scoring systems are geared towards ascertaining prognosis, appraising disease activity and establishing optimised treatment strategies in IBD patients.

Lewis (2011) demonstrated the potential role of biomarker assays in the care of patients with suspected or established IBD (figure 6.1). Biomarkers might be used in all phases of the care. For patients with suspected IBD (Panel A), biomarkers can be used to select which patients are unlikely to have IBD and could forgo further testing. Once patients are diagnosed, biomarkers can be used to determine which patients have CD or UC (Panel B) and to predict disease course (Panel C). Biomarkers might be used to determine which patients are most likely to respond to therapies (Panel D) and determine prognosis (Panel E) and to identify those who require more aggressive therapies (Panel F). In patients with recurrent symptoms (Panel G), biomarkers can differentiate patients with active inflammation from those likely to have symptoms from other causes (Panel H).

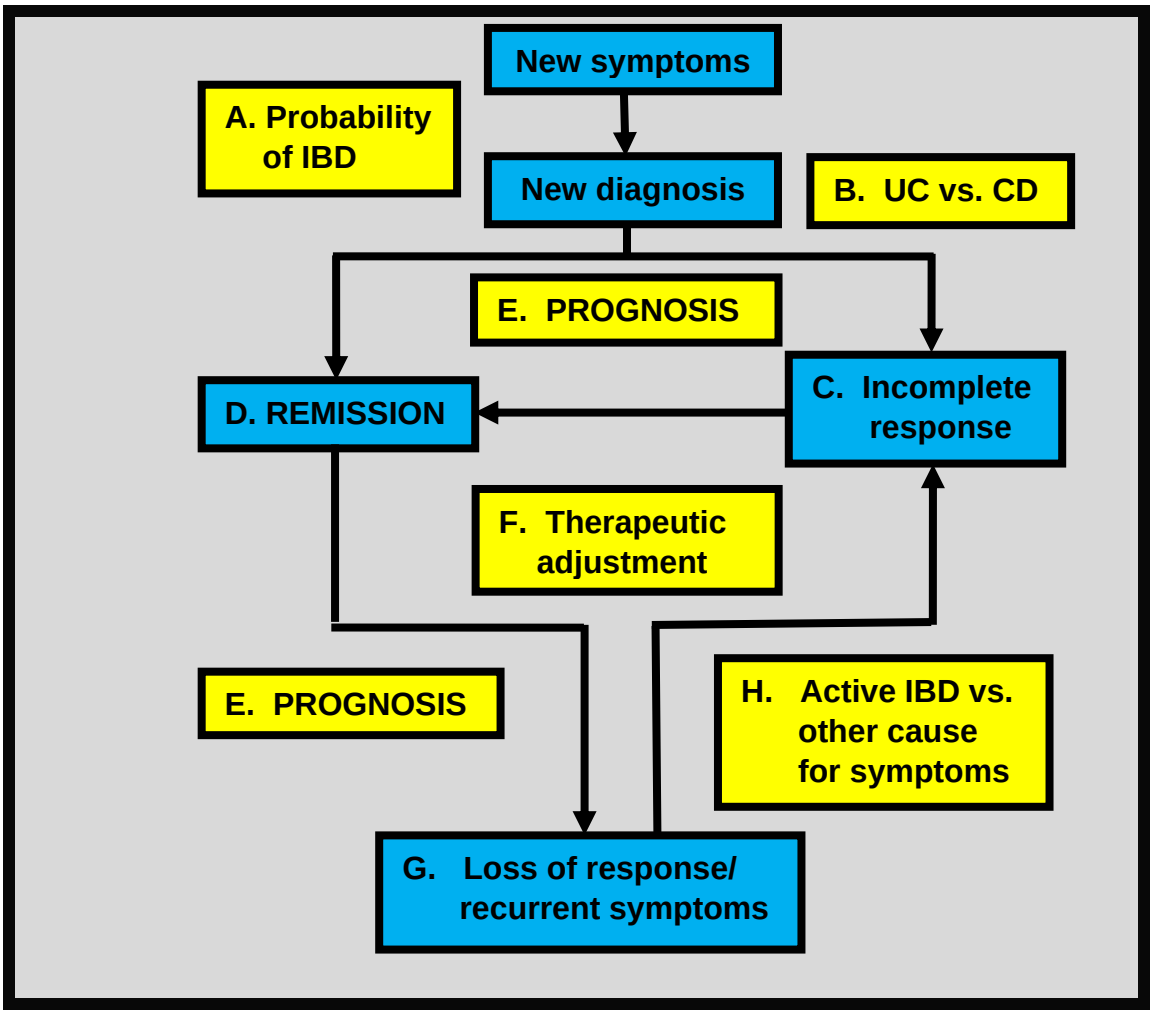

Figure 6.1 – A clinician's guide to management of IBD patients using endoscopy, radiology, and biomarkers. The potential role of biomarker assays in the care of patients with suspected or established IBD. Modified from: Lewis JD (2011) The utility of biomarkers in the diagnosis and therapy of inflammatory bowel disease. *Gastroenterology,* 140 (6): 1817 – 1826.

Generally, IBD patients with severe disease have greater changes in serum inflammatory biomarkers compared to those without inflammation or with low–grade inflammation. ESR, CRP, serum albumin, α_1–proteinase inhibitor, cholinesterase and haematocrit have all been shown to correlate with endoscopic activity in monitoring disease activity in IBD patients (Day et al., 2017; Tromm et al., 1992; Sachar et al., 1986). These blood–based biomarkers,

however, are not specific, thereby limiting their prognostic utility in IBD assessment (Sands, 2015; Choden et al., 2018; Sachar et al., 1990).

Serum CRP is appealing because it is inexpensive, quick to result and non–invasive. It, however, has only modest correlation with clinical and endoscopic findings in IBD patients (Solem et al., 2005; Karoui et al., 2007; Tilakaratne et al., 2010) but has a better correlation for transmural inflammation (Fagan et al., 1982; Vermeire et al., 2005; Saverymuttu et al., 1986b). As serum CRP is not a specific biomarker for intestinal inflammation, it may be falsely low despite active mucosal inflammation (Chang et al., 2015) and may be elevated due to other reasons such as infection or extra–intestinal inflammation (Chang et al., 2015; Fengming and Jianbing, 2014).

The need, therefore, for a validated non–invasive laboratory biomarker to monitor response to treatment in IBD is appealing. Whilst multiple biomarkers in stool (e.g., calprotectin and lactoferrin) and blood (e.g., CRP, ESR, albumin, platelets, haemoglobin, and leucocyte count) have been tested (Panes et al., 2016; Kalla et al., 2017), the primary focus of the present study is the evaluation of serum calprotectin compared to faecal calprotectin, CRP and platelets in IBD assessment.

Calprotectin, a member of the S100 protein family, is a biomarker of inflammation (van Rheenen et al., 2010; Kaiser et al., 2007). Calprotectin is a heterodimeric calcium–binding protein of the S100 family (S100A8 or MRP8 and S100A9 or MRP14), which inhibits metalloproteinases, and has pro–apoptotic activities and antimicrobial properties (Johne et al., 1997; Steinbakk et al., 1990). It is found mainly in neutrophils and monocytes or macrophages where it constitutes up to 60% of the total cytosolic protein.

Faecal calprotectin has advantages of a high stability at room temperature, resistance to degradation and a homogenous distribution in stools, as previously described. Faecal calprotectin for IBD has a sensitivity and specificity of 93% and 96% respectively in adults and a sensitivity of 92% and specificity of 76% in children. Van Rheenen et al (2010) concluded that faecal calprotectin is useful in excluding patients who do not require further investigation thereby reducing the necessity for expensive endoscopy or radiological investigations. Faecal calprotectin, however, is limited by difficulty in patients' sampling, delays in laboratory testing (Wright et al., 2015; Meuwis et al., 2013; Ho et al., 2009), intra–individual variability in active UC and optimal timing for sampling (Lasson et al., 2014; Calafat et al., 2015). Unlike the systemic circulation, the faecal stream is in close interaction with the intestinal mucosa which can take up biomolecules functioning as biomarkers of intestinal inflammation. Among these, calprotectin is the most extensively used biomarker for assessing the disease activity in IBD (Schoepfer et al., 2013; Jahnsen et al., 2008).

Diverse opinions exist on the value of serum calprotectin in assessing the disease burden and prognosis of IBD (Townsend et al., 2019; Day et al., 2019; Kalla et al., 2016; Leach et al., 2007), prognosis of UC (Hare et al., 2013), identifying disease relapse in CD (Lügering et al., 1995), complementing hsCRP and faecal calprotectin in predicting disease relapse in CD (Meuwis et al., 2013), as a sole sensitive biomarker for inflammatory activity in IBD (Ferrer et al., 2019; Okada et al., 2019), and identifying a role in adolescents with IBD (Carlsen et al., 2019).

Serum calprotectin, however, may be a useful biomarker for monitoring IBD. It presents no practical issues of intra–individual and within–day variability and

sampling inconvenience as patients are most likely to favour its routine use. It predicts exacerbation and deterioration of function in inflammatory diseases such as rheumatic diseases and cystic fibrosis (Mariani et al., 2014; Obry et al., 2014; Abildtrup et al., 2015; Gray et al., 2010; Reid et al., 2015), and predicts radiological disease progression in rheumatoid arthritis (Hammer et al., 2010). Serum calprotectin could, therefore, be used to identify disease recurrence following IBD therapy. In combination with faecal calprotectin and hsCRP, serum calprotectin predicted disease recurrence following anti–TNF–α withdrawal in CD patients (Meuwis et al., 2013).

Most laboratory blood–based biomarkers, such as ESR, albumin, haemoglobin, and platelets, used in IBD studies show poor analytical performance (Chang et al., 2015; Solem et al., 2005). The poor sensitivity and specificity, and lack of correlation with intestinal inflammation, makes these biomarkers poor predictors of disease recurrence and response to therapy (Tibble et al., 2000a; Langhorst et al., 2008). Serum calprotectin may identify patients at greater risk of recurrence and those with symptoms of intestinal inflammation that may benefit from therapeutic adjustment. In addition to the reduced cost of testing, morbidity, and mortality risks to patients, non–invasive blood–based biomarkers like serum calprotectin may have a role as either an alternative or a supplement to faecal calprotectin in IBD assessment.

Given the limitations of routine systemic and faecal biomarkers of inflammation, there is need to investigate the utility of serum calprotectin to replace or supplement faecal calprotectin and compare its performance to conventional inflammatory blood biomarkers, serum CRP and platelets, in discriminating between active and inactive disease activity in IBD in an outpatient setting.

6.1.1 Aim of this chapter

The aim of the study presented in this chapter was to investigate serum calprotectin compared to faecal calprotectin and the conventional inflammatory blood biomarkers, serum CRP and platelets in discriminating between active and inactive disease in IBD.

The purpose of data analysis was to investigate the potential of serum calprotectin as a biomarker to replace or supplement faecal calprotectin and other conventional inflammatory blood biomarkers in discriminating between active and inactive IBD.

6.2 Analytical Materials and Methods

The analytical materials and methods were the same as those set out in sections 4.2 – 4.4.

6.2.1 IBD Patient Cohort and Demographics

One hundred and seventy–five patients (58% male) with IBD attending the twice–weekly IBD clinic at New Cross Hospital Wolverhampton were recruited, with mean (SD) of 48 (16.3) years.

The exclusion and inclusion criteria in the study were the same as those set out in Chapter 5 under sub section 5.2.2.1 (i.e., patients with inflammatory bowel disease).

6.3 Method

Faecal and serum calprotectin were measured using the methodology detailed in Chapter 4 under sub sections 4.5.1 (i.e., extraction and measurement of faecal calprotectin) and 4.5.2 (i.e., measurement of serum calprotectin in IBD

and control patients). Serum calprotectin results above the measuring range of the assay were diluted with Incubation Buffer (Code: B–MRP8/14–IB, for the Bühlmann BMN®-Cp assay) or Sample Buffer (Cat. No. K 6935; Label: SAMPLEBUF, for the Immunodiagnostik™ IDK®-Cp assay) as appropriate, and re–assayed to enable quantitation.

Platelets were measured on the Sysmex XN–10® analyser using methods and reagents supplied by the Sysmex Corporation, Kobe, Japan.

CRP was measured using the Abbott Immuno–turbidimetric assay on the Abbott c16000 Turbidimetric Chemistry Analyser developed by Abbott Laboratories Inc., Abbott Diagnostics, Abbott Park, IL 60064, USA. The package insert for the assay is provided in Appendix H. The reference range is ≤ 5 mg/L for both serum and plasma samples (Datti et al., 2001; Schlebusch et al., 2002) while the intra– (within assay) and inter–assay (between assay) CVs were 1.4% and 1.2% respectively (Kennedy et al., 1999).

To evaluate the agreement between the results of serum calprotectin measured with the BMN®-Cp assay and results obtained with the IDK®-Cp assay, 175 serum samples were tested with both assays. These samples consisted of IBD serum calprotectin samples that spanned the dynamic range of the assay. Each sample was tested according to the methodology detailed in Chapter 4 under sub section 4.5.2.

Serum calprotectin concentrations obtained with the BMN®-Cp assay were plotted against those determined using the IDK®-Cp assay, a Spearman's rank–order coefficient of correlation was calculated followed by a Passing–Bablok linear regression analysis (Analyse–it® Software, 2016; Passing and Bablok, 1983; Passing and Bablok, 1984; Bablok et al., 1988). A 45–degree line of

equality was included in the plot to assess bias. Subsequent analysis of data was performed using the Bland–Altman approach to assess any between–method bias (Bland and Altman, 1986; Bland and Altman, 1995; Bland and Altman, 1999).

The clinical study involved the evaluation of serum calprotectin, with or without other markers, to categorise patients into those with active or inactive IBD in a cohort of 175 patients. Clinical information from the hospital notes was used to categorise disease type into CD, UC and unclassified IBD (IBDU). The classification of patients into active and inactive IBD over the course of the study period was obtained from the hospital notes and this was largely based on clinical evaluation (Schoepfer et al., 2012) and where available endoscopy (Kim et al., 2016; Khanna et al., 2016; Niv et al., 2014; Hall et al., 2014; Hebuterne et al., 2013; Travis et al., 2012), radiology (Pariente et al., 2015; Gilletta et al., 2015; Fiorino et al., 2015; Panes et al., 2013; Panes et al., 2011) and faecal calprotectin.

Disease locations within the small intestine included ileal and upper, and colonic and ileocolonic sites for CD; and the large intestine included proctitis (E1), left–sided or distal colitis (E2, limited to the sigmoid and descending colon) and extensive colitis or pan proctitis (E3) sites for UC (Meier and Strum, 2011) were recorded.

To construct a prognostic model for serum calprotectin, measurements of the traditional laboratory parameters (i.e., faecal calprotectin, CRP and platelets) were included in the ROC curves and AUC analyses for IBD monitoring. This entailed a comparative analysis of the prognostic utility of serum calprotectin in relation to these biomarkers: faecal calprotectin, CRP, and platelets, in IBD

assessment. ROC curves and AUC analyses used the reference ranges supplied by the ELISA kits manufacturers and those of the in–house methods at New Cross Hospital Wolverhampton, to define the optimal cut–off points (i.e., highest sum of sensitivity + specificity) for the prognostic model for serum calprotectin.

6.3.1 Statistical Analysis

Data processing and statistical analysis were performed as described in Chapter 4 under sub section 4.5.5.

The Kolmogorov–Smirnov (KS) and Shapiro–Wicks (SW) tests were used to assess the normality of data. Faecal calprotectin, serum calprotectin, CRP and platelets data were non–parametric and are presented as medians and interquartile ranges (IQR). Other data were parametric and were presented as numbers and percentages. Fecal calprotectin results < 20 or > 1932 µg/g were arbitrarily assigned values of 20 and 1932 µg/g respectively, for statistical purposes.

Spearman rank–order correlation was used to measure the degree of association between variables. A *p*–value of < 0.05 was considered statistically significant. Linearity was assessed by linear regression analysis and Passing–Bablok (Passing and Bablok, 1983).

The ROC curve was constructed as previously described in Chapter 5 under section 5.5 (Area under the curve) to determine the accuracy of serum calprotectin measured by the BMN®-Cp and IDK®-Cp assays as a prognostic test in monitoring the response to therapy in IBD, and to discriminate between active and inactive IBD. An assay with an AUC value of ≥ 0.7 (DeLong et al., 1988), was deemed as statistically relevant for use in monitoring response to

treatment in IBD. A coefficient of correlation (i.e., Spearman's rank–order value, *r*) that lie between 0.5 and 1.0 would indicate a good correlation for statistical analysis purpose in the present study.

6.4 Results

Table 6.1 summarises the stool and blood parameters of 175 IBD patients that were recruited in the study. Median concentrations of serum BMN®-Cp and serum IDK®-Cp of 4239 and 3727 ng/mL respectively were similar (p = 0.11).

Parameters	fCAL® (µg/g)	BMN®-Cp (ng/mL)	IDK®-Cp (ng/mL)	CRP (mg/L)	Platelet (x10E9/L)
N	175	175	175	175	175
Median	328	4239	3727	3	276
IQR	102–1014	2923–6429	2368–6166	1–8	228–335
Reference range	**> 200[a]**	**400–3900[b]**	**< 3000[b]**	**≤ 5[b]**	**150–400[a]**

Table 6.1 – Faecal calprotectin, serum calprotectin, CRP and platelet concentrations for 175 IBD patients. [a]In–house reference. [b]Reference range supplied by kit manufacturer. N = number; IQR = Interquartile range.

6.4.1 Serum Calprotectin Method Comparison

Serum calprotectin measured by the BMN®-Cp assay and by the IDK®-Cp assay was highly correlated (r = 0.889) (figure 6.2). Linear regression analysis confirmed a linear relationship between the serum concentrations of calprotectin obtained with the IDK®-Cp and BMN®-Cp assays, and a Passing–Bablok test identified a constant (α = 0.973; 95% CI: 0.9185–1.022) and proportional bias (β = –435.3; 95% CI: –620.3 to –248.1) between the two methods. The Bland–Altman plot comparison between the BMN®-Cp assay and the IDK®-Cp assay illustrates that the BMN®-Cp assay has a 19% positive proportional bias relative to the IDK®-Cp assay (figure 6.3).

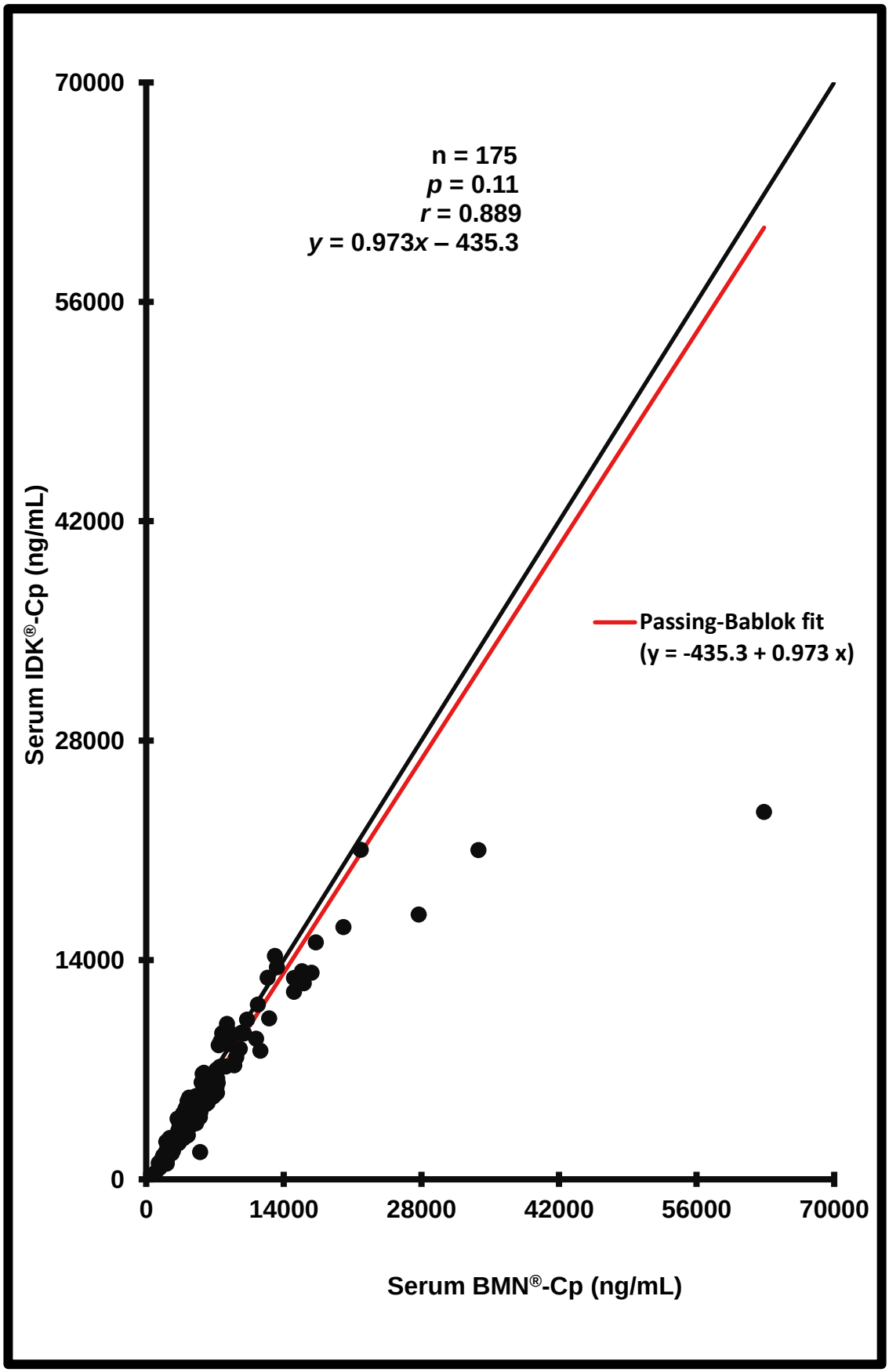

Figure 6.2 – Passing–Bablok method comparison between the BMN®-Cp and IDK®-Cp assays in 175 IBD samples. A line of equality (45° line) was included in the plot.

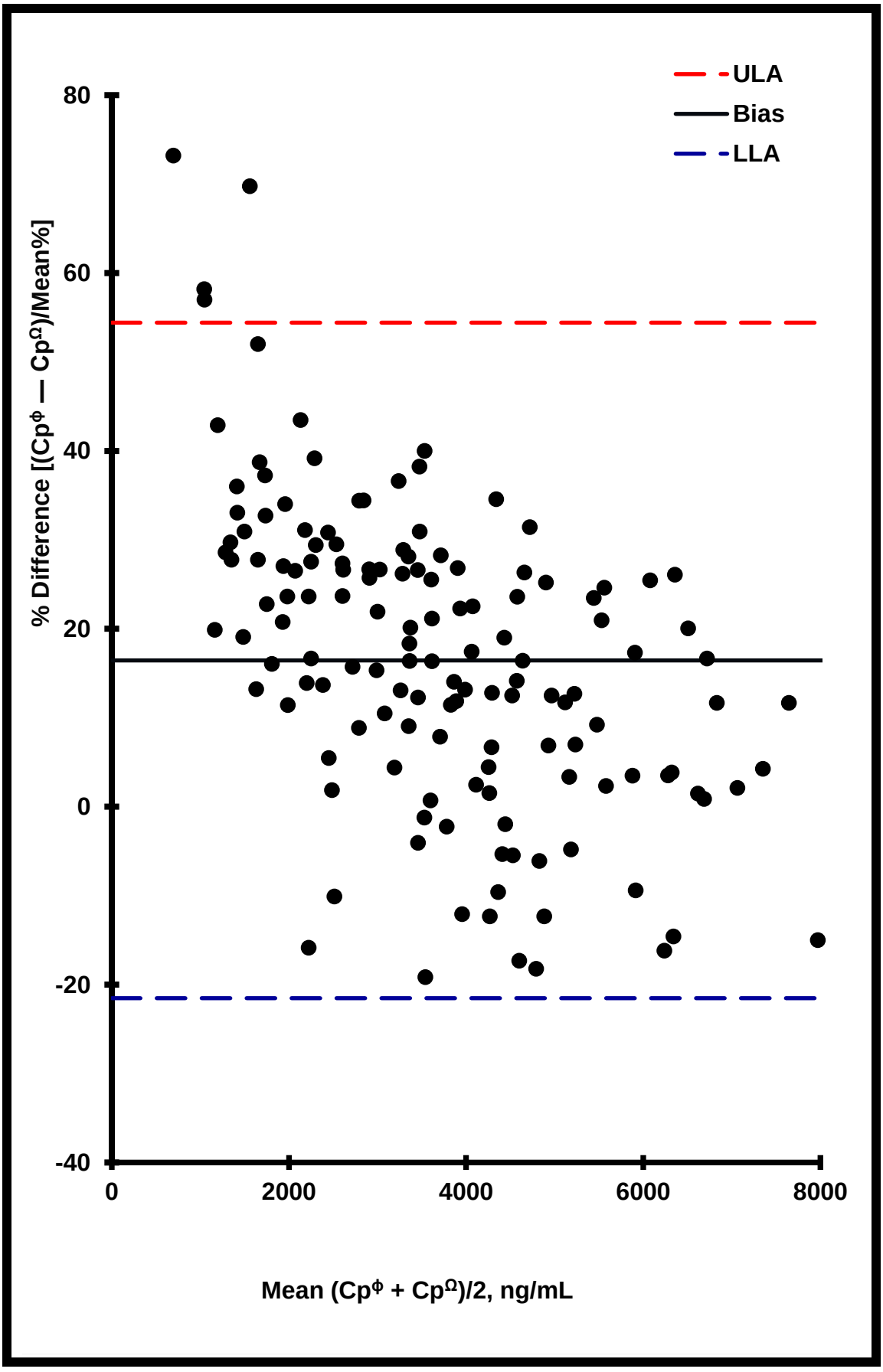

Figure 6.3 – A Bland–Altman plot comparing the BMN®-Cp and IDK®-Cp assays in 175 IBD samples.

6.4.2 Correlations

Correlations between faecal calprotectin, serum calprotectin, serum CRP and platelets are shown in figures 6.4 – 6.13 and table 6.2. In summary, faecal calprotectin correlated strongest with serum CRP (r = 0.51) and weakly with platelets (r = 0.20), serum IDK®-Cp (r = 0.30) and serum BMN®-Cp (r = 0.32). Serum BMN®-Cp and serum IDK®-Cp correlated strongest with serum CRP (r = 0.65 for serum BMN®-Cp and r = 0.60 for serum IDK®-Cp), and weakly with platelets (r = 0.34 for serum BMN®-Cp and 0.37 for serum IDK®-Cp). The correlation of serum CRP with platelets was weak (r = 0.22). Serum BMN®-Cp significantly correlated with serum IDK®-Cp (r = 0.97, p < 0.00001).

Assay		***r***	***r²***	***p***	***y = mx + c***
BMN®-Cp (ng/mL) vs.	**fCAL™ (µg/g)**	0.32	0.10	0.00001	2.5549*x* + 4321.2
	CRP (mg/L)	0.65	0.42	0.00001	298.77*x* + 3504
	Platelets (x10E9/L)	0.34	0.12	0.00001	21.898*x* + 433.41
IDK®-Cp (ng/mL) vs.	**fCAL™ (µg/g)**	0.30	0.09	0.00005	1.7098*x* + 3929.3
	CRP (mg/L)	0.60	0.36	0.00001	176.55*x* + 3570
	Platelets (x10E9/L)	0.37	0.14	0.00001	17.519*x* – 81.091
CRP (mg/L) vs.	**fCAL™ (µg/g)**	0.51	0.26	0.00001	12.124*x* + 520.41
	Platelets (x10E9/L)	0.22	0.05	0.00732	0.0355*x* – 2.2515
Platelets (x10E9/L) vs.	**fCAL™ (µg/g)**	0.20	0.04	0.00752	1.4518*x* + 197.77
BMN®-Cp (ng/mL) vs.	**IDK®-Cp (ng/mL)**	0.97	0.95	0.00001	0.5679*x* + 1635.3

Table 6.2 – Coefficient of correlation (Spearman's *p*) between serum calprotectin measured by the BMN®-Cp and IDK®-Cp assays and faecal calprotectin (fCAL™) and blood parameters. *r* = correlation coefficient. Correlation is significant at the 0.01 level (2–tailed).

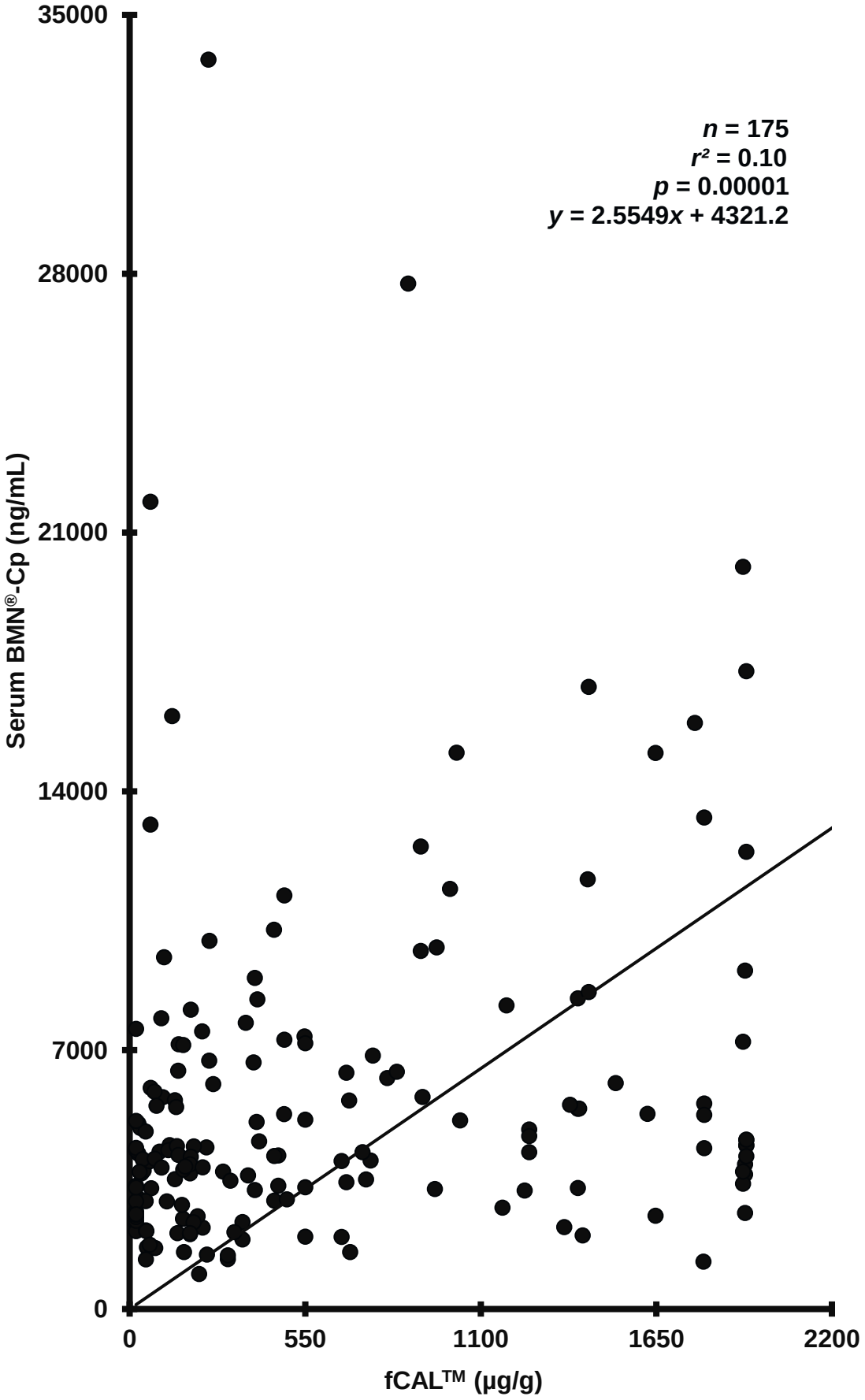

Figure 6.4 – Correlation of serum BMN®-Cp versus fCAL™ assay in monitoring IBD.

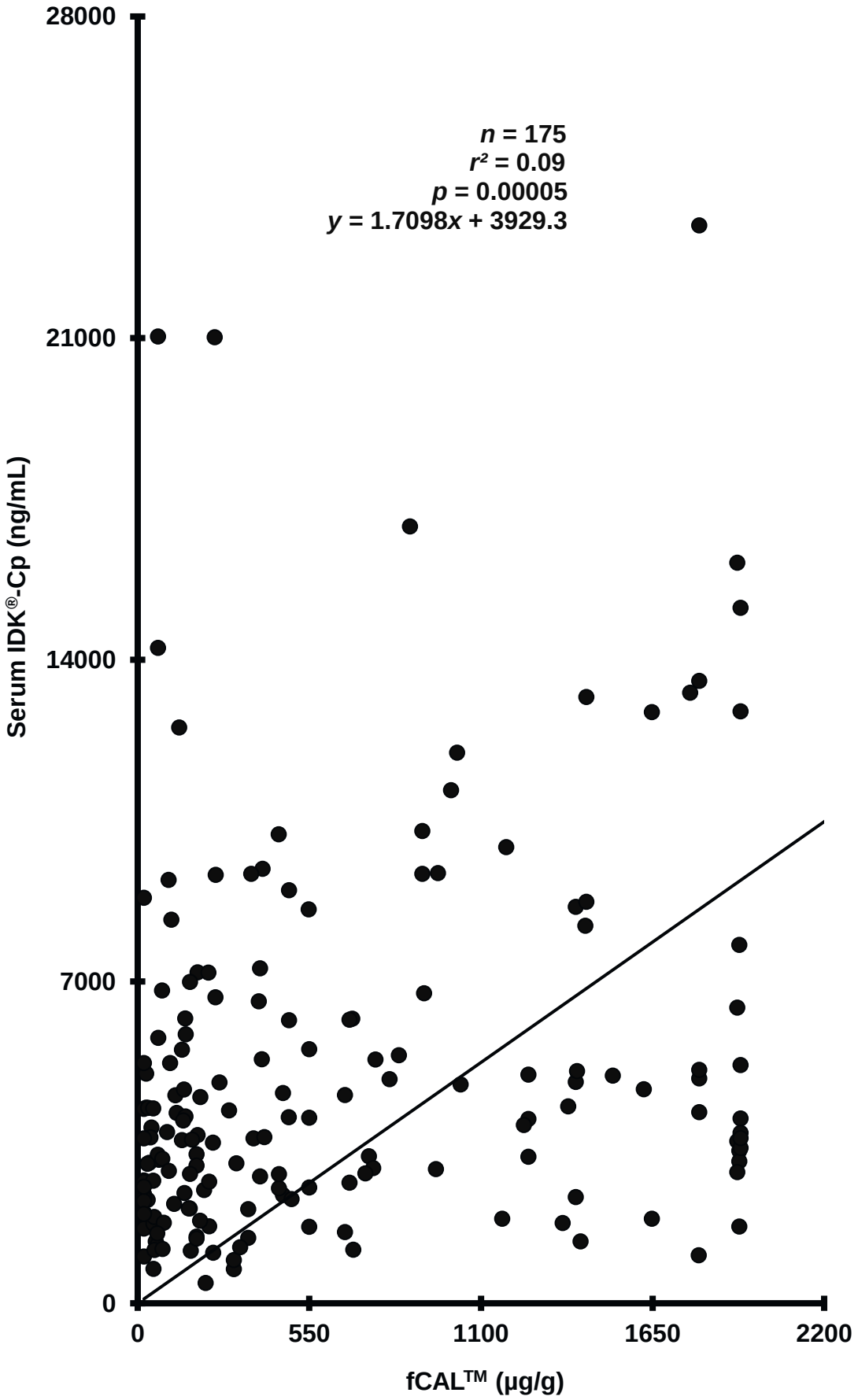

Figure 6.5 – Correlation of serum IDK®-Cp versus fCAL™ assay in monitoring IBD.

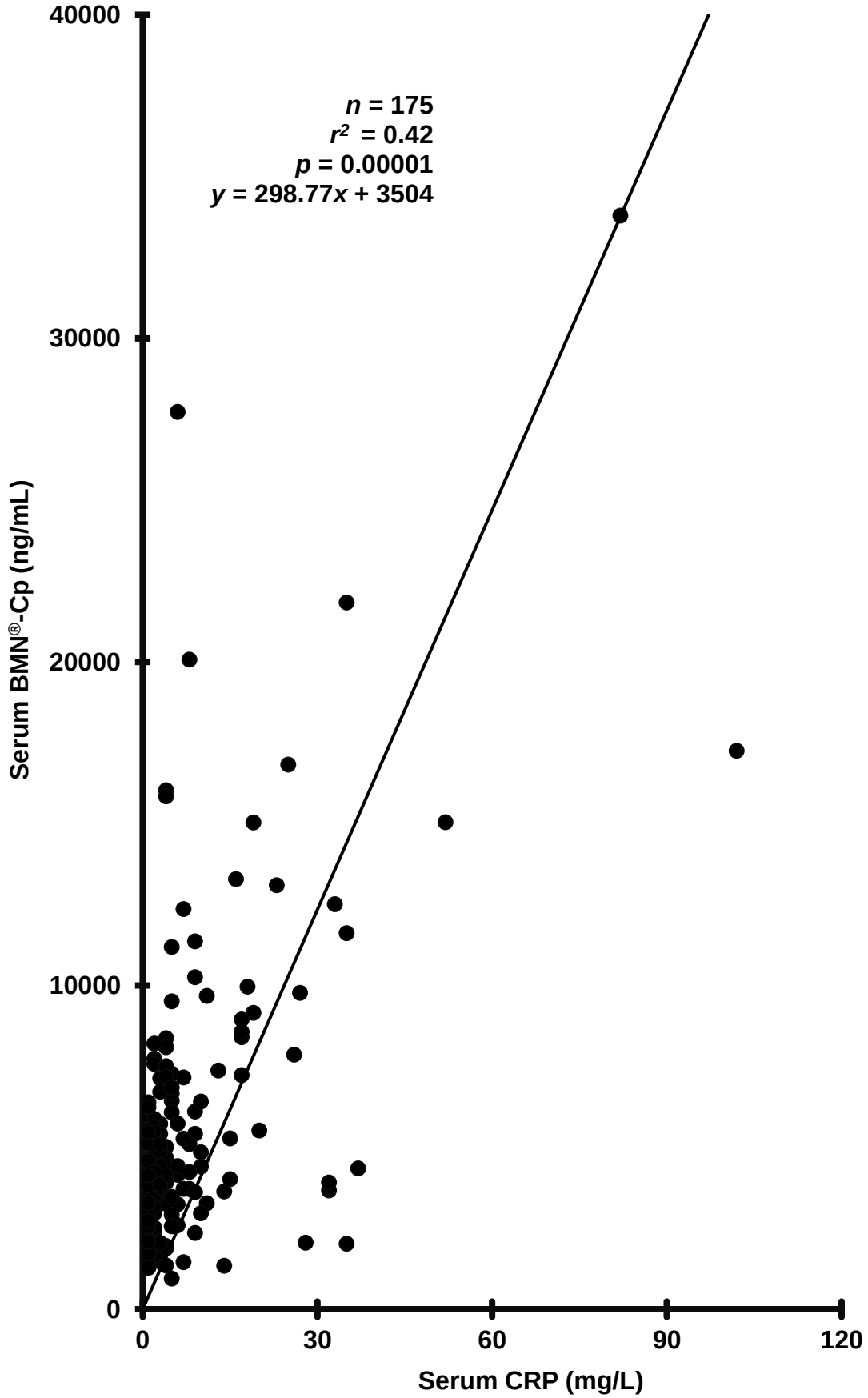

Figure 6.6 – Correlation of serum BMN®-Cp versus serum CRP in monitoring IBD.

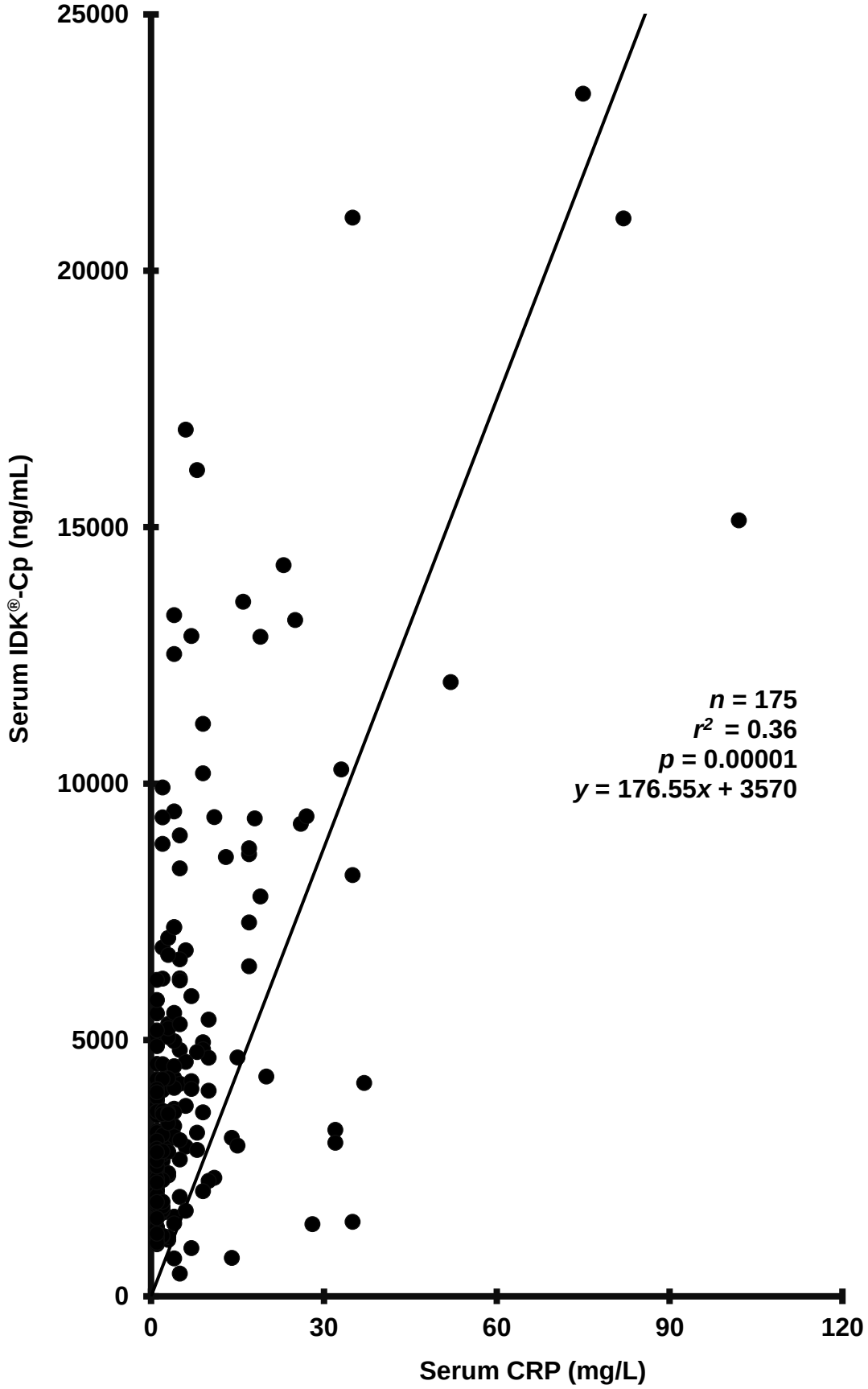

Figure 6.7 – Correlation of serum IDK®-Cp versus serum CRP in monitoring IBD.

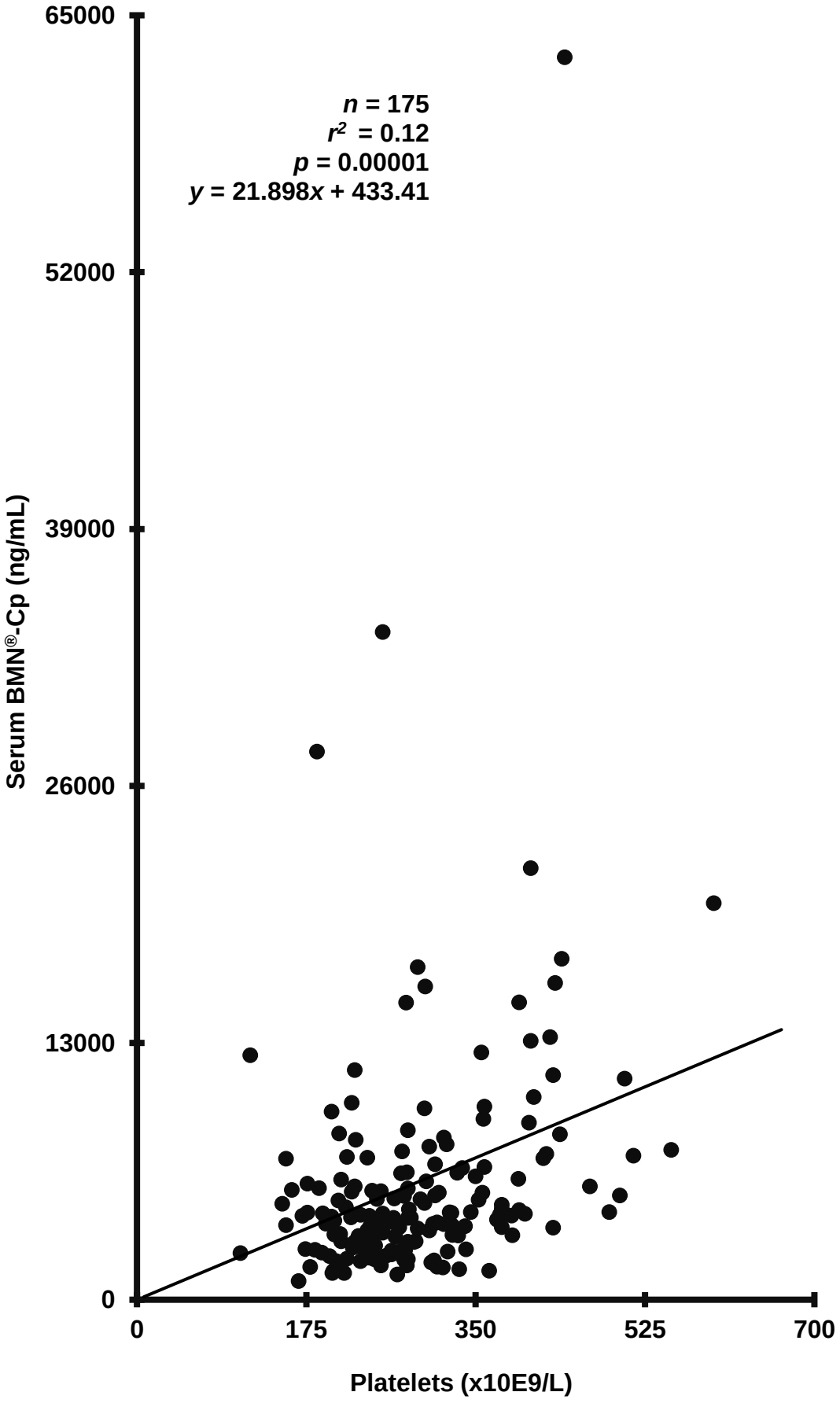

Figure 6.8 – Correlation of serum BMN®-Cp versus Platelet count in monitoring IBD.

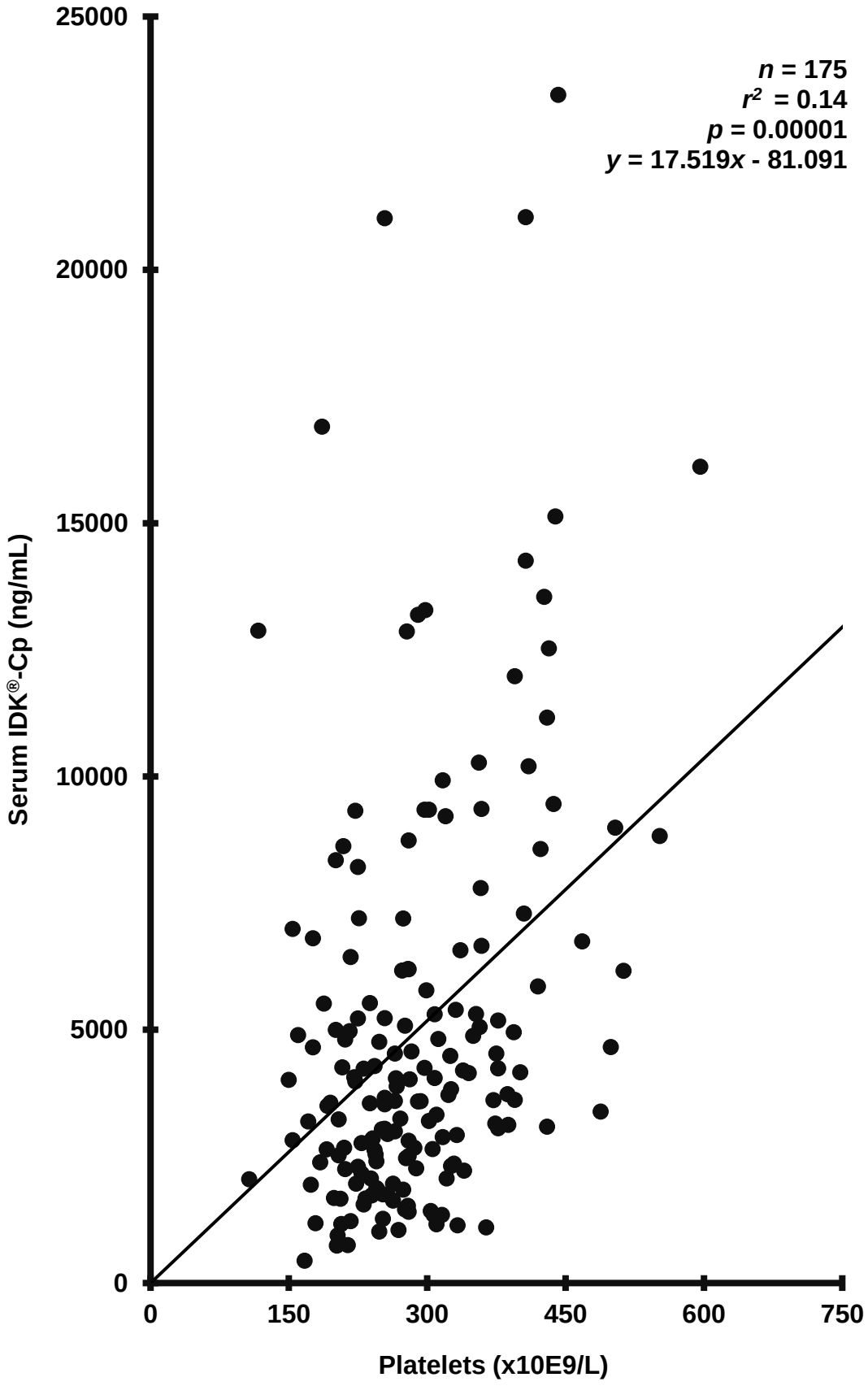

Figure 6.9 – Correlation of serum IDK®-Cp versus Platelet count in monitoring IBD.

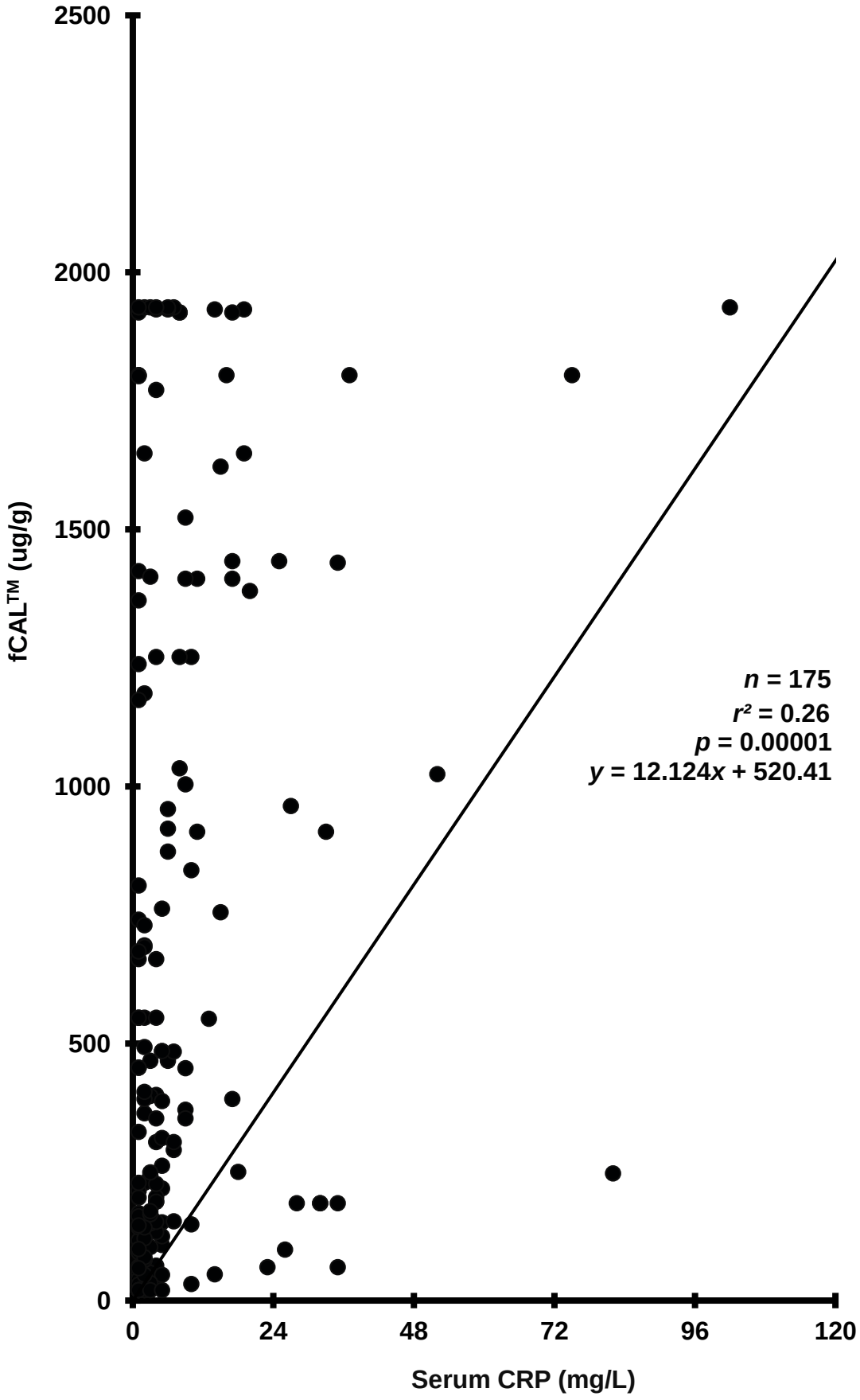

Figure 6.10 – Correlation of fCAL™ versus serum CRP in monitoring IBD.

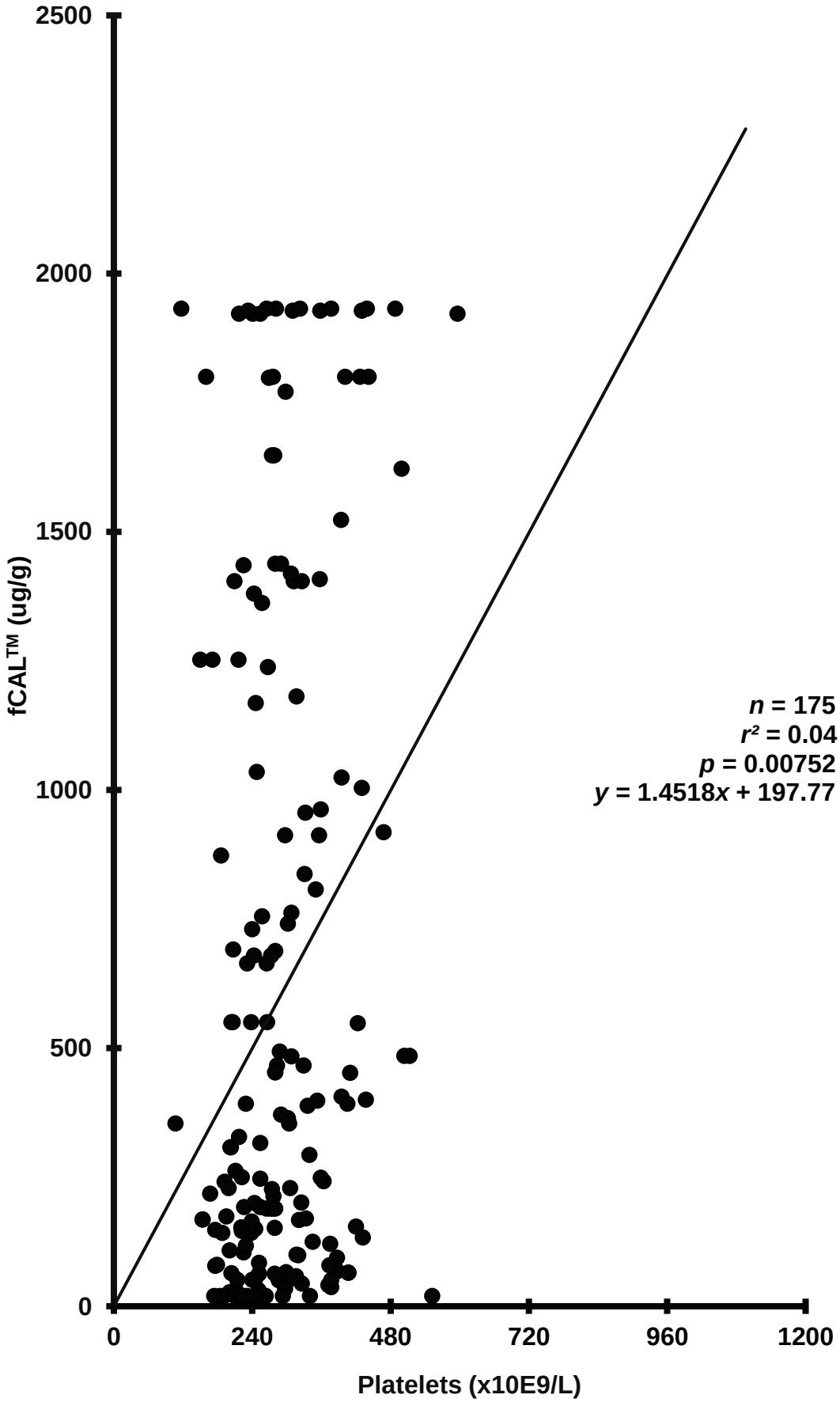

Figure 6.11 – Correlation of fCAL™ versus Platelet count in monitoring IBD.

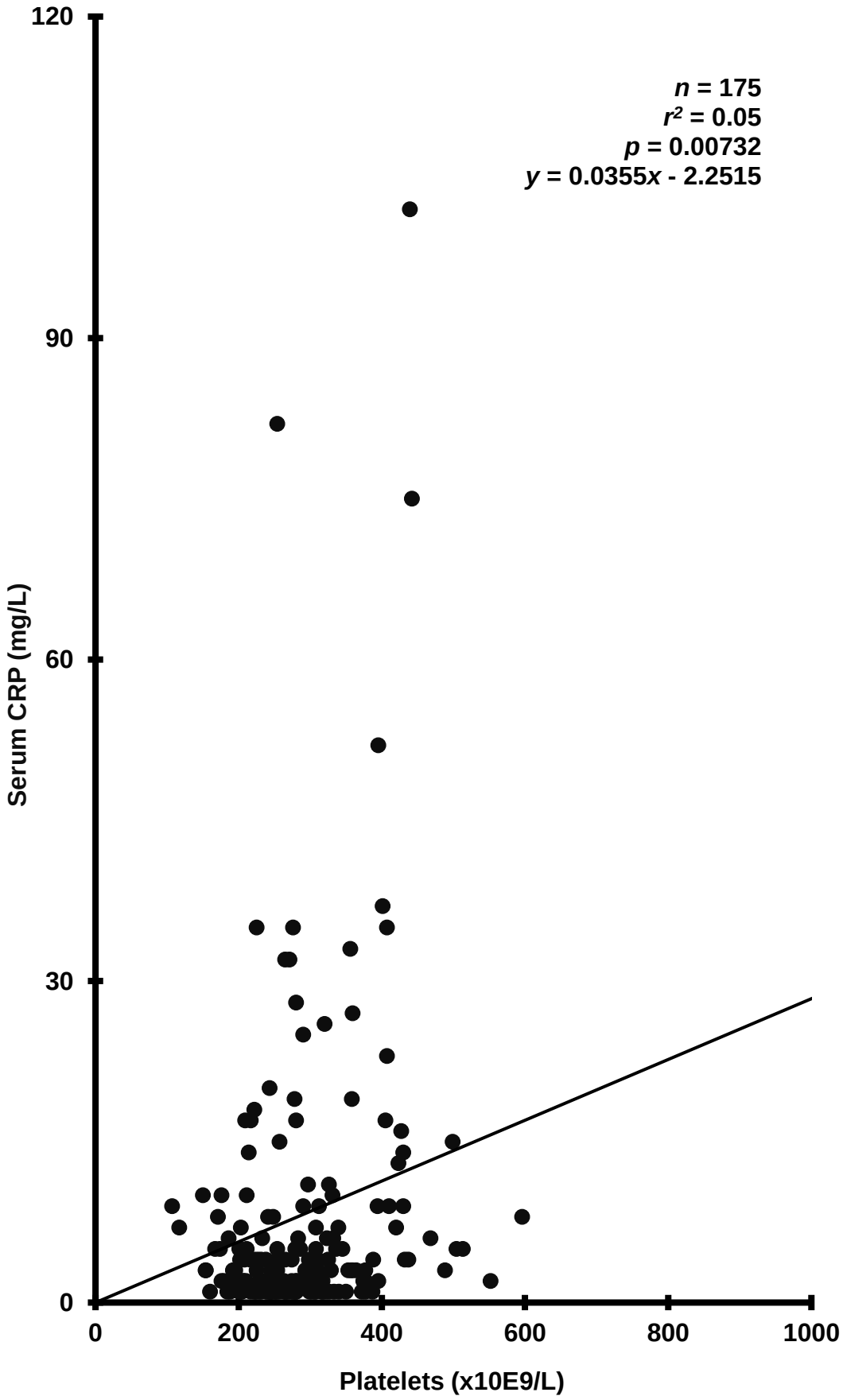

Figure 6.12 – Correlation of serum CRP versus Platelet count in monitoring IBD.

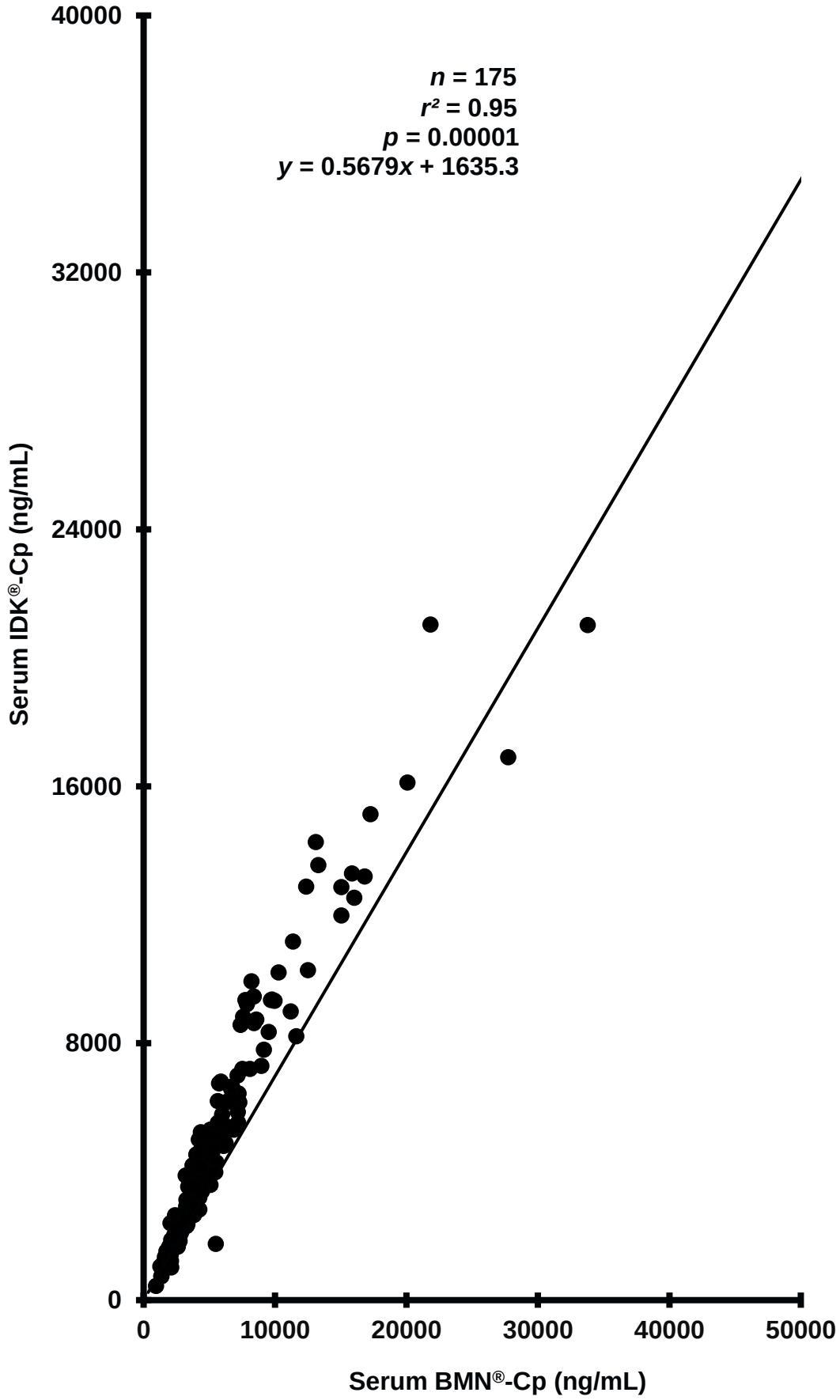

Figure 6.13 – Correlation of serum BMN®-Cp versus serum IDK®-Cp count in monitoring IBD.

6.4.3 Baseline patient characteristics and medical information

In the study cohort of 175 IBD patients; 101 (57.7%) patients had CD, 71 (40.6%) had UC and 3 (1.7%) had indeterminate colitis or inflammatory bowel disease unclassified (IBDU) (figure 6.14).

Classification of disease activity among the 175 cohort of IBD patients based on clinical notes diagnosis indicated that 99 (56.6%) cases were inactive, 73 (41.7%) cases were active, and 3 (1.7%) cases were deemed to be indeterminate colitis or IBDU (figure 6.15). The classification of active or inactive IBD from the notes was almost exclusively based on faecal calprotectin results.

6.4.4 Biomarkers in active and inactive (quiescent) IBD

The results of biomarkers in active and quiescent CD and UC are shown in table 6.3.

In summary, faecal calprotectin concentrations in active CD were higher (p = 0.0001) than in quiescent CD, and similarly in active UC were higher (p = 0.0001) compared to quiescent UC. Serum BMN®-Cp and serum IDK®-Cp concentrations in active CD were higher ($p < 0.006$) than in quiescent CD but were similar ($p > 0.1$) in active UC compared to quiescent UC (figures 6.16 – 6.17). Serum CRP in active CD was higher (p = 0.0095) compared to quiescent CD but similar (p = 0.0638) in active UC compared to quiescent UC. Platelets were similar (p = 0.0579) in active and quiescent CD and similar (p = 0.8055) in active and quiescent UC.

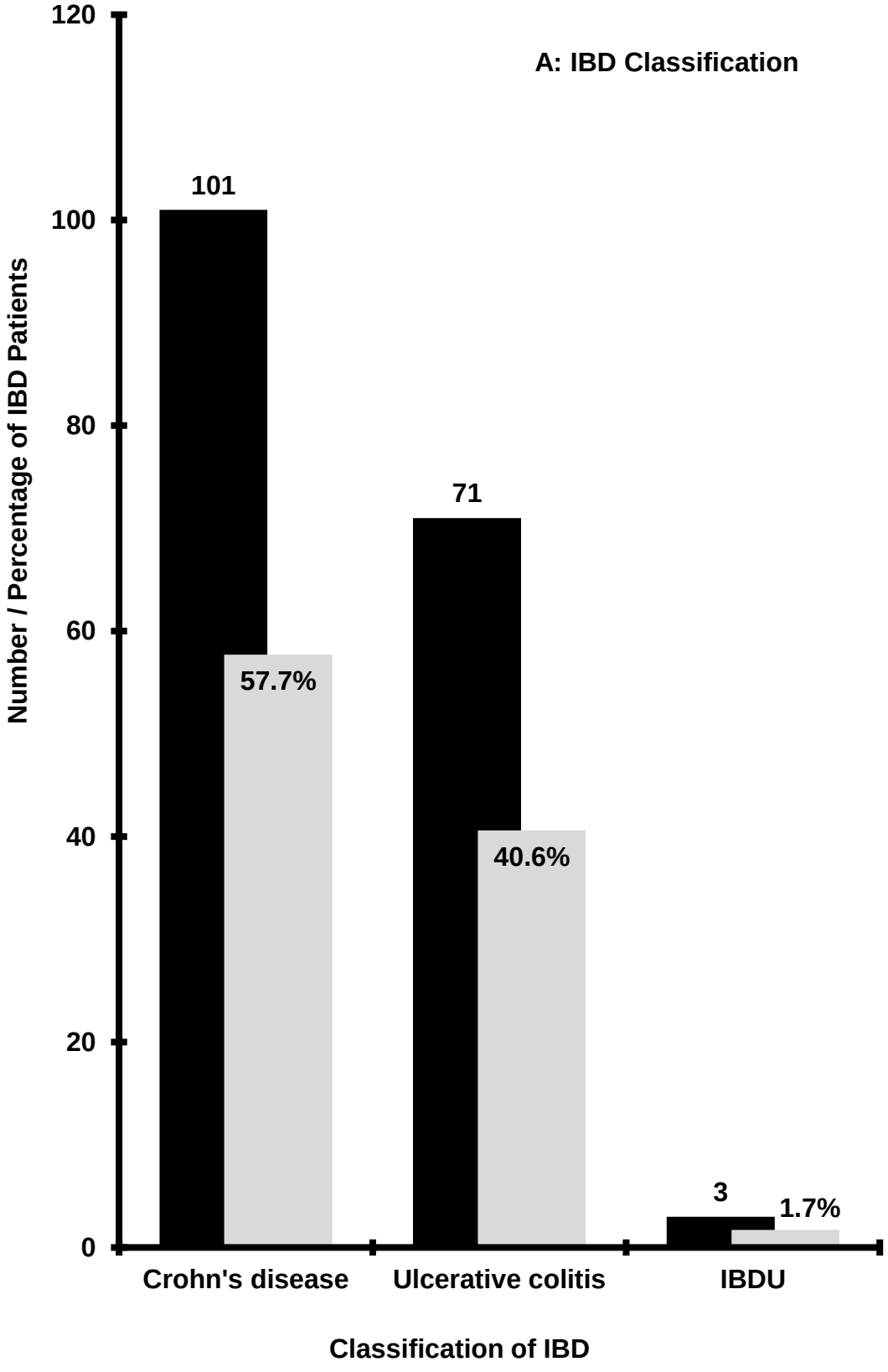

Figure 6.14 – A histogram plot of IBD patients (n = 175; black bars) expressed as percentage (silver bars) of CD, UC and IBDU. IBDU means inflammatory bowel disease unclassified or indeterminate.

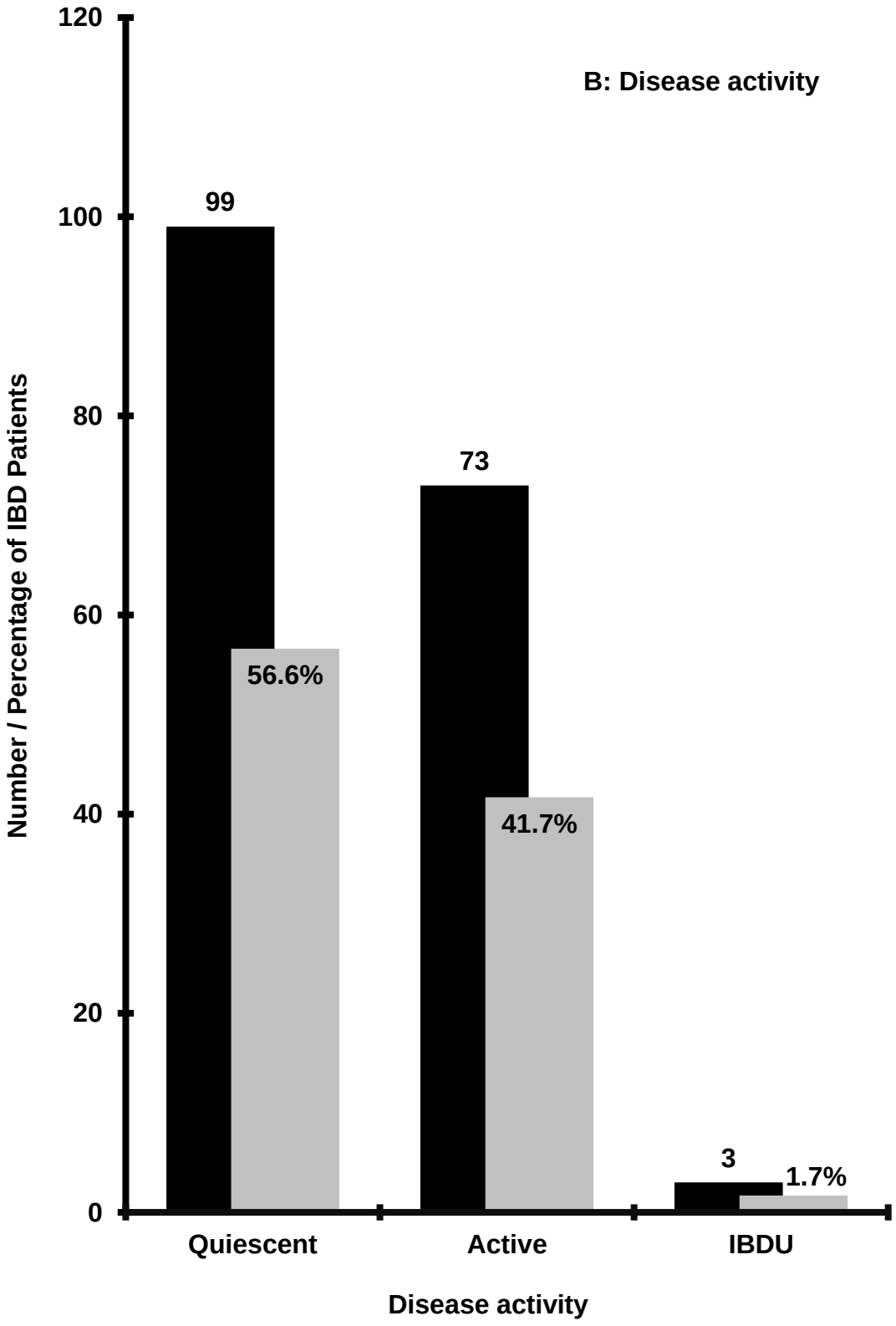

Figure 6.15 – A histogram plot of IBD 175 patients (n = 175; black bars) expressed as percentage (silver bars) of disease activity. IBDU means inflammatory bowel disease unclassified or indeterminate.

Parameters	Crohn's Disease					Ulcerative Colitis				
	Active Disease		Inactive Disease		*p* value	Active Disease		Inactive Disease		*p* value
	Median	IQR	Median	IQR		Median	IQR	Median	IQR	
fCAL™ (µg/g)	1131	524 – 1484	182	53 – 252	–	1004	466 – 1922	151	55 – 280	–
BMN®-Cp (ng/mL)	5507	4107 – 9750	3823	2496 – 4923	0.0055	4534	3387 – 6416	4031	2401 – 5414	0.1825
IDK®-Cp (ng/mL)	5131	3487 – 9325	2967	1925 – 4691	0.0012	4531	2920 – 6433	3307	2104 – 4789	0.1065
CRP (mg/L)	9	2 – 17	3	1 – 5	0.0095	4	2 – 8	2	1 – 4	0.0638
Platelets (x10E9/L)	290	243 – 397	260	213 – 322	0.0579	269	233 – 331	280	227 – 325	0.8055
N	**44**		**57**			**29**		**42**		

Table 6.3 – Median and Interquartile range (IQR) for levels of faecal calprotectin, serum calprotectin, serum CRP and platelets in 172 patients with active and quiescent CD and UC. *p* value not given for fCAL™ as it was may have been used in some cases to distinguish between active and quiescent disease.

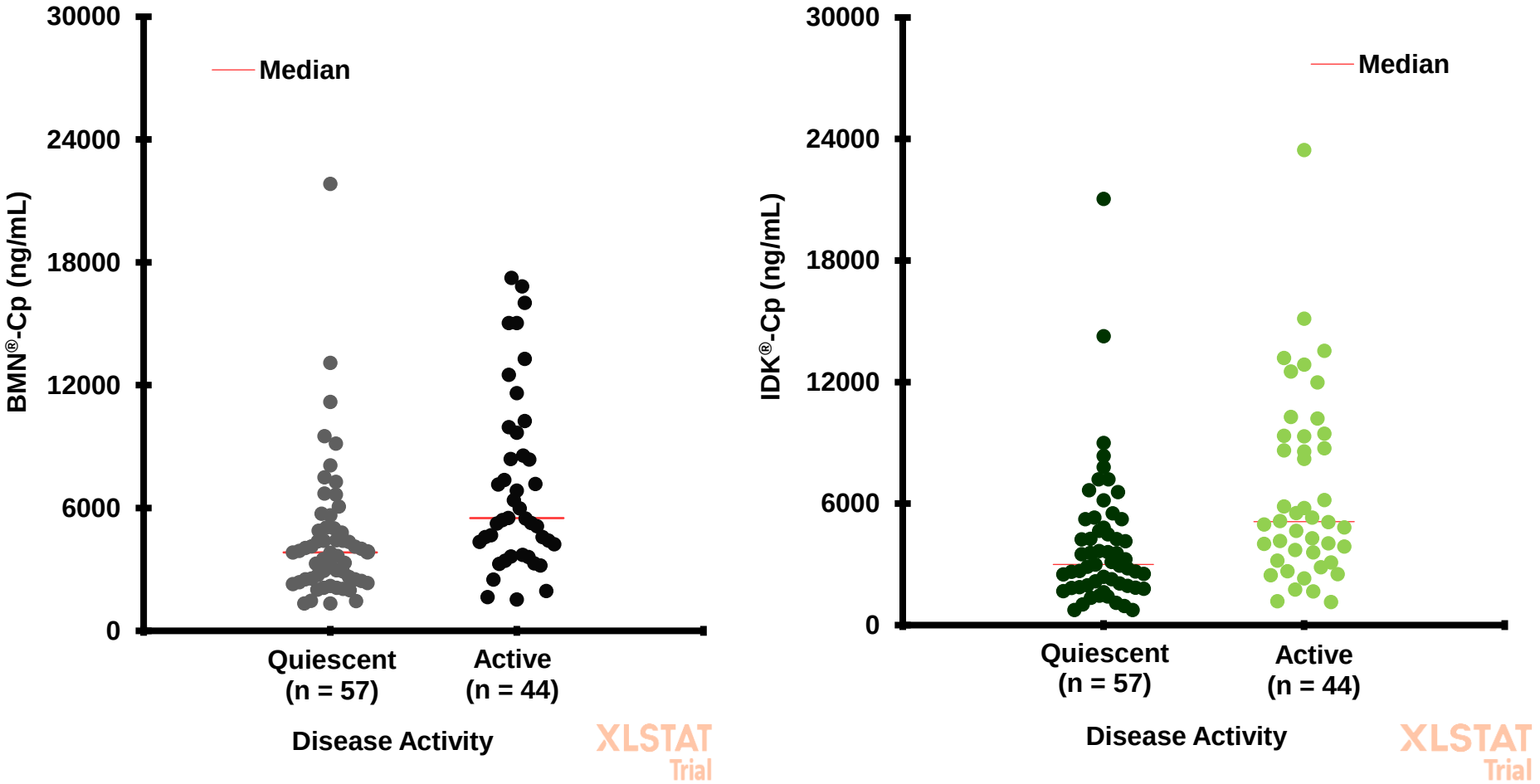

Figure 6.16 – Serum calprotectin concentrations measured by BMN®-Cp and IDK®-Cp methods in quiescent and active CD.

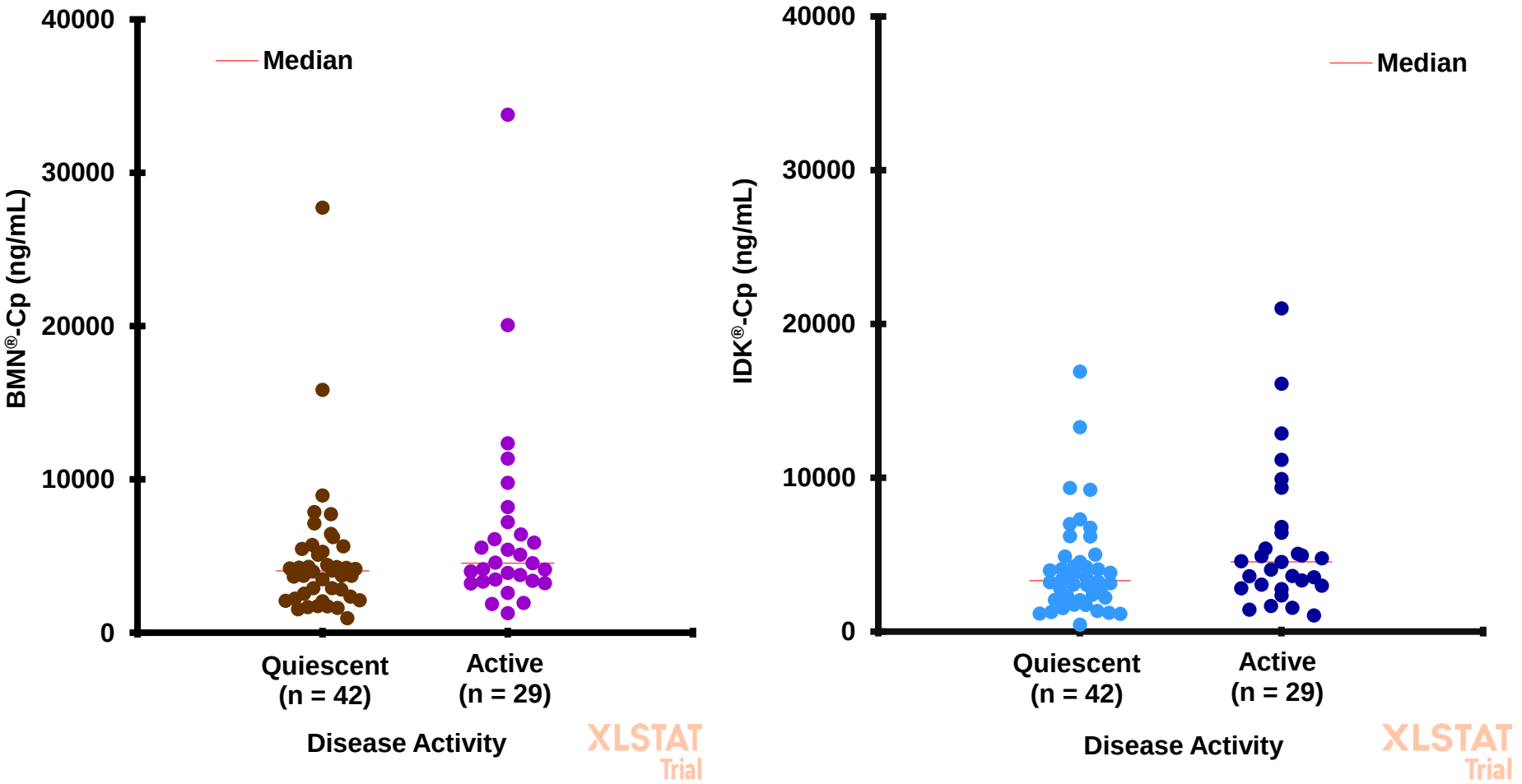

Figure 6.17 –Serum calprotectin concentrations measured by BMN®-Cp and IDK®-Cp methods in quiescent and active UC.

6.4.5 Serum calprotectin, serum CRP and platelets in active and inactive CD and UC according to site of disease

Serum BMN®-Cp and serum IDK®-Cp concentrations were higher ($p < 0.05$) in active CD than quiescent CD at the ileal and upper GI, and the colonic and ileo–colonic sites of the ileum (table 6.4, and figures 6.18 and 6.19). Serum BMN®-Cp and serum IDK®-Cp concentrations were similar ($p > 0.05$) in active UC and quiescent UC involving the rectum, distal colon and pancolon (table 6.5, and figures 6.20 and 6.21).

Disease Site	**Disease Activity**	**Statistics**	**Parameters**			
			Serum Calprotectin (ng/mL)		**CRP (mg/L)**	**Platelets (x10E9/L)**
			BMN®-Cp	**IDK®-Cp**		
Ileal and Upper GI (A1/A2)	Active (n = 25)	Median	5264	4866	8	277
		IQR	3632 – 11842	3029 –10219	1 –17	243 – 395
	Inactive (n = 33)	Median	3306	2656	2	245
		IQR	2518 – 4552	1925 – 4670	1 – 4	210 – 307
	p value		*0.028*	*0.014*	*0.005*	*0.054*
Colonic and Ileo–colonic (A3)	Active (n = 19)	Median	6416	5541	9	308
		IQR	4654 – 8377	4216 – 8797	4 –15	232 – 383
	Inactive (n = 24)	Median	4047	3495	4	275
		IQR	2392 – 5488	1930 – 4729	2 –11	247 – 338
	p value		*0.009*	*0.005*	*0.031*	*0.522*

Table 6.4 – Serum calprotectin, serum CRP and platelets levels for a common set of samples used in discriminating between active and inactive CD in 101 patients. Except for platelets, the difference in medium serum calprotectin and serum CRP levels between the active and quiescent course of the disease is statistically significant, $p < 0.05$ at all the disease sites. IQR = Interquartile range.

Disease Site	Disease Activity	Statistics	Parameters			
			Serum calprotectin (ng/mL)		CRP (mg/L)	Platelets (x10E9/L)
			BMN®-Cp	IDK®-Cp		
Proctitis (E1)	Active (n = 2)	Median	20999	15471	5	373
		IQR	14604 – 27395	12697 – 18246	4 – 7	345 – 402
	Inactive (n = 2)	Median	5867	5123	6	293
		IQR	5564 – 6149	4584 – 5661	5 – 7	286 – 301
	p value		–	–	–	–
Distal Colitis (E2)	Active (n = 13)	Median	4065	3589	4	269
		IQR	3333 – 6414	2816 – 5394	3 – 7	254 – 329
	Inactive (n = 21)	Median	3766	3528	1	280
		IQR	2914 – 5636	2667 – 6193	1 – 3	239 – 326
	p value		*0.739*	*0.586*	*0.122*	*0.994*
Pan Colitis (E3)	Active (n = 14)	Median	4816	4666	4	256
		IQR	3934 – 5797	3394 – 5027	2 – 7	229 – 345
	Inactive (n = 19)	Median	3502	2826	2	252
		IQR	2063 – 4294	1656 – 4213	1 – 4	223 – 329
	p value		*0.358*	*0.294*	*0.274*	*0.999*

Table 6.5 – Serum calprotectin, serum CRP, and platelets levels for a common set of samples used in discriminating between active and inactive UC in 71 patients. The difference in medium serum calprotectin, serum CRP, and platelets levels between the active and quiescent course of the disease is not statistically significant, $p > 0.05$ at all the disease sites. IQR = Interquartile range.

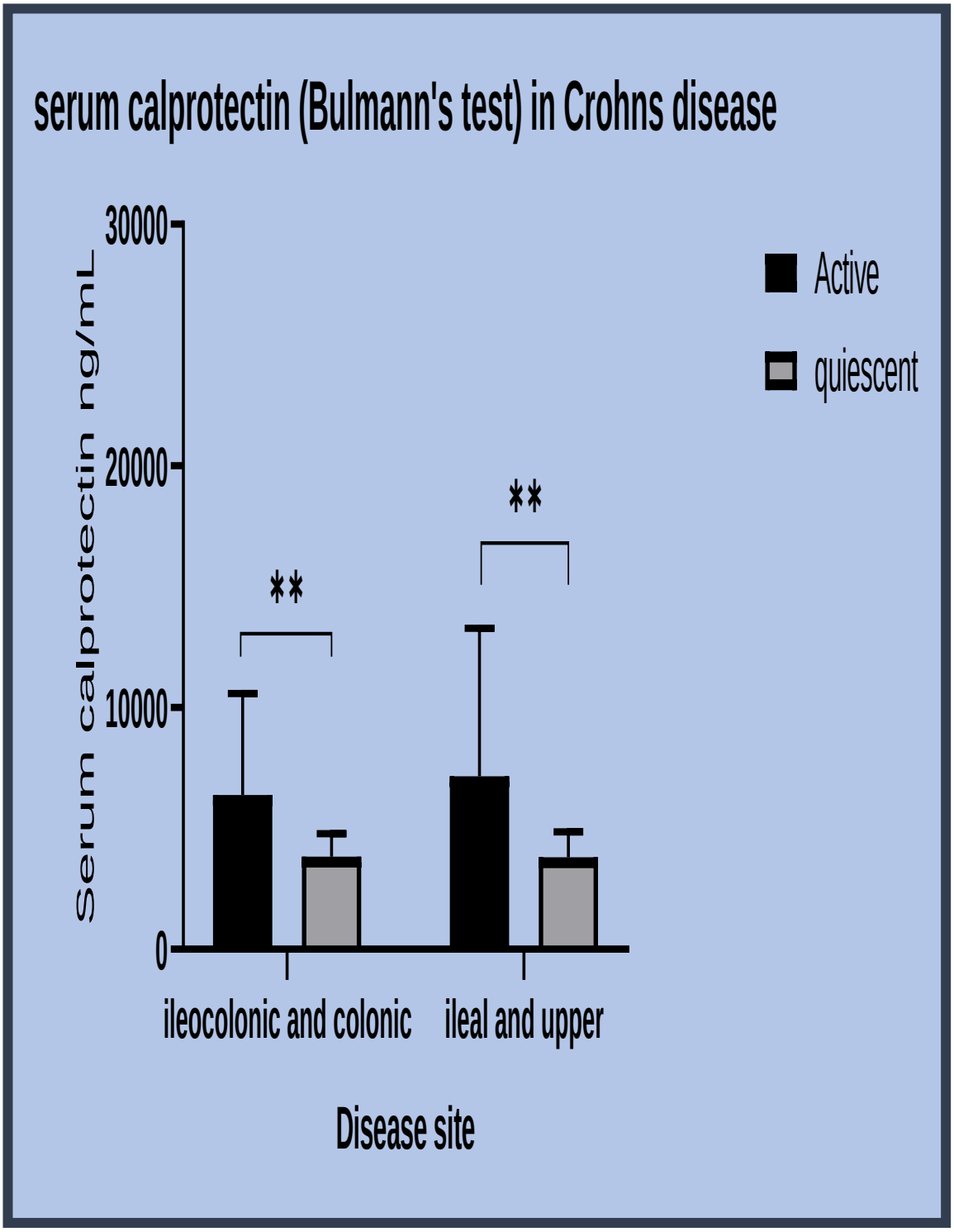

Figure 6.18 – Serum BMN®-Cp at ileo–colonic and colonic, and ileal and upper GI sites of the small bowel in CD patients with active CD (black bars) and quiescent CD (grey bars). ***p* = 0.009 at the ileo–colonic and colonic site and *p* = 0.028 at the ileal and upper GI site.

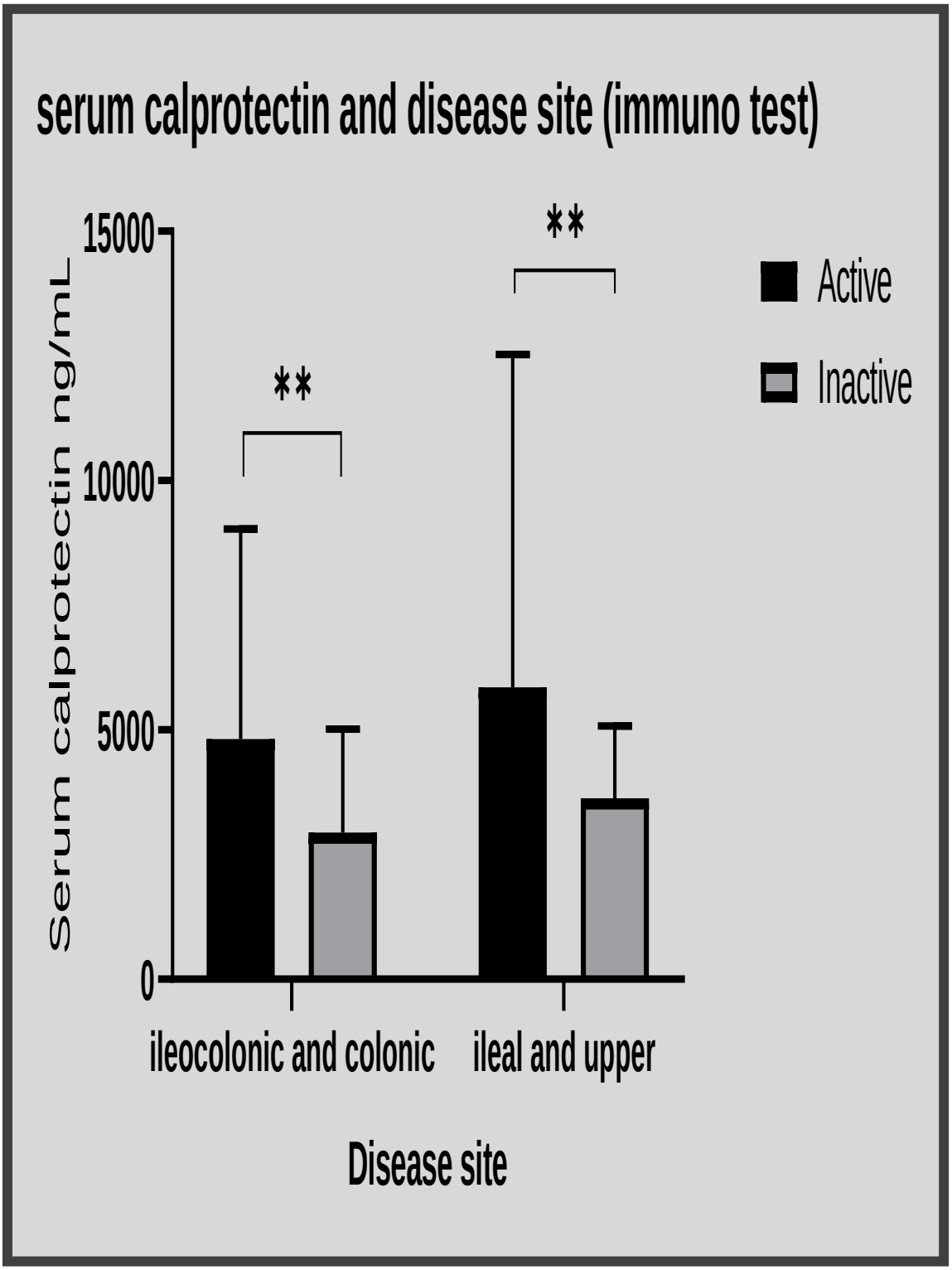

Figure 6.19 – Serum IDK®-Cp at ileo–colonic and colonic, and ileal and upper GI sites of the small bowel in CD patients with active CD (black bars) and quiescent CD (grey bars). ***p* = 0.005 at the ileo–colonic and colonic site and *p* = 0.014 at the ileal and upper GI site.

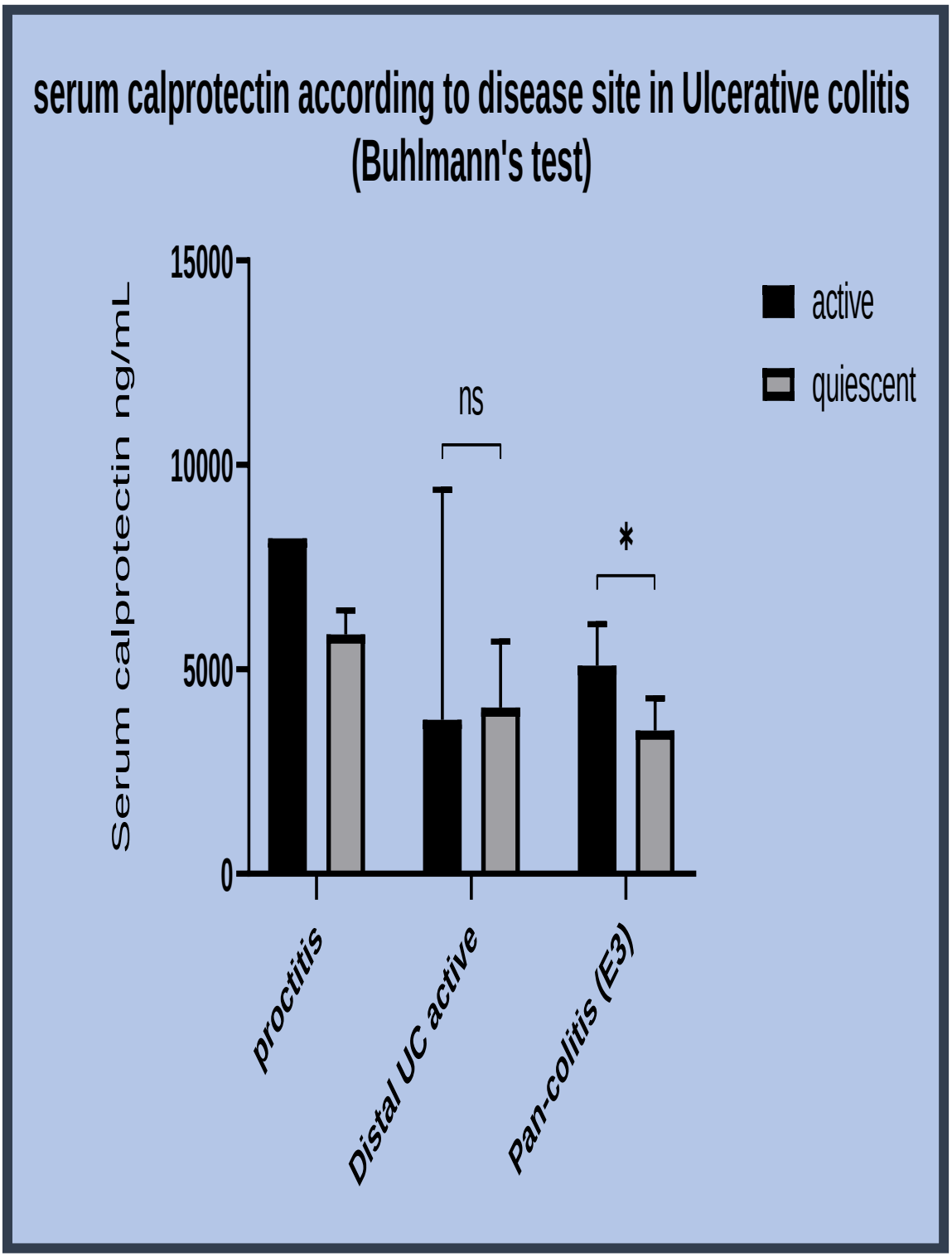

Figure 6.20 – Serum BMN®-Cp at Proctitis, Distal UC and Pancolitis sites of the large bowel in UC patients with active UC (black bars) and quiescent UC (grey bars). **p* = 0.36 at the proctitis site, *p* = 0.74 at the distal UC site and *p* = 0.36 at the pancolitis site. ns = not statistically significant.

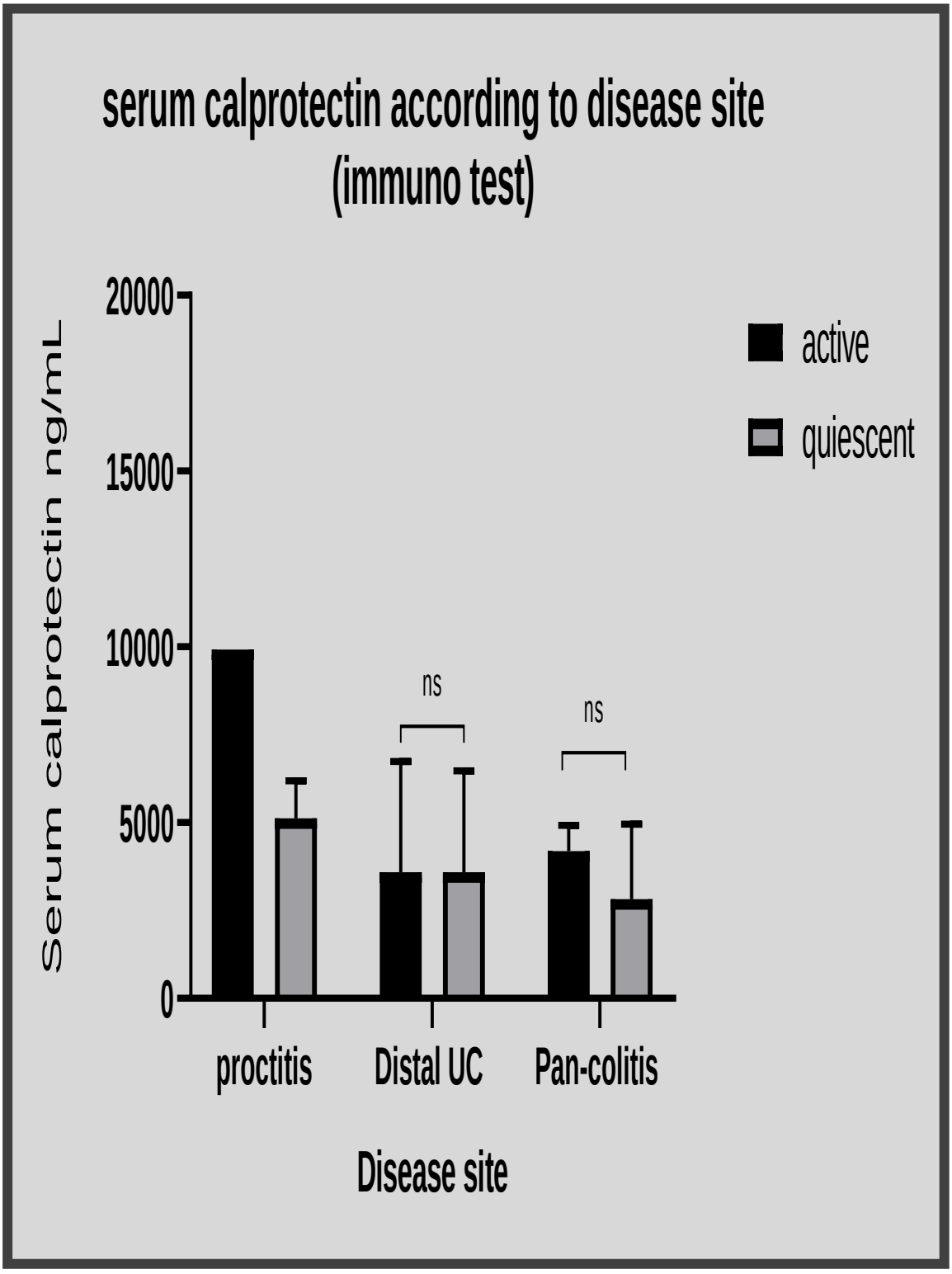

Figure 6.21 – Serum IDK®-Cp at Proctitis, Distal UC and Pancolitis sites of the large bowel in UC patients with active UC (black bars) and quiescent UC (grey bars). *p* = 0.21 at the proctitis site, *p* = 0.59 at the distal UC site and *p* = 0.29 at the pancolitis site. ns = not statistically significant.

6.4.6 Utility of serum calprotectin, serum CRP and platelets in discriminating active and inactive IBD

Receiver operator characteristic (ROC) curves were constructed for serum BMN®-Cp, serum IDK®-Cp, serum CRP and platelets in the discrimination between active and inactive IBD, CD and UC (figures 6.22 – 6.24).

In all patients with IBD, the performance of serum CRP was marginally superior to that of serum BMN®-Cp and serum IDK®-Cp, and these were superior to platelets (table 6.6). In patients with CD, none of the blood biomarkers performed well (table 6.7). In patients with UC , the performance of serum CRP was marginally superior to that of serum BMN®-Cp and serum IDK®-Cp, and these were superior to platelets (table 6.8).

Area Under the Curve

Test Result Variable(s)	Area Under the Curve (AUC)	Standard Error of the Mean (SEM)[a]	95% Confidence Interval (95%CI)	
			Lower Bound	Upper Bound
BMN-Cp® (ng/mL)	0.662	0.046	0.572	0.751
IDK®-Cp (ng/mL)	0.656	0.047	0.563	0.748
CRP (mg/L)	0.699	0.045	0.610	0.788
Platelets (x10E/9L)	0.547	0.050	0.449	0.646

Table 6.6 – The Area Under the Curve (AUC) analysis of serum BMN®-Cp (blue), IDK®-Cp (red) assays), serum CRP (green) and platelet (orange) in discriminating active and inactive disease in 175 patients with IBD. [a]Under the non–parametric assumption.

Area Under the Curve

Test Result Variable(s)	Area Under the Curve (AUC)	Standard Error of the Mean (SEM)[a]	95% Confidence Interval (95%CI)	
			Lower Bound	Upper Bound
BMN-Cp® (ng/mL)	0.560	0.071	0.420	0.699
IDK®-Cp (ng/mL)	0.556	0.068	0.422	0.690
CRP (mg/L)	0.585	0.067	0.454	0.716
Platelets (x10E/9L)	0.609	0.071	0.470	0.747

Table 6.7 – The Area Under the Curve (AUC) analysis of serum BMN®-Cp (blue), IDK®-Cp (red) assays), serum CRP (green) and platelet (orange) in discriminating active and inactive disease in 101 patients with CD. [a]Under the non–parametric assumption.

Area Under the Curve

Test Result Variable(s)	Area Under the Curve (AUC)	Standard Error of the Mean (SEM)[a]	95% Confidence Interval (95%CI)	
			Lower Bound	Upper Bound
BMN-Cp® (ng/mL)	0.670	0.067	0.539	0.801
IDK®-Cp (ng/mL)	0.660	0.069	0.525	0.794
CRP (mg/L)	0.752	0.065	0.623	0.880
Platelets (x10E/9L)	0.487	0.074	0.341	0.633

Table 6.8 – The Area Under the Curve (AUC) analysis of serum BMN®-Cp (blue), IDK®-Cp (red) assays), serum CRP (green) and platelet (orange) in discriminating active and inactive disease in 71 patients with UC. [a]Under the non–parametric assumption.

ROC Curve of serum calprotectin, serum CRP and Platelets in IBD

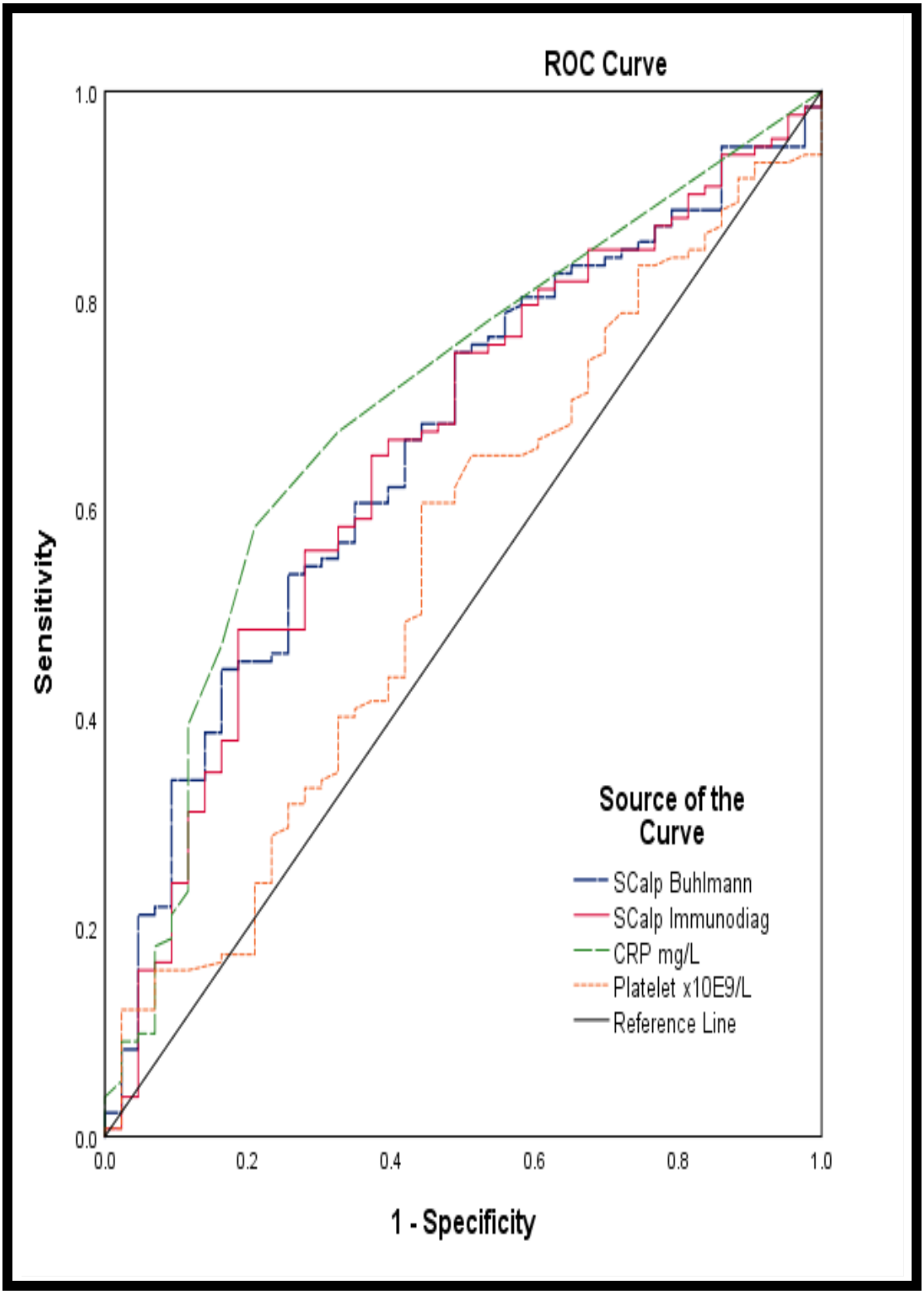

Figure 6.22 – Receiver operating characteristic (ROC) curve analysis of serum calprotectin (measured with the BMN®-Cp (blue), IDK®-Cp (red) assays), serum CRP (green) and platelet (orange) in discriminating between active and inactive IBD in 175 patients. The reference line is indicated by the black line that runs diagonally across the curve.

ROC Curve of serum calprotectin, serum CRP and Platelets in CD

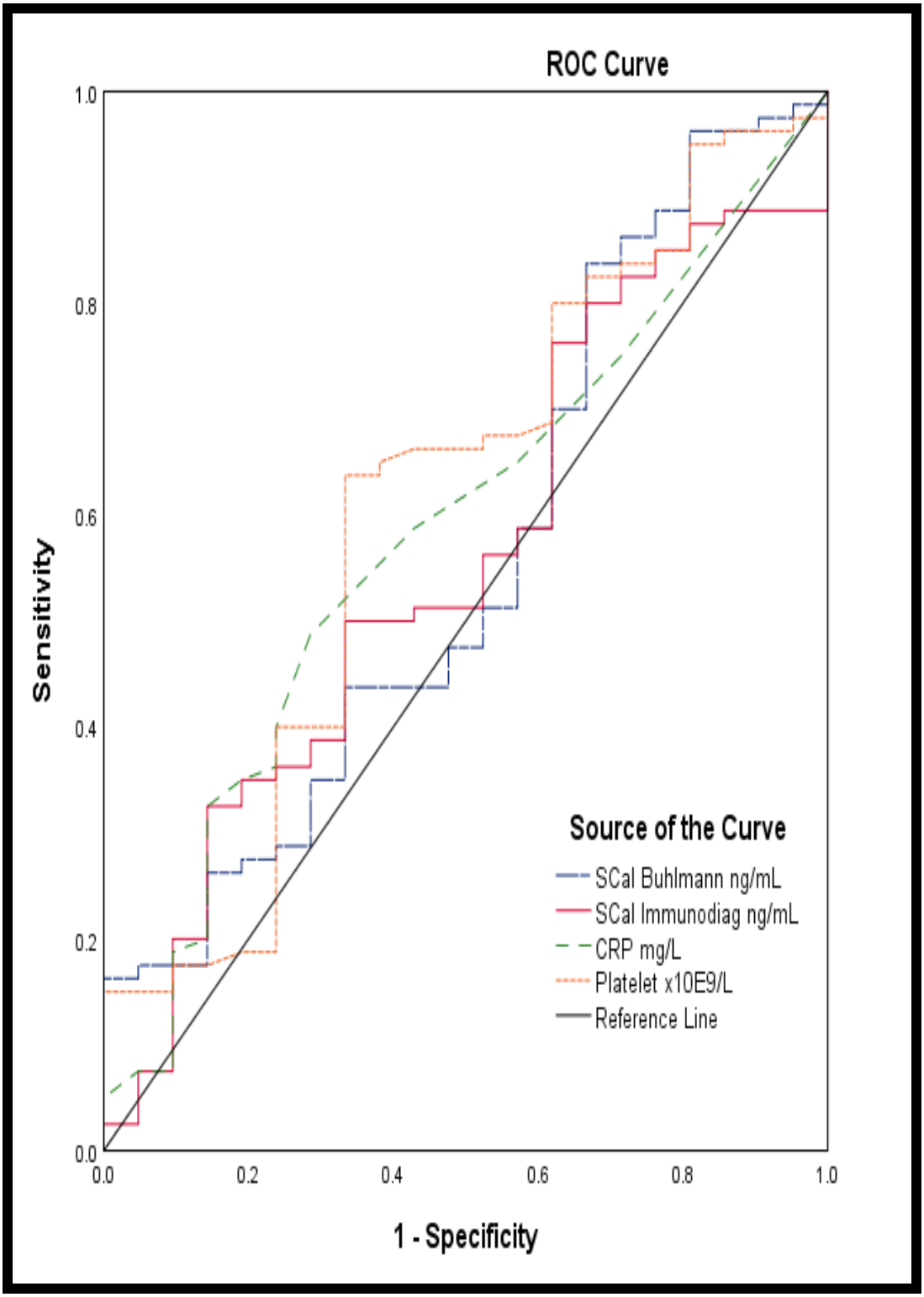

Figure 6.23 – Receiver operating characteristic (ROC) curve analysis of serum calprotectin (measured with the BMN®-Cp (blue), IDK®-Cp (red) assays), serum CRP (green) and platelet (orange) in discriminating between active and inactive CD in 101 patients. The reference line is indicated by the black line that runs diagonally across the curve.

ROC Curve of serum calprotectin, serum CRP and Platelets in UC

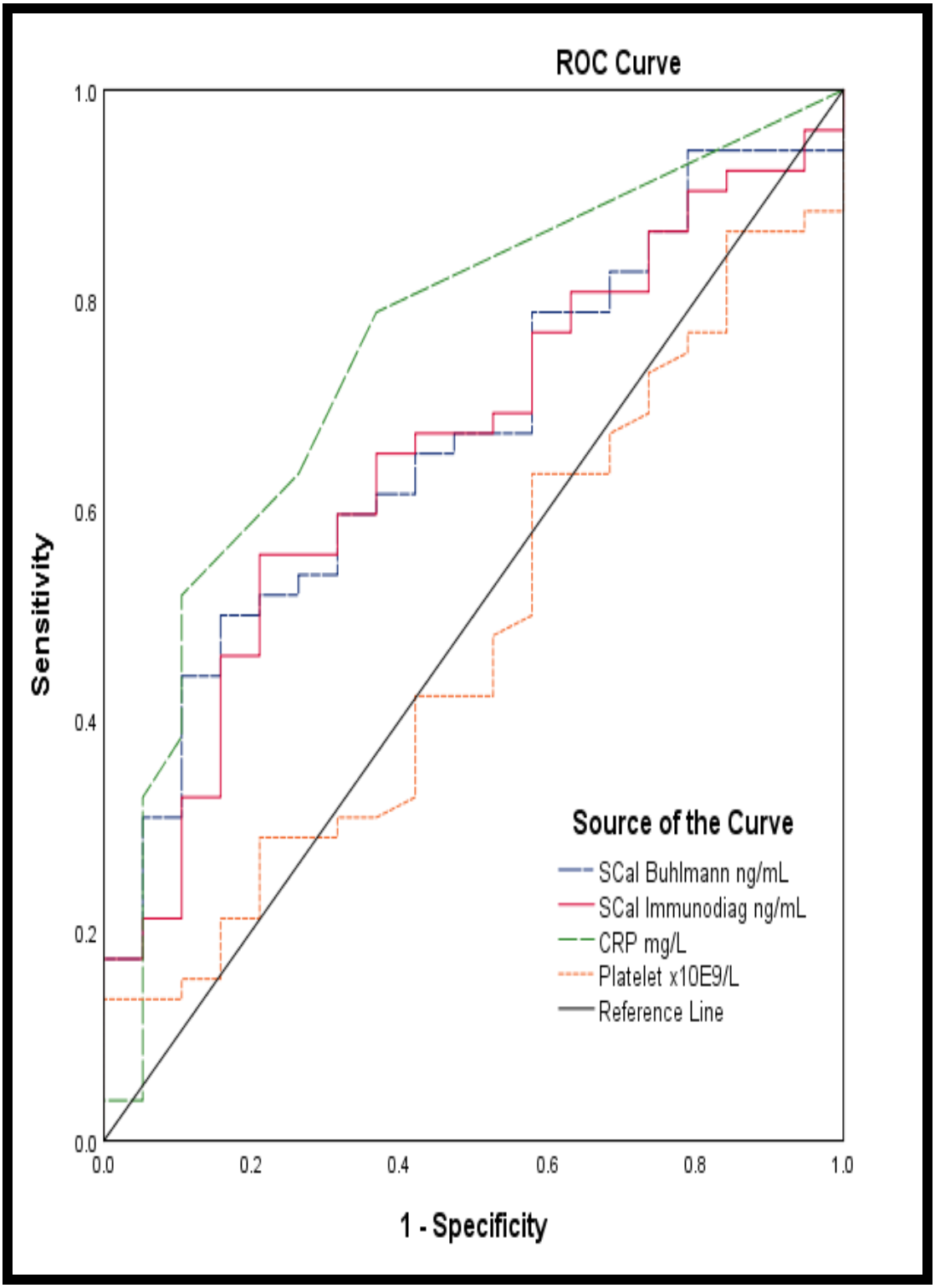

Figure 6.24 – Receiver operating characteristic (ROC) curve analysis of serum calprotectin (measured with the BMN®-Cp (blue), IDK®-Cp (red) assays), serum CRP (green) and platelet (orange) in discriminating between active and inactive UC in 71 patients. The reference line is indicated by the black line that runs diagonally across the curve.

6.4.7 Sensitivity and specificity

From the ROC curve analysis, the sensitivity and specificity using manufacturer recommended and in–house cut–offs of the four biomarkers for discriminating between active and inactive IBD, CD and UC are shown in table 6.9.

Test Result Variable(s)	Cut–off value	Test Performance	Disease Classification		
			IBD	CD	UC
BMN®-CP (ng/mL)	> 3900 ng/mL[a]	Sensitivity (%)	63	61	62
		Specificity (%)	58	43	58
		PLR	1.5	1.1	1.5
		NLR	0.6	0.9	0.7
IDK®-Cp (ng/mL)	> 3000 ng/mL[a]	Sensitivity (%)	68	66	69
		Specificity (%)	51	38	47
		PLR	1.4	1.1	1.3
		NLR	0.6	0.9	0.7
CRP (mg/L)	> 4 mg/L[a]	Sensitivity (%)	47	49	39
		Specificity (%)	84	71	89
		PLR	2.9	1.7	3.5
		NLR	0.6	0.7	0.7
Platelet (x10E9/L)	> 400 x10E9/L[b]	Sensitivity (%)	13	15	77
		Specificity (%)	93	90	53
		PLR	1.9	1.5	1.6
		NLR	0.9	0.9	0.4

Table 6.9 – Test performance described in terms of the sensitivity and specificity of the BMN®-Cp, IDK®-Cp, serum CRP and Platelet assays at different cut–off values for discriminating active and inactive disease in IBD, CD and UC. [a]Cut–off value supplied by kit manufacturer. [b]In–house cut–off value. PLR = Positive Likelihood Ratio; NLR = Negative Likelihood Ratio (Bolin and Lam, 2013). (For definitions of PLR and NLR, see Appendix I: Definition of terms; The validity of a diagnostic test; The diagnostic value of serum calprotectin data in IBD study).

6.5 Discussion

Colonoscopy and biopsy remains the gold standard in the assessment of the response to treatment in IBD by discriminating between active and inactive IBD (Lehmann et al., 2015; Panes et al., 2017; Kim et al., 2016; Travis et al., 2012; Hebuterne et al., 2013; Khanna et al., 2016; Hall et al., 2014; Niv et al., 2014; Panes et al., 2013; Henderson et al., 2014; Van Rheene et al., 2010). Colonoscopy, however, is invasive, relatively expensive, and inconvenient for patients. The development, therefore, of convenient, inexpensive, and non–invasive biomarkers are appealing in the assessment of IBD.

Faecal calprotectin is routinely used in the management of IBD patients and in this study may have been used in some patients to distinguish active from inactive disease in both CD and UC cohorts (Hanai et al., 2003). Despite acceptable sensitivity and specificity of faecal calprotectin in monitoring disease activity in IBD (D'Inca et al., 2007; Røseth et al., 2004; Jones et al., 2008; Schoepfer et al., 2009; Schoepfer et al., 2010; Sipponen et al., 2008a; Sipponen et al., 2008b; Sipponen et al., 2008c; Sipponen et al., 2010; Solem et al., 2005; Lewis, 2011; Saverymuttu et al., 1986b; Farup et al., 1995; Fagerberg et al., 2007; Choden et al., 2018; Chang et al., 2015; Meier and Strum, 2011; Smith and Gaya, 2012; Gallo et al., 2014; Judd et al., 2011; Vermeire et al., 2006; Lehmann et al., 2015; Manceau et al., 2017; Schoepfer et al., 2012; Schoepfer et al., 2013; Naismith et al., 2014; Fukunaga et al., 2018; Gaya et al., 2005; Boschetti et al., 2015; D'Haens et al., 2012; Kopylov et al., 2014; Konikoff and Denson, 2006; Angriman et al., 2007; Mindemark and Larsson, 2012; Tibble et al., 2000b; Hanai et al., 2004; Røseth et al.,

1997), patients have difficulty collecting stool samples and therefore more convenient biomarkers are desirable.

The present study evaluated the performance of serum calprotectin, using two different commercially available assays, as a biomarker to replace or supplement faecal calprotectin in distinguishing active and inactive IBD. We also compared serum calprotectin to serum CRP and platelets.

Blood biomarkers of acute phase response (APR) such as CRP, ESR, albumin and platelets have been widely investigated in IBD. They, however, are not specific to IBD and although their concentrations increase in active IBD, they also increase in response to various inflammatory disorders including infections, other autoimmune disorders, and malignancy (Menees et al., 2015; Cakal et al., 2009; Rodgers and Cummins, 2007; Lewis, 2005; Solem et al., 2005; Fagan et al., 1982; Bell and Wilson, 2014; Moran et al., 1995; Cellier et al., 1994; Macfarlane et al., 1986; Yamada, 1999; Lannergård et al., 2005; Duzova et al., 2003; Jaye and Waites, 1997; Vermeire et al., 2005; Vermeire et al., 2004; Chenillot et al., 2000; Pincus et al., 2014).

Similarly, serum calprotectin is a non–specific marker of APR (Chapter 7) and would be expected to have the same limitations as other biomarkers of inflammation. In this study, all biomarkers had varying degrees of correlations with each other. The correlation of faecal calprotectin was best with serum CRP and less with platelets and serum calprotectin, irrespective of assay. Serum calprotectin, irrespective of assay, correlated best with serum CRP and both correlated less with platelets. These results corroborate findings of Suarez–Ferrer and colleagues who reported correlations between serum calprotectin with both serum CRP and faecal calprotectin (Suarez–Ferrer et

al., 2019). These findings are also consistent with those studies reporting a correlation between serum calprotectin and faecal calprotectin (Meuwis et al., 2013; Hare et al., 2013), but not others reporting no such correlation (Azramezani et al., 2019; Carlsen et al., 2019; McCann et al., 2017). Studies have also, similarly, reported a correlation between serum calprotectin and serum CRP (Azramezani et al., 2019; Carlsen et al., 2019; Okada et al., 2019). We also report a correlation between serum calprotectin and platelets which differs from findings in a previous study (Okada et al., 2019). Differences between these studies may reflect differences in study cohorts, study methodology and analytical methods.

Serum calprotectin, irrespective of assay, and serum CRP (but not platelets) were significantly different in patients with active and inactive CD, and this was irrespective of site of disease. Conversely, serum calprotectin, irrespective of assay, and serum CRP and platelets were not significantly different in patients with active and inactive UC, and this was irrespective of site of disease. Receiver operating characteristic curve (ROC) analyses, however, indicated that the blood biomarkers performed better in UC than in CD and of these serum CRP performed best; none however performed well consistent with other studies (Kalla et al., 2016; D'Haens et al., 2020; Boschetti et al., 2015). Our results suggest that serum calprotectin (and serum CRP) may have a role in predicting exacerbations in CD but not UC. Our data are, therefore, consistent with studies indicating that serum calprotectin may have a role in predicting relapse and disease burden in CD (Kalla et al., 2016; Hare et al., 2013; Lügering et al., 1995; Meuwis et al., 2013). Serum calprotectin, however,

appears to offer no advantage over serum CRP, which is readily available and less expensive.

We suggest that the different serum calprotectin and serum CRP results in CD and UC reflect the underlying disease processes and their associated faecal and systemic inflammatory biomarkers. Calprotectin produced by the gut epithelial cells and mucosal neutrophils during intestinal inflammation is released into the gut lumen leading to increased levels of faecal calprotectin whereas serum calprotectin is a biomarker of systemic inflammation rather than intestinal–specific inflammation. CD is a transmural disease and therefore during disease exacerbations may appear more likely to provoke a systemic inflammatory response in addition to intestinal inflammatory response leading to an increase in both serum calprotectin (and serum CRP) and faecal calprotectin respectively. UC, however, is a mucosal disease and therefore disease exacerbations are typified by intestinal inflammatory response with increasing faecal calprotectin but, unless acute and severe (Hare et al., 2013), may appear less likely to provoke a systemic inflammatory response. This may explain why serum calprotectin (and serum CRP) may not be increased in active disease relative to faecal calprotectin.

Serum calprotectin measured by the BMN®-Cp assay and the IDK®-Cp assay were highly correlated. The BMN®-Cp assay had a 19% positive constant proportional bias relative to the IDK®-Cp assay which is reflected in their respective reference intervals. Both assays performed equally.

The strength of this study was a large patient sample allowing use of a range of statistical tests analyses without incurring the risk of type 2 statistical errors. The present study, however, has limitations. The clinical diagnosis of active

and inactive IBD was based on clinical evaluation and where available supported by faecal calprotectin, imaging and endoscopy. Colonoscopy and biopsy is the gold standard in discriminating between active and inactive IBD. Clinical activity scoring such as Mayo disease severity score for UC (includes endoscopy component) and the Harvey–Bradshaw clinical index for CD have also been used to assess relapse of IBD. Ideally, therefore faecal calprotectin and blood biomarkers should have been evaluated for active and inactive IBD defined by endoscopic biopsy findings or less preferably clinical scoring systems. Unless clinically indicated, however, endoscopy is not used to distinguish active and inactive IBD and clinical disease activity scoring systems are rarely used in routine clinical practice. This study was, however, designed to assess whether serum calprotectin could replace or supplement faecal calprotectin in predicting relapse in patients with IBD. Other limitations include small number of patients with IBDU and proctitis (UC) that precluded evaluating utility of blood biomarkers in assessing active versus inactive disease in these patient cohorts.

6.6 Conclusion

We found no significant relationship between serum calprotectin and disease activity in patients with UC. Serum calprotectin, for both the BMN®-Cp and IDK®-Cp assays, had a significant association with disease activity in patients with CD irrespective of site of disease.

Serum calprotectin had a moderate positive correlation with serum CRP and only a weak positive correlation with platelets and faecal calprotectin. Serum CRP demonstrated a modest benefit over serum calprotectin based on ROC curves analyses for CD and UC and a greater correlation with faecal

calprotectin. In our patient cohort, therefore, serum calprotectin offers no advantages over serum CRP, which is easily available as point–of–care testing (POCT) or near–patient testing (NPT) and less expensive.

CHAPTER 7

CLINICAL STUDY III: AN INVESTIGATION OF THE ROLE OF SERUM CALPROTECTIN IN THE ACUTE PHASE RESPONSE

7.1 Introduction

The process of inflammation may be accompanied by large number of behavioural, biochemical, physiological and nutritional changes that are both local and distant, from the site or sites of inflammation, and involves the participation of many organ systems. These changes are events that lead to wide–ranging systemic response, and have been described as acute phase response (APR), even though they can also become prolonged and accompany both acute and chronic inflammatory disorders (Bell and Wilson, 2014; Suffredini et al., 1999; Pannen and Robotham, 1995; Baumann and Gauldie, 1994).

Two interrelated processes that are characterised by fever and leucocytosis, distinguish APR. These are changes in the concentrations of numerous plasma proteins (table 7.1) that are upregulated in the presence of acute phase stimulus (Vermeire et al., 2004). Following changes in the concentrations of many plasma proteins and the initiation of the APR, the liver and other tissues undergo dramatic upregulation and synthesis of a variety of proteins which can mediate or inhibit inflammatory processes, function as transport proteins and participate in tissue repairs (Gabay and Kushner, 1999).

Proteins whose plasma concentrations increase (▲), i.e., positive acute phase proteins →	**Complement System →**	C1s; C2; C3; C4; C5; C9; Factor B; C1 inhibitor; C4b–binding protein; Mannose–binding lectin.
	Coagulation and Fibrinolytic System →	Fibrinogen; Plasminogen; Tissue plasminogen activator; Protein S; von Willebrand factor; Urokinase; Vitronectin; Plasminogen–activator inhibition 1; Prothrombin; Factor VIII.
	Antiproteases →	α_1–Protease inhibitor; Pancreatic secretory trypsin inhibitor; Inter–α– trypsin inhibitors; α_2–Antiplasmin; Antithrombin III; α_2–Macroglobulin; α_1–Antichymotrypsin.
	Transport proteins →	Albumin; Ceruloplasmin; Haptoglobin; Hemopexin; Ferritin.
	Participants in inflammatory responses →	Secreted phospholipase A_2; Lipopolysaccharide-binding protein; Interleukin–1–receptor antagonist; Granulocyte colony–stimulating factor (GCSF).
	Others →	CRP; Serum amyloid A; α_1–Acid glycoprotein; Fibronectin; Ferritin; Angiotensinogen; Gc globulin.
Proteins whose plasma concentrations decrease (▼), i.e., negative acute phase proteins →	Albumin; Transferrin; Transthyretin; α_2–HS glycoprotein; α–fetoprotein; Thyroxine–binding globulin; Insulin–like growth factor 1; Factor XII.	

Table 7.1 – Acute phase proteins in humans. The resulting changes in acute phase proteins in systemic circulation are most often indications of onset of inflammatory processes which may not necessarily reflect the incidence of IBD, rheumatoid arthritis or bone disease. APR, in a restricted and more often used definition refers to changes in concentrations of large number of plasma proteins, predominantly the acute phase proteins that are associated with this response. These changes are primarily the result of variations in the configuration of protein synthesis in hepatocytes. Modified from Pannen and Robotham (1995); Ceciliani et al (2002) and Campbell et al (1982).

The other involves a large number of behavioural, biochemical, physiological and nutritional changes that counteract the underlying challenge in order to restore homeostasis as soon as possible (table 7.2). Both processes are accomplished by isolating and destroying infective organisms, or removing harmful molecules, and activating the repair process (Gabay and Kushner, 1999). Some examples of acute phase phenomena depict acute phase reactions that involve a wide range of changes due to neuroendocrine, haematopoietic, metabolic, hepatic and other changes arising from interactions of molecules of nonprotein constituents in plasma (Gabay and Kushner, 1999).

Neuroendocrine changes →	Fever, Somnolence and Anorexia; Increased secretion of corticotropin–releasing hormone, corticotropin and cortisol; Increased secretion of arginine vasopressin; Decreased production of insulin–like growth factor 1; Increased adrenal secretion of catecholamines.
Haematopoietic changes →	Anaemia of chronic diseases; Thrombocytosis; Leucocytosis.
Metabolic changes →	Loss of muscle and negative nitrogen balance; Decreased gluconeogenesis; Osteoporosis; Cachexia; Increased hepatic lipogenesis; Increased lipolysis in adipose tissue; Decreased lipoprotein lipase activity in muscle and adipose tissue.
Hepatic changes →	Increased metallothionein, inducible nitric oxide synthase, heme oxygenase, manganese superoxide dismutase and tissue inhibitor of metalloproteinase–1; Decreased phosphoenolpyruvate carboxylase activity.
Changes in non–protein plasma constituents →	Hypozincaemia, hypoferremia and hypercupraemia; Increased plasma retinol and glutathione concentrations.

Table 7.2 – Other acute phase phenomena. Synthesis of acute phase proteins is instigated by tissue injury that precedes inflammation as the most important element of APR which, occurs prior to the onset of a specific immune response and also before the onset of clinical signs. In this way APR becomes one of the first indicators that a pathological process is occurring. Modified from Bell and Wilson (2014).

Changes attributable to APR are coordinated severally by the innate immune defense system's response mechanism to tissue injury that are evoked in response to inflammation (Ceciliani et al., 2002). These changes are collectively modulated by acute phase proteins – a group of proteins that are largely synthesised by the hepatocytes, (although other cells can also produce these proteins) that circulate in the blood. Concentrations of circulating acute phase proteins are characterized by at least 25 per cent increase (positive acute phase proteins) or decrease (negative acute phase proteins) in response to tissue injury (Bell and Wilson, 2014; Gabay and Kushner, 1999). Synthesis of acute phase proteins is instigated by tissue injury that precedes inflammation as the most important element of APR which, occurs prior to the onset of a specific immune response and also before the onset of clinical signs. In this way APR becomes one of the first indicators that a pathological process is occurring.

7.1.1 Acute phase proteins in the disease process

Serum concentration of major acute phase proteins including albumin, orosomucoid, serum amyloid A (SAA) and CRP are vital to understanding of the disease process because of their ease of testing and sensitivities as biomarkers of acute and chronic inflammation (Bell and Wilson, 2014). Low albumin levels are common in active disease states (Moran et al., 1995). Orosomucoid, with a half–life of approximately 4 to 5 days in serum, may increase up to 4– to 5–folds during severe inflammation in both adult and paediatric populations (Cellier et al., 1994; Macfarlane et al., 1986). SAA levels in serum increases with age in adults compared to paediatric population, and has been found to be a better

biomarker of inflammation and predicting response to treatment outcome with colchicine dosing in familial Mediterranean fever than CRP, ESR, fibrinogen and ferritin (Yamada, 1999; Lannergård et al., 2005; Duzova et al., 2003).

CRP has been used extensively in the paediatric population as an indicator of APR in inflammation or tissue damage, and as an indicator or predictor of disease course (Henderson et al., 2015; Rhodes et al., 2010; Ridker et al., 2000; Jaye and Waites, 1997). CRP synthesis by hepatocytes is stimulated by cytokines, primarily IL–1β, IL–6 and TNF–α. Like CRP, the production of cytokines by the hepatocytes may vary between individuals due to genetic polymorphism or individual variability inside the genes (MacGregor et al., 2004; Zhang and Ni, 2011; Berger et al., 2002; Brull et al., 2003; Zee and Ridker, 2002).

Some studies have evaluated CRP assay that reports a reference value of ≤ 5 or 8 mg/L in different APR situations and diseases including IBD. A major drawback is the low level of CRP assay sensitivity (Vermeire et al., 2005; Vermeire et al., 2004; Cellier et al., 1994). However, the availability of high–sensitivity (hs–CRP) assay that measures as low as 0.2 mg/L CRP has addressed this limitation, with mean CRP levels of 0.37 and 0.98 mg/L in healthy children and healthy adults respectively (Chenillot et al., 2000).

However, low specificity of acute phase proteins for disease processes is a limitation which may even be underestimated and could contribute to delays in diagnosis and treatment (Pincus et al., 2014; Parkes et al., 2018).

A comparison of some biochemical properties of CRP and SAA with calprotectin is presented in table 7.3.

Characteristics	Acute Phase Proteins		
	Calprotectin	**C–reactive protein (CRP)**	**Serum Amyloid A (SAA)**
Origin / Source	Abundant in neutrophils	Synthesised by hepatocytes	Mostly synthesised by hepatocytes
Family background	S100 family of calcium–binding proteins	Pentraxin superfamily	Apolipoprotein family
Chromosomal location / (Encoded by)	Chromosome 1 (1q 12 – 21)	Chromosome 1 (1q 23 – 24)	Chromosome 11 (11:p15.1)
Structure (i.e., monomeric / polymeric constituents)	Heterocomplex: light and heavy chain.	Five monomers	Three isotypes in plasma: SAA1, SAA2 and SAA4.
Molecular weight (kDa)	36	118	12
Half–life (hours)	5	19	1.5
Incremental pattern in infectious diseases	40 to 130–fold	100 to 1000–fold	100 to 1000–fold
Period for upregulation	Within hours	Within hours	Within hours

Table 7.3 – The common biochemical characteristics of the traditional acute phase proteins, CRP and SAA compared to calprotectin. Modified from: MacGregor et al (2004); Yamada (1999); Lannergård et al (2005); Duzova et al (2003).

7.1.2 C–reactive protein as an inflammatory biomarker in surgery

After major surgery, characteristic homeostatic changes such as fever, leucocytosis, tachypnoea and tachycardia occur, and have been described as the postoperative APR (Berger et al., 1997; Neumaier et al., 2015). IL–6, mainly released by monocytes, is the central signal molecule of the APR mechanism, and is understood to be the most potent stimulator of hepatic synthesis of fibrinogen and CRP (Mooren et al., 2006). However, IL–1 gene polymorphism known to affect the inflammatory response are highly related to plasma levels of CRP and fibrinogen in patients referred for coronary angiography (Berger et al., 2002; Brull et al., 2003; Zee and Ridker, 2002).

First described in human acute phase serum at the Rockefeller Institute by Tillett and Francis (1930), based upon observations on human acute phase serum agglutinating certain members of *Streptococcus pneumoniae* by binding to a soluble extract of C–polysaccharide (the characteristic that subsequently gave CRP its name), the production of CRP (figure 7.1) occurs almost exclusively in the liver by the hepatocytes as part of the APR mechanism upon stimulation by IL–1, IL–6 and TNF–α originating at the site of inflammation (Vermeire et al., 2004).

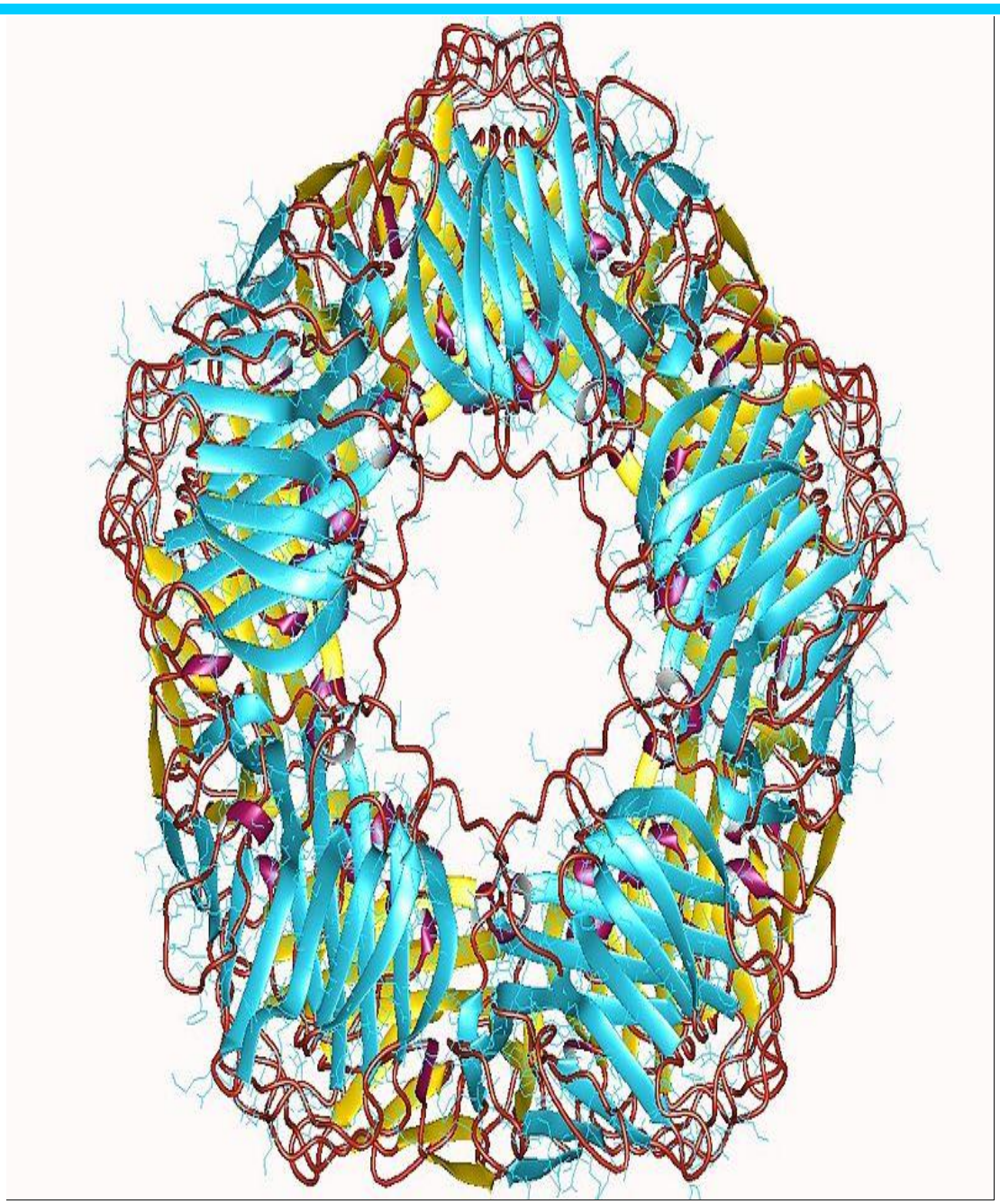

Figure 7.1 – The molecular cyclic pentametric structure of C–reactive protein complexed with phosphocholine. Phosphocholine along with the two calcium ions (Ca^{2+}), are necessary for ligand binding and are located at the binding sites of each promoter. Each of the five identical non–covalently attached subunits has an intra–disulphide bond and the molecular weight of each subunit is approximately 23 kDa (Modified from: Lv et al (2018); Thompson et al (1999) and Shrive et al (1996).

With a 50 to 60% homology share with SAA, CRP is considered superior to conventional parameters (i.e., ESR, leucocyte count etc), and newer parameters such as IL–1, IL–6, TNF–α and procalcitonin (Douraiswami et al., 2012; Oberhofer et al., 2012) in detecting surgical complications with bacterial infection (Choudhry et al., 1992; Ellitsgaad et al., 1991; Ettinger et al., 2015; Meyer et al., 1995), and in reflecting the extent of surgical trauma (Neumaier et al., 2006). Even though postoperative CRP concentrations are considered as risk factors for the postoperative outcome, and having the kinetic property to increase several hundred–fold within 24 hours after tissue injury from a normal concentration of about 5 mg/L, it is however, thought to be a rudimentary biomarker for monitoring postoperative patients (Foglar and Lindsey, 1998; Neumaier and Scherer, 2008).

In the presence of acute phase stimuli, primarily the production of IL–6, and enhanced in combination with IL–1 and TNF–α, CRP synthesis is rapidly (within hours) upregulated and may reach concentrations that are 500– to 1000–fold higher than under basal circumstances. With a short half–life of about 19 hours that is independent of any physiological or pathophysiological circumstances, or of its concentration in the serum, the rate of production of CRP almost exclusively by the hepatocytes is the only factor that determines its serum concentration. Thus, only liver failure or therapies affecting the acute phase stimulus may decrease CRP concentration (Vermeire et al., 2004). This is another characteristic which ensures that CRP concentration quickly decreases once the acute phase stimulus disappears. This makes CRP a very valuable biomarker to detect and follow–up in inflammation, and this characteristic is in contrast to other acute phase protein such as fibrinogen, plasminogen, prothrombin and von Willebrand factor (Kushner, 1990).

7.1.3 C–reactive protein in response to post–operative trauma

An increase in CRP levels is seen as expression of inflammatory response after trauma/operation (Foglar and Lindsey, 1998). This has been demonstrated in the events of slightly elevated CRP levels of about 5– to 10–fold above normal levels. Similar patterns are common after orthopaedic surgery and therapy for femoral fracture where CRP concentration rose up to 136 mg/L within 48 hours postoperatively, before a continuous decline to normal levels in about two weeks (Neumaier et al., 2015).

In some postoperative cases of total hip or knee arthroplasty, CRP levels of about 10 – 16 mg/L has been recorded (Aalto et al., 1984; Niskanen et al., 1996; Okafor and Maclellan, 1998; White et al., 1998). There are also examples of CRP concentrations of about 67 mg/L and 45 mg/L in fracture treatment of tibial shaft (Lindström et al., 1997) and in malleolar fracture treatment (Buttenschoen et al., 2000) respectively.

Margheritini et al (2001) reported CRP levels as high as 95 mg/L in arthroscopically assisted anterior cruciate ligament reconstruction. The concentration of CRP during fracture treatment in spine surgery is however dependent on the extent of operation. Whilst CRP levels of about 65 mg/L is common in dorsal stabilisation, higher values of about 174 mg/L has been obtained in ventral fusion operation (Scherer et al., 2001). Furthermore, CRP levels are also dependent on various trauma regions and could reflect the degree of surgical trauma. Kristiansson et al (1999) and Sietses et al (1999) reported from their studies in abdominal surgery that CRP levels were significantly decreased following laparoscopic procedure for the treatment of gastro–

oesophageal reflux (i.e., fundoplication) or the surgical removal of the gallbladder (i.e., cholecystectomy) compared to after conventional operation.

Similar trends whereby 48 hours postoperative peak CRP levels depend on the extent of surgery were obtained in cervical neck fractures (about 135 mg/L), osteosynthesis (about 100 mg/L) and hip replacement (about 150 mg/L) (Neumaier et al., 2006). Kruidenier et al (2018) reported a sharp increase in CRP levels, with a peak after 48 to 72 hours, followed by a rapid decline, returning to normal levels after 2 to 3 weeks in postoperative CRP levels in patients undergoing uncomplicated cases of orthopaedic (trauma) surgery. CRP kinetics follow the same pattern of decline after different types of surgical procedures. Unless in the case of stagnation or secondary increase, an underlying complication may be present (Scherer et al., 2001; Beloosesky et al., 2004; Sastre et al., 2006; Neumaier and Scherer, 2008). Moreover, the type of trauma and surgical intervention correlates with CRP levels; with the highest CRP levels recorded in femur fractures where mean peak CRP values were between 136 and 154 mg/L. This contrasted with ankle fractures, with a mean peak of 34 to 39 mg/L in uncomplicated cases (Neumaier and Scherer, 2008; Scherer et al., 2001).

In all, the CRP response to post–surgical trauma was only useful in estimating the extent of tissue damage and invasiveness of a procedure including a reflection of the degree of postoperative stress for the patient.

7.1.4 Serum calprotectin as a biomarker of acute phase response

Unlike procalcitonin, ESR and CRP, calprotectin directly reflects the leucocyte counts in the inflamed joints rather than systemic inflammatory activity. Thus, calprotectin appears to be a considerably more sensitive biomarker of local disease activity (Kane et al., 2003b; Frosch et al., 2000; Hammer et al., 2011).

Calprotectin, an acute phase protein and a heterodimeric complex formed by two S100 calcium–binding, myeloid–related protein 8 (MRP8 or S100A8) and MRP14 (or S100A9), is an important member of the S100 family protein representing about 60% of the soluble cytosolic protein in polymorphonuclear leucocytes, in addition to being a major monocyte/macrophage protein (Foell and Roth, 2004).

Calprotectin is synthesised by activated monocytes and neutrophils in the systemic circulation and inflamed tissues; but released primarily during inflammatory responses predominantly from the activated leucocytes locally at the site of joint inflammation (Johne et al., 1997). Thus, calprotectin level directly reflects local ongoing joint inflammatory activities rather than systemic inflammatory response, as occurs with acute phase proteins used in clinical practice, such as CRP and ESR (Siemons et al., 2014; Johne et al., 1997). Recently, calprotectin has been categorised as one of the so–called damage–associated molecular patterns (DAMPs) that signal tissue and cell damage (Bianchi, 2007).

Calprotectin as a biomarker in inflammatory diseases, appears to be more sensitive than CRP in being able to detect minimal residual inflammation (Sunahori et al., 2006). The synthesis and subsequent release of calprotectin promotes the migration of phagocytic cells to the sites of inflammation and further intensifies the inflammation cascade (Sunahori et al., 2006). Increased

calprotectin concentrations have been shown to support the diagnoses of many inflammatory diseases (Ometto et al., 2017; Manceau et al., 2017; Abildtrup et al., 2015; Pepper et al., 2015; Nilsen et al., 2014; van Rheene et al., 2010; Zali et al., 2008; Nisapakultorn et al., 2001); and following injuries or surgical procedures (Robinson et al., 2017; Pous–Serrano et al., 2017; Simioni et al., 2017; Abildtrup et al., 2015).

Calprotectin helps to stratify disease activity in rheumatoid arthritis patients who are being treated with TNF–α inhibitors (TNFi) more accurately than CRP and ESR, regardless of composite index (i.e., disease activity score, clinical disease activity index or simplified disease activity index) used to assess disease activity (Inciarte–Mundo et al., 2016). There is a positive correlation between calprotectin and other inflammatory indices, including CRP, ESR and SAA (Andres–Cerezo et al., 2011; Inciarte–Mundo et al., 2015; Inciarte–Mundo et al., 2016); and an inverse correlation between calprotectin and TNFi trough concentrations in serum (Inciarte–Mundo et al., 2016).

Earlier, several investigators have reported increased serum levels of calprotectin in rheumatoid arthritis patients, its correlation with disease activity and decreased levels of calprotectin after effective treatment (Andres–Cerezo et al., 2011; Brun et al., 1992; Brun et al., 1994). Calprotectin has also been shown to correlate with ultrasound–determined synovitis, which is considered to be a more sensitive assessment of joint inflammation than clinical examination (Hammer et al., 2011). Lately, it was demonstrated that calprotectin is a better predictor of ultrasound synovitis than CRP, and this suggests that calprotectin might be a more reliable tool for evaluating joint inflammatory activity (Hurnakova et al., 2015).

Calprotectin, being stable at room temperature, appears to be a promising candidate biomarker for the follow–up of disease activity in many autoimmune disorders, where it can potentially predict response to treatment or disease relapse (García–Arias et al., 2013; Hammer et al., 2007; Frosch et al., 2004). Also, there is evidence that a number of immunomodulators, including TNFi, may reduce calprotectin expression (Ometto et al., 2017).

The implication of serum calprotectin in the inflammatory process was demonstrated over 20 years ago (Haga et al., 1993). The effect of the APR on the clinical assessment of different acute phase proteins during postoperative laboratory investigation is well known (Saul et al., 2021; Lee et al., 2019; Qureshi et al., 2018; Schutz et al., 2018; Shin et al., 2018; Chapman et al., 2016; Fakler et al., 2016; Medina–Fernández et al., 2016; Neumaier et al., 2015; Adamina et al., 2015; Wang et al., 2014; Platt et al., 2012; Al–Jabi and El–Shawarby, 2010; Kang et al., 2010; Dupont et al., 2008; Neumaier and Scherer, 2008; Welsch et al., 2007; Beloosesky et al., 2004). In orthopaedic patients, the trauma of surgery induces APR that adds to the response of chronic inflammation which may already be present in such patients (Berntzen et al., 1991c; Neumaier et al., 2006; Neumaier et al., 2015).

Whereas some biomarkers that are released during inflammation, e.g., procalcitonin and CRP, have been considered as helpful biomolecules to estimate the outcomes of sepsis in postoperative patients (Hegazy et al., 2014; Martin–Loeches et al., 2015; Mickiewicz et al., 2015), there is still no commonly used biomarker for monitoring the development of sepsis in postoperative orthopaedic patients, and little is known about the course of serum calprotectin

levels over time and its relationship with postoperative patients (Huang et al., 2016).

Hitherto, no studies have focused on the ability of calprotectin to reliably reflect active inflammatory response in postoperative orthopaedic patients either in general or specific conditions of low concentrations of traditionally measured acute phase proteins such as CRP and ESR.

7.1.5 Aim of this chapter

The aim of the study presented in this chapter was to prospectively evaluate the effect of the APR, as provoked by elective orthopaedic surgery, on serum calprotectin analysed on two commercially available immunoassays and compared the incremental serum responses to those of serum C–reactive protein.

7.2 Patients Recruitment and Study Design

Patients undergoing elective knee or hip surgery were recruited at their pre–operative assessment. Patients who were less than 18 years, pregnant, difficult to bleed, received a blood transfusion within the last three months, or had acute inflammatory illness were excluded. Subjects gave written informed consent to participate in this study which was approved by the National Research Ethics Service (NRES Committee South Central – Hampshire B; Study Code 14/SC/1396).

Blood samples were collected into Sarstedt S–monovette sample tubes (Sarstedt, Nümbrecht, Germany) on the morning of and 48 hours after surgery. Potassium ethylene diamine tetra acetic acid (K_2EDTA) tubes were used to count

neutrophils and serum gel tubes were used to measure calprotectin, CRP, albumin and total protein. Neutrophils were counted immediately after collection. Serum was separated within two hours of collection, aliquoted and frozen at –80°C until analysed in single batches.

7.3 Analytical Materials and Methods

The analytical materials and methods were the same as those set out in Chapter 4 under sections 4.2 – 4.4

In summary, calprotectin was measured by ELISA supplied by Bühlmann (Bühlmann Laboratories AG, Baselstrasse 55, CH – 4124 Schönenbuch, Switzerland) and Immunodiagnostik™ (Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany). Serum CRP (immunoturbidimetry), serum albumin (bromocresol purple) and serum total protein (biuret) were measured using methods and reagents supplied by Abbott Diagnostics on the Abbott ARCHITECT c16000 Analyser (Abbott Diagnostics, Abbott Park, IL, USA). Neutrophils were counted using flow cytometry (Sysmex XN–10®, Sysmex Corporation, Kobe, Japan). The intra–assay CVs are 3.1% for Bühlmann calprotectin (serum BMN®-Cp), 2.9% for Immunodiagnostik™ calprotectin (serum IDK®-Cp), 1.24% for CRP, 0.9% for albumin and 1.1% for total protein.

7.3.1 Statistical Analysis

Data processing and statistical analysis were performed as described in Chapter 4 and presented as summarised in Chapter 6 under sub section 6.3.1.

In summary, the Kolmogorov–Smirnov (KS) test assessed normality of data. CRP data were non–parametric but were normally distributed following logarithmic transformation. Other data were parametric. Student's t–test was, therefore, used to assess the significance of differences between raw and logarithmically transformed parametric data. Pearson's linear correlation assessed the significance of association between raw or logarithmically transformed parametric variables. Data processing and statistical analyses were performed using GraphPad Prism version 7.00 for windows (GraphPad Software, La Jolla, California, USA). Data (including pre–transformed raw CRP) are expressed as means with standard deviation (SD) in parentheses.

7.4 Results

Thirty patients (16 female) aged 70.0 (9.4) years were recruited. Co–morbidities were osteoarthritis (fourteen patients), obesity (thirteen patients), hypertension (ten patients), cardiorespiratory disease (nine patients), hypercholesterolaemia (four patients), cancer (three patients), gout (one patient), inactive rheumatoid arthritis (one patient) and treated hypothyroidism (one patient).

The pre– and post–operative data are shown in the table 7.4 and figures 7.2 – 7.4. Following surgery, serum CRP, serum BMN®-Cp, serum IDK®-Cp and neutrophils increased ($p < 0.0001$); serum albumin and serum total protein decreased ($p < 0.0001$) (Table 7.4). The mean (SD) post–operative increase in serum BMN®-Cp (3.0 (1.9) fold) and serum IDK®-Cp (2.8 (1.8) fold) were similar ($p = 0.6575$) but these were both lower ($p < 0.0001$) than serum CRP (82.0 (60.8) fold).

Logarithmically transformed serum CRP correlated positively with serum BMN®-Cp ($r = 0.64$; $p < 0.0001$), serum IDK®-Cp ($r = 0.65$; $p < 0.0001$) and neutrophil

count (r = 0.66; p < 0.0001), and negatively with serum total protein (r = –0.43; p = 0.0008) and serum albumin (r = –0.70; p < 0.0001). Serum BMN®-Cp correlated positively with serum IDK®-Cp (r = 0.97; p < 0.0001) and neutrophil count (r = 0.68; p < 0.0001), and negatively with serum albumin (r = –0.54; p < 0.0002). Serum IDK®-Cp correlated positively with neutrophil count (r = 0.67; p < 0.0001), and negatively with serum albumin (r = –0.55; p < 0.0001). There was no correlation between serum total protein and either serum BMN®-Cp or serum IDK®-Cp.

Serum calprotectin measured by the BMN®-Cp assay had a 19% positive proportionate bias compared to the IDK®-Cp assay (figure 7.5).

Analyte (Unit)		**Before Surgery Mean (SD)**	**After Surgery Mean (SD)**
***CRP (mg/L)**		4.76 (7.59)	179.70 (61.89)
Serum Calprotectin	**BMN®-Cp (µg/mL)**	4.44 (2.57)	14.87 (6.72)
	IDK®-Cp (µg/mL)	3.81 (2.22)	11.81 (4.80)
Neutrophils (x10^9/L)		4.44 (1.58)	8.60 (2.49)
Albumin (g/L)		39.0 (3.09)	32.7 (2.37)
Protein (g/L)		69.5 (6.04)	62.6 (4.90)

Table 7.4 – The Mean (SD) laboratory data in 30 patients before and 48 h after elective orthopaedic surgery. *Pre–transformed data shown but logarithmic data analysed.

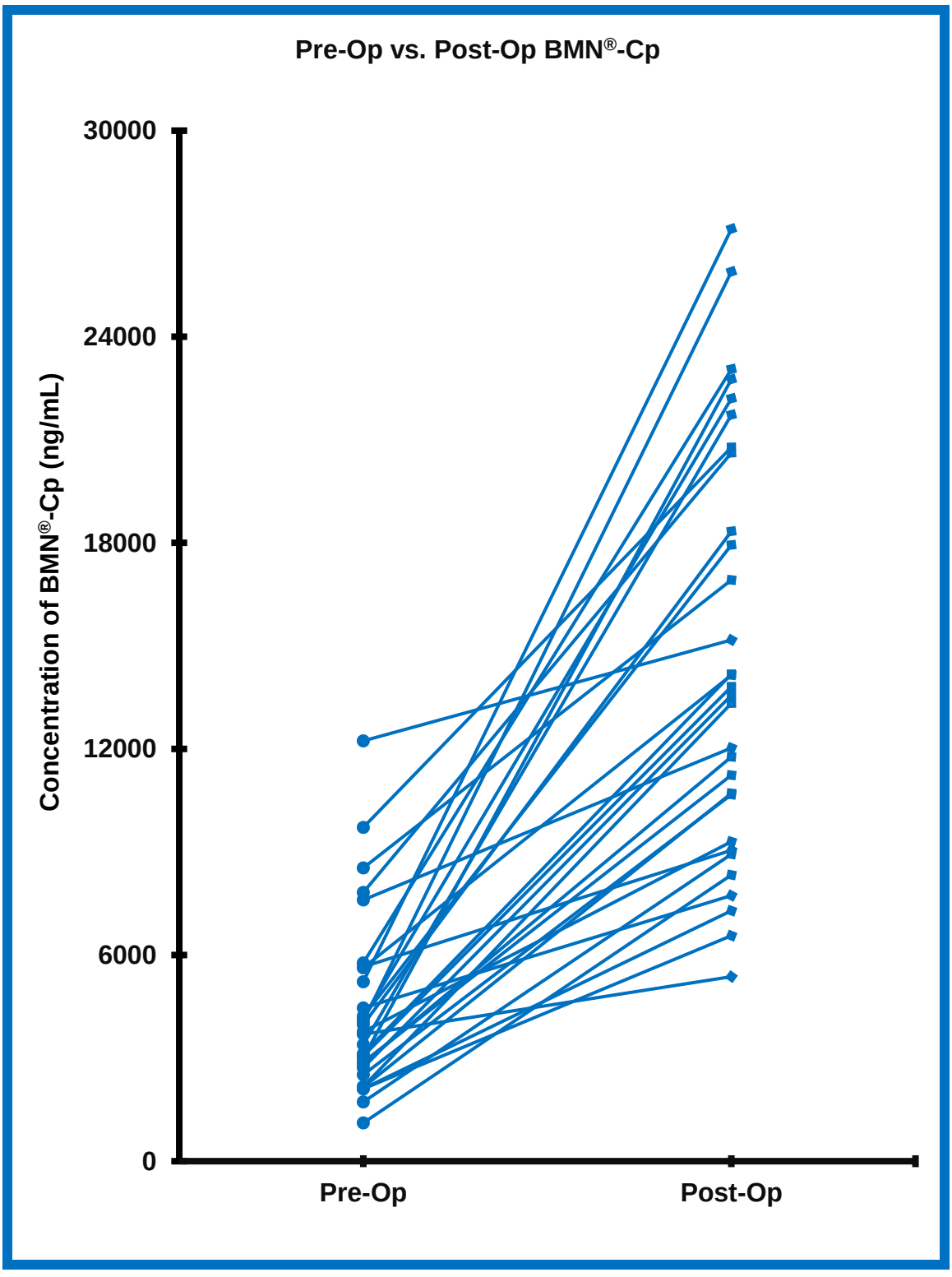

Figure 7.2 – A line plot of individual change in serum calprotectin concentration measured with the BMN®-Cp assay in 30 orthopaedic patients before (pre–operative) and after (48 hours post–operative) undergoing knee and hip surgery. The round (begin arrow) and square (end arrow) points indicate pre–operative and 48 hours post–operative values for serum calprotectin, respectively.

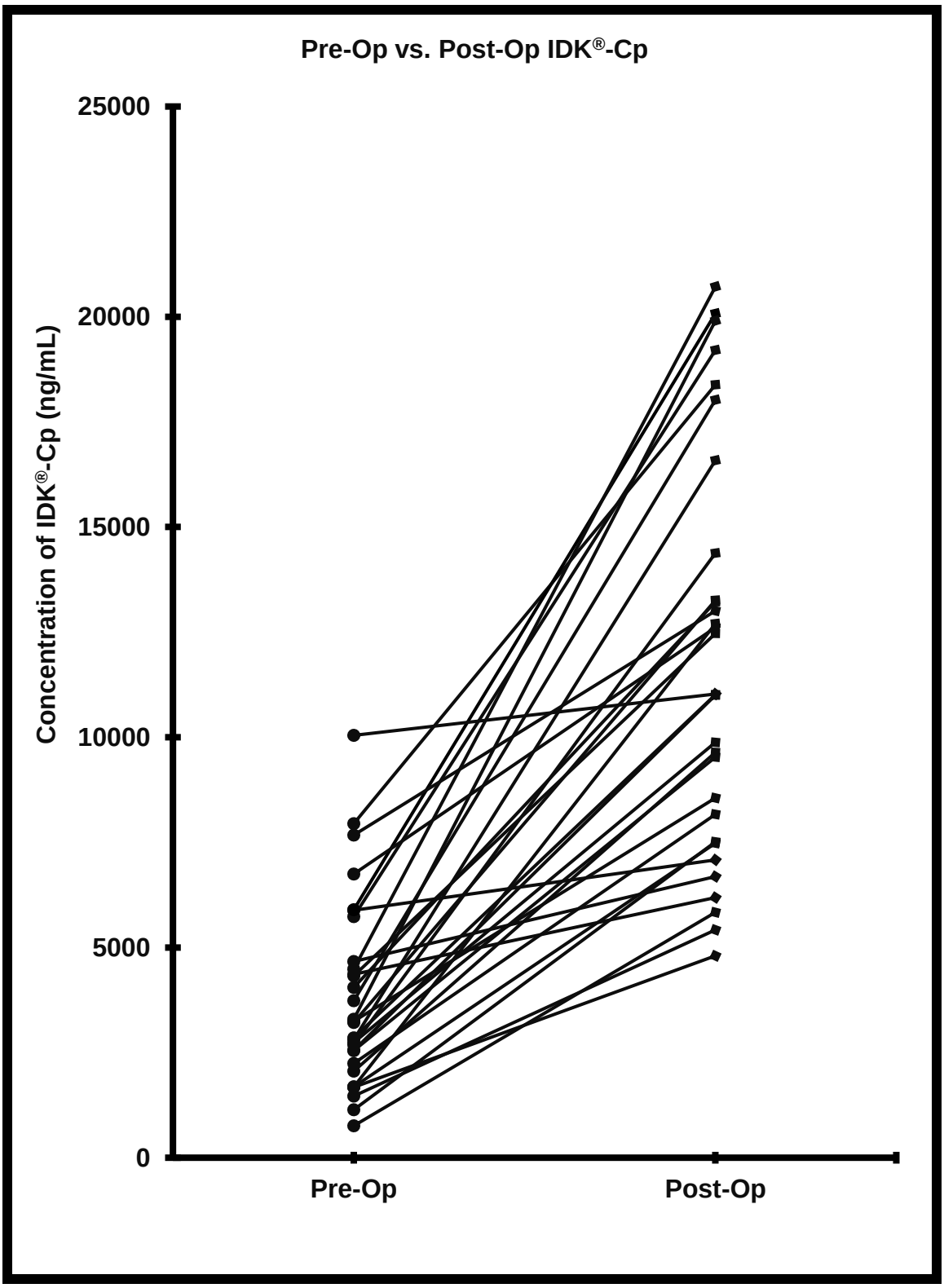

Figure 7.3 – A line plot of individual change in serum calprotectin concentration measured with the IDK®-Cp assay in 30 orthopaedic patients before (pre–operative) and after (48 hours post–operative) undergoing knee and hip surgery. The round (begin arrow) and square (end arrow) points indicate pre–operative and 48 hours post–operative values for serum calprotectin, respectively.

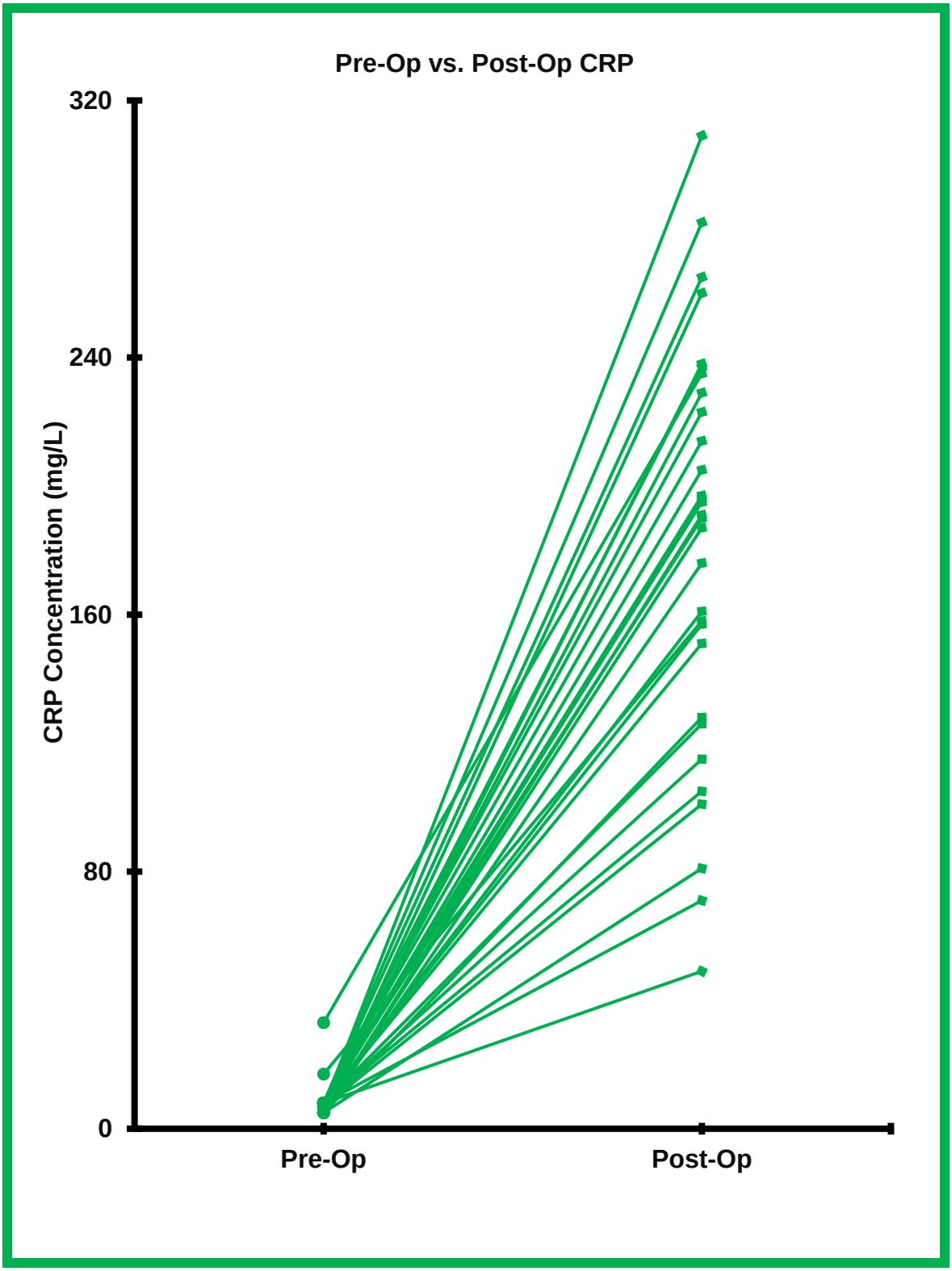

Figure 7.4 – A line plot of individual change in serum CRP concentration measured with the Abbott ARCHITECT c16000 Analyser in 30 orthopaedic patients before (pre–operative) and after (48 hours post–operative) undergoing knee and hip surgery. The round (begin arrow) and square (end arrow) points indicate pre–operative and 48 hours post–operative values for serum calprotectin, respectively.

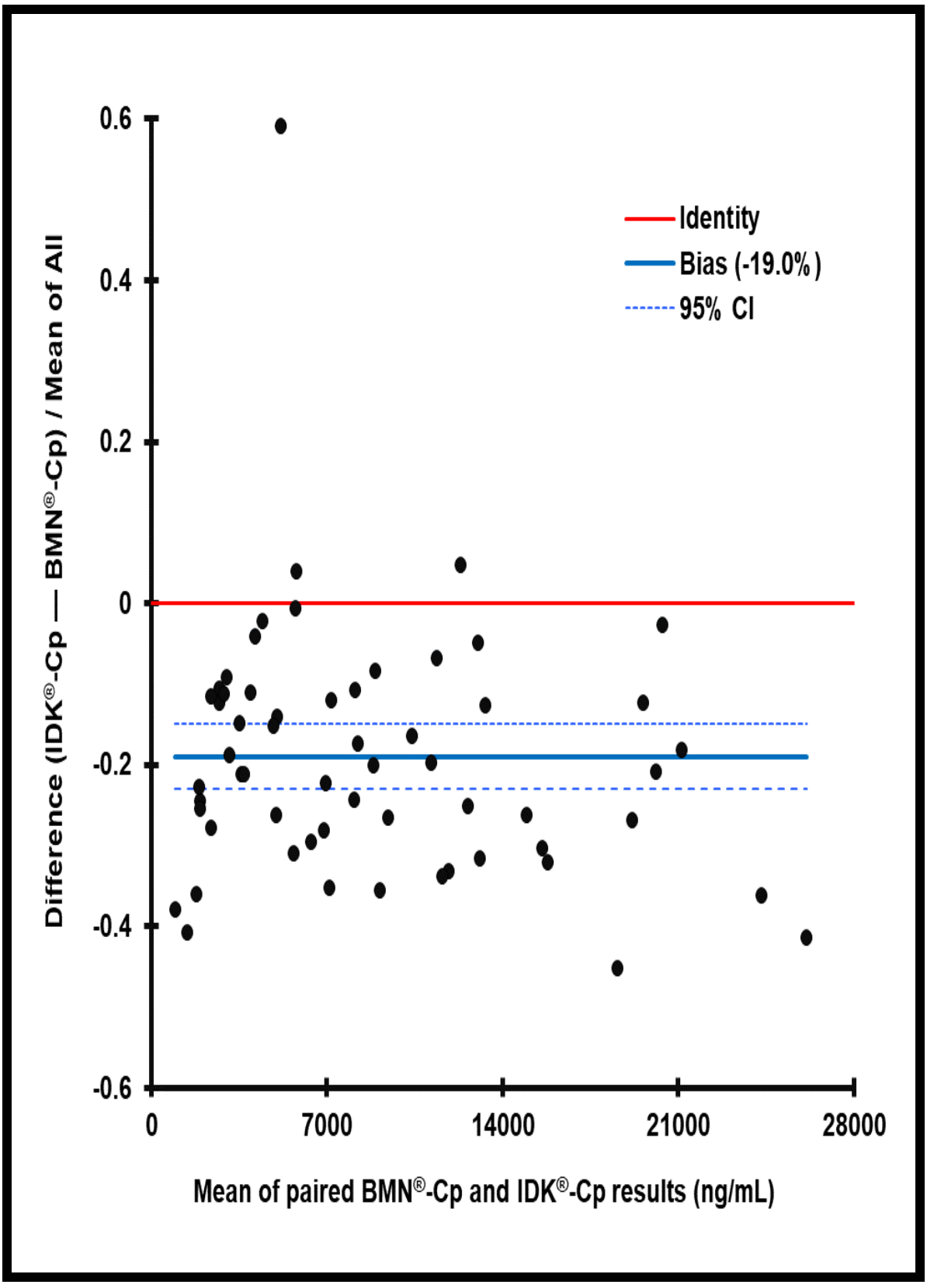

Figure 7.5 – The Bland–Altman (Mean–Difference or Limits of Agreement) Plot and analysis of the relationship between serum calprotectin measured by the Bühlmann (BMN®-Cp) and the Immunodiagnostik™ (IDK®-Cp) assays.

7.5 Discussion

The increase in serum CRP and decrease in serum albumin following surgery are consistent with an APR, which was provoked solely by surgery as no patient developed post–operative infection or other acute inflammatory disease. The increase in serum CRP following surgical insult is well recognised (Berntzen et al., 1991c; Foglar and Lindsey, 1998; Neumaier et al., 2015). Neumaier et al (2015), for example, reported a 27–fold increase in serum CRP levels within 48 hours after orthopaedic surgery which then declined to baseline levels within 14 days.

The concomitant increase in serum calprotectin is consistent with its role as an acute phase protein (MacGregor et al., 2004; Yamada, 1999; Lannergård et al., 2005; Duzova et al., 2003; Cellier et al., 1994; Macfarlane et al., 1986). The effect on serum calprotectin by an APR elicited by orthopaedic surgery reported in this study may, however, not be applicable to APRs triggered by other inflammatory insults such as sepsis and systemic inflammatory diseases. It, however, indicates that serum calprotectin is a non–specific biomarker of inflammation.

The smaller incremental response of serum calprotectin compared to serum CRP after surgery may reflect their different origins, sites of production and half–lives. CRP is synthesised by hepatocytes, and its concentration reflects short–term inflammation with a peak at 48 hours after stimulation. Calprotectin synthesis occurs in activated neutrophils, macrophages, epithelial cells and monocytes (Meuwis et al., 2013) and in this study is supported by the correlation between serum calprotectin and neutrophil count. In addition, there may be differences in production rate and half–lives (19 hrs for CRP and 5 hrs for calprotectin) or

clearance from the circulation (Louis et al., 2012). These factors may explain the different incremental responses 48 hours after elective orthopaedic surgery.

The Bühlmann assay is recognised to give higher serum calprotectin results than the Immunodiagnostik™ assay (Burkhardt et al., 2001; Stríz et al., 2001; Healy et al., 2006; Altwegg et al., 2007; Börekçi et al., 2009; Heller et al., 2011; Lönnkvist et al., 2011), and this is reflected in their cited reference intervals (400 – 3900 versus < 3000 µg/mL respectively). In this study and those previously reported in Chapter 5 and Chapter 6, serum calprotectin measured with the BMN®-Cp assay had a 19% positive proportionate bias compared to the serum IDK®-Cp assay. The excellent correlation between the two serum calprotectin assays, however, indicates that they perform equally in response to an inflammatory insult.

Inflammation typically provokes an APR that results in increased concentrations of CRP, SAA, caeruloplasmin, haptoglobin, α1–acid glycoprotein, α2–macroglobulin, complement components, coagulation proteins such as factors V and VIII, and fibrinogen, and decreased concentrations of albumin and α1–apolipoprotein (Germolec et al., 2018). Through altering homeostasis, these biomarkers can initiate or support defensive and/or adaptive processes that facilitate short–term healing. Prolonged inflammation, in response to cytokine signals from the site of inflammation, may promote chronic inflammation, and tissue damage (Khalil and Al–Humadi, 2020; Germolec et al., 2018). CRP is a sensitive, reliable and commonly used diagnostic biomarker for the APR. CRP levels rise and fall rapidly to reflect the disease process relative to other blood proteins, e.g., leukocytes (Sproston and Ashworth, 2018). Serum calprotectin is considered an accurate biomarker in discriminating inflammatory disease activity

in RA patients receiving tumour necrosis factor inhibitors (TNFi) (Inciarte–Mundo et al., 2016), and tocilizumab (TCZ) (Inciarte–Mundo et al., 2015). However, efforts to explore the ability of serum calprotectin to reliably reflect the active inflammatory response in postoperative orthopaedic patients remains work in progress.

This study has limitations. The a priori sample size was powered on changes in acute phase protein biomarkers (Vermeire et al., 2004; Gabay and Kushner, 1999; Ceciliani et al., 2002; Campbell et al., 1982; Suffredini et al., 1999; Pannen and Robotham, 1995; Baumann and Gauldie, 1994; Bell and Wilson, 2014; Berczi et al., 2009; Berczi and Szentivanyi, 2003), but the study group was relatively small and may have been under–powered for assessing changes in inflammatory biomarkers and therefore subject to type 1 and type 2 statistical errors respectively. The effect on serum calprotectin was studied two days after elective surgery, and the shorter– and longer–term effect of an APR on serum calprotectin remains to be clarified.

7.6 Conclusion

In a prospective study, we confirm that serum calprotectin is a positive acute phase protein. The Bühlmann and Immunodiagnostik™ serum calprotectin assays perform comparably during an APR. At two days following an inflammatory insult, serum CRP may be a better discriminatory biomarker of the APR than serum calprotectin based on a much greater incremental response.

CHAPTER 8

SUMMARY, CONCLUSIONS AND FUTURE PERSPECTIVES

In this study, a two–site sandwich ELISA for the quantitation of serum calprotectin and serum S100A12 was developed, and optimised. During optimization, it was established that incubation of standards and samples with orbital shaking at low to medium speed did not result in much lower and more variable reactions. It may be that there was not higher density of macromolecules in serum samples than in standard solutions and that with orbital shaking at low to medium speed, no other macromolecules push for antigen molecules from a favourable position for antibody binding before a bond can be determined. This meant that shaking during incubation of controls, standards and samples was not discontinued.

Furthermore, the influence of several parameters was analysed. Different concentrations of primary antibody, secondary antibody and horseradish–peroxidase–labelled streptavidin were compared. Different washing buffers and washing protocols were analysed and the effects of using different buffers to dilute the reagents were studied. Various incubation times and protocols for calibrators and samples were also evaluated. In the interest of space none of the results of these experiments are presented here, and only the optimized ELISA procedure is described.

A reproducible standard curve (see figure 3.4 in Chapter 3 under sub section 3.2.4) was generated with the use of a 4–parameter curve fit: $y = (A – D)/[1 + (x/C)B] + D$, where D is the y value corresponding to the asymptote at high values on the x–axis; A is the y value corresponding to the asymptote at low

values on the *x*–axis; C is the *x* value corresponding to the midpoint between A and D; and B describes how rapidly the curve makes its transition from the asymptotes in the centre. All 4 parameters are calculated with an algorithm based on the Levenberg–Marquardt method (SOFTMAX PRO; Molecular Devices).

In summary, the BMN®-Cp (Bühlmann Laboratories AG, Baselstrasse 55, CH – 4124 Schönenbuch, Switzerland), IDK®-Cp (Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) and IDK®-A12 (Immunodiagnostik™ AG, Stubenwald–Allee 8a, D–64625 Bensheim, Germany) assays developed, optimised and validated for use in serum were analytically sensitive and specific, linear, precise, accurate, reproducible with a wide working range, and have the ability to discriminate between patients without or with inflammatory diseases such as IBD. Further studies then evaluated the clinical utility of the validated serum BMN®-Cp, serum IDK®-Cp and serum IDK®-A12 assays in the assessment of inflammatory disorders, in particular IBD.

In the first clinical study, we investigated the diagnostic potential of serum calprotectin and serum S100A12 as biomarkers to replace or supplement faecal calprotectin considered the ‘gold standard’ biomarker for the diagnosis of IBD. Receiver operating characteristic curve and concordance tables indicated that serum IDK®-Cp (AUC = 0.793; k_w = 0.33) had similar diagnostic performance as serum BMN®-Cp (AUC = 0.771; k_w = 0.32). Serum IDK®-Cp assay had the best diagnostic efficiency at a cut–off value of >2500 ng/mL giving a sensitivity of 80% and specificity of 65%. The diagnostic performance of serum S100A12 (AUC = 0.700) was poor. Our data, however, indicate that

serum biomarkers, particularly serum S100A12, are unlikely to replace faecal calprotectin in the diagnosis and exclusion of IBD in patients presenting with symptoms of chronic diarrhoea. A raised serum BMN®-Cp or serum IDK®-Cp, however, may have a role identifying which patients require endoscopy without recourse to faecal calprotectin but a normal serum calprotectin does not exclude IBD.

In a second clinical study, we investigated serum BMN®-Cp and serum IDK®-Cp and the conventional inflammatory blood biomarkers CRP and platelets compared to faecal calprotectin, effectively the gold standard in this study, in discriminating between active and inactive disease in an IBD cohort consisting of patients with CD and UC. Although the differentiation between active and inactive disease obtained from the patient records was the clinical classification, it became apparent that this almost wholly was based on faecal calprotectin results. Serum BMN®-Cp, serum IDK®-Cp and serum CRP but not platelets were significantly different in patients with active and inactive CD, irrespective of disease site. All blood biomarkers, however, were similar in patients with active and inactive UC irrespective of disease site.

Receiver operating characteristics (ROC) curve analyses indicated that serum CRP performed best in discriminating between active and inactive disease in IBD, CD and UC; however, none of the blood biomarkers performed well. Our results indicated that serum CRP, serum BMN®-Cp and serum IDK®-Cp may have a role in predicting exacerbations in CD but not UC. We suggest that this difference reflects the underlying disease processes. UC is a disease of large bowel limited to the mucosa and inflammation of which would increase faecal calprotectin; whereas CD is a transmural bowel disease and inflammation of

which would more likely provoke a systemic inflammatory response increasing blood inflammatory biomarkers in addition to intestinal inflammation increasing faecal calprotectin. Whilst serum CRP, serum BMN®-Cp and serum IDK®-Cp are not likely to replace faecal calprotectin, they could supplement its prognostic utility in IBD assessment, particularly in patients with CD. In this patient cohort neither serum BMN®-Cp or serum IDK®-Cp offered no advantages over serum CRP, which is easily available and less expensive.

In a third clinical study, we studied the effect of acute phase response provoked by elective orthopaedic surgery on serum calprotectin. An increase in serum CRP and decrease in serum albumin 48 hours following elective knee and hip surgery confirmed an acute phase protein response (APPR), which was provoked solely by surgery as no patient developed post–operative infection or other acute inflammatory disease. A concomitant increase in serum BMN®-Cp and serum IDK®-Cp is consistent with the role of serum calprotectin as an acute phase protein and confirms that serum calprotectin is a non–specific biomarker of inflammation irrespective of the aetiology of the inflammatory response. The smaller incremental response of serum calprotectin (3–fold) compared to serum CRP 48 hours after surgery may largely reflect their half–lives following a single inflammatory insult. CRP and calprotectin have half–lives of 19 hrs and 5 hrs respectively which may explain the different incremental responses 48 hours after elective orthopaedic surgery.

In all three clinical studies, the excellent correlation between the two serum calprotectin assays indicates that they perform equally well in response to an inflammatory insult including IBD and uncomplicated lower limb joint

replacement orthopaedic surgery. Serum BMN®-Cp results, however, had a 19% positive proportionate bias compared to serum IDK®-Cp results. The absolute results from these different serum calprotectin assays, therefore, cannot be used inter–changeably. The manufacturer provided upper reference limits (URL) for serum BMN®-Cp (>3900 ng/mL) and serum IDK®-Cp (>3000 ng/mL) assays, however, reflect this 19% positive bias and therefore serum BMN®-Cp and serum IDK®-Cp results relative to the appropriate URLs may be usefully compared.

Our studies, however, have limitations. The most important limitation was in the second clinical study where the clinical diagnosis of active and inactive IBD was almost exclusively based on a faecal calprotectin cut–off value of > 200µg/g supported by imaging and clinical evaluation. Colonoscopy and biopsy is the gold standard in discriminating between active and inactive IBD. Clinical activity scoring such as Mayo disease severity score for UC (includes endoscopy component) and the Harvey–Bradshaw clinical index for CD have also been used to assess relapse of IBD. Ideally, therefore, faecal calprotectin and blood biomarkers should have been evaluated for active and inactive IBD defined by endoscopic biopsy findings or less preferably clinical scoring systems.

Unless clinically indicated, however, endoscopy is not used to distinguish active and inactive IBD since it is invasive, relatively expensive, and inconvenient for patients. Clinical disease activity scoring systems are rarely used in routine clinical practice as they are time consuming within limited time for patient consultations. The study was, therefore, designed to assess whether serum BMN®-Cp and serum IDK®-Cp could replace or supplement

faecal calprotectin in predicting relapse in patients with IBD and the results showed them to be of limited value.

Further work, therefore, on the prognostic utility of serum BMN®-Cp and serum IDK®-Cp in IBD studies preferably in a large multicentre cohort of scrupulously phenotyped IBD patients will be required to interrogate the failure of serum calprotectin to predict disease activity in patient with UC and confirm its potential in assessing disease activity in patients with CD.

Adaptation of Immunodiagnostik™ S100A12 ELISA K 6938 assay for the *in–vitro* determination of S100A12 in stool for the measurement of S100A12 in serum was not practical. The analytical performance and diagnostic accuracy of serum S100A12 assay in IBD is poor and unsatisfactory, and therefore should not be recommended as a biomarker in IBD assessment. Further optimisation and analytical validation studies, are required to explore its potential in the assessment of IBD. Other immunoassay technologies and mass spectrometry techniques could be evaluated as future assay methodologies for the *in–vitro* measurement of S100A12 in serum.

Colonoscopy and biopsy remains the gold standard in the assessment of the response to treatment in IBD by discriminating between active and inactive IBD. Colonoscopy, however, is invasive, relatively expensive, and inconvenient for patients. Faecal biomarkers of intestinal inflammation, in particular calprotectin, have therefore been established as the 'gold standard biomarker' in the assessment of IBD. Collection of stool specimens, however, is often unappealing and inconvenient for patients. The development, therefore, of convenient, inexpensive, and minimally invasive blood biomarker remain appealing for the assessment of IBD.

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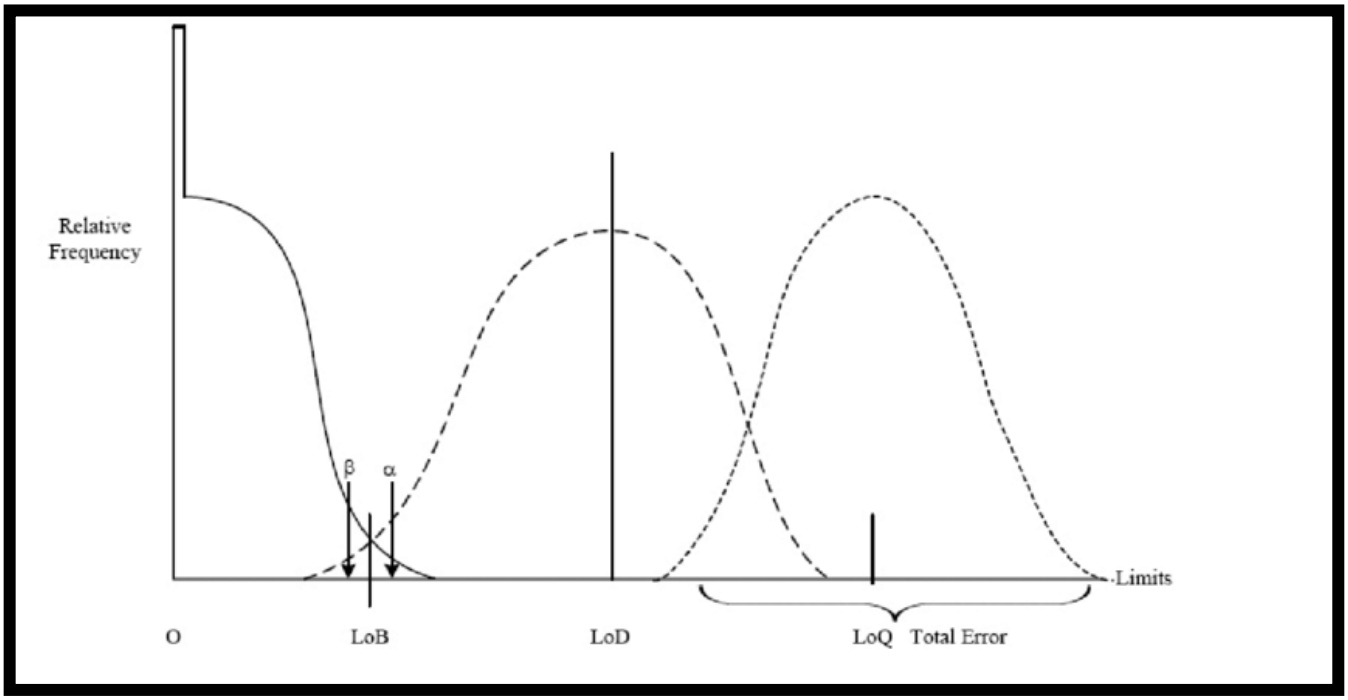
APPENDIX A: THE INTERRELATIONSHIP BETWEEN ANALYTICAL SENSITIVITY (Limit of the Blank, LoB and Lower Limit of Detection, LLoD) AND FUNCTIONAL SENSITIVITY (Limit of Quantitation, LoQ)



REPORT

Not detected, <LLoD	**Detected, <LLoD**	**Detected, <LLoD**	**As Measured**

α = Prob (False Positive) ——— Distribution of Blank
β = Prob (False Negative) - - - - - Distribution of sample at LLoD
Total Error < Laboratory Goal ——— Distribution of sample at LoQ

Illustrated Format Of Analytical Sensitivity. The relationship between limit of the blank (LoB), lower limit of detection (LLoD) and limit of quantitation (LoQ) based on the approved guideline of the Protocols for Determination of Limits of Detection and Limits of Quantitation as published in the National Committee for Clinical Laboratory Standards (NCCLS) publication of EP17–A. The solid line defines the LoB and represents the distribution of results for a blank specimen. As modern analyzers seldom report results of less than zero, the frequency of "zero" results is artificially increased as illustrated. The LoB shown excludes a small proportion of blank results (α). The dashed line defines the LoD and represents the scatter (imprecision) of results for a specimen of low concentration. The LoD is then set so that only a small proportion (β) of these results will fall below LoB. The dotted line defines the LoQ and represents the distribution of results for a specimen of low concentration meeting the target for total error (imprecision and bias). It may be that this LoQ total target error is met by a specimen at the LoD concentration in which case LoQ = LoD. Otherwise, LoQ will have to be set after testing a specimen of higher concentration. Modified figure taken from: Armbruster and Pry (2008) Limit of Blank, Limit of Detection and Limit of Quantitation. *Clinical Biochemistry Review*, 29 (Suppl. 1): S49 – S52.

APPENDIX B: MATERIALS (REAGENTS) SUPPLIED BY BUHLMANN AG LABORATORIES AND IMMUNODIAGNOSTIK™ AG AND THEIR PREPARATION (RECONSTITUTION)

Reagents Supplied and Preparation

I. Bühlmann MRP8/MRP14 Calprotectin S100A8/S100A9 ELISA in Serum and Plasma

Reagents	Quantity	Code	Reconstitution
Microtitre Plate (precoated with anti–MRP8/MRP14 mAb	12 x 8–wells	B–MRP8/MRP14–MP	Ready to use
Plate Sealer	3 pieces		
Wash Buffer Concentrate (x10) with preservatives	1 bottle 100 mL	B–MRP8/MRP14–WB	Dilute with 900 mL of deionised water
Incubation Buffer with preservatives	1 bottle 100 mL	B–MRP8/MRP14–IB	Ready to use
*Calibrators A to E MRP8/MRP14 in a buffer matrix with preservatives	5 Vials 1 mL	B–MRP8/MRP14–CASET	Ready to use
Control Low / High Human serum matrix with preservatives	2 Vials 1 mL	B–MRP8/MRP14–CONSET	Ready to use
Enzyme Label Anti–MRP8/MRP14 Ab conjugated to HRP	1 Vials 11 mL	B–MRP8/MRP14–EL	Ready to use
TMB–Substrate TMB in citrate buffer with H_2O_2	1 Vials 11 mL	B–TMB	Ready to use
Stop Solution 0.25 M Sulfuric acid	1 Vials 11 mL	B–STS	Ready to use; Corrosive agent

***The actual concentration of the standards A to E are 4, 12, 40, 120 and 240 ng/mL MRP8/MRP14, respectively. Serum samples will be diluted 1:100, therefore the calibrators A – E are labelled as follows: 0.4, 1.2, 4.0, 12.0 and 24.0 µg/mL.**

STORAGE AND SHELF LIFE OF REAGENTS

Storage and shelf life of reagents. Unopened Reagents: store at 2 – 8˚C. Do not use past kit expiration date printed on the labels. Open / Reconstituted Reagents: store at 2 – 8˚C until expiration date (for Incubation Buffer, Controls, Calibrators, Enzyme Label, TMB–Substrate and Stop Solution). For Diluted Wash Buffer, store up to 6 months at 2 – 8˚C. For Microtitre Plate, return unused strips immediately to the foil pouch containing the desiccant packs and reseal along the entire edge of zip–seal. Store until expiration date at 2 – 8˚C.

II. Immunodiagnostik™ (IDK®) Calprotectin (MRP8/MRP14 S100A8/S100A9) ELISA K 6935 in Serum and Plasma

Reagents	Quantity	Cat. No.	Label	Reconstitution
Microtitre Plate (holder with precoated strips)	12 x 8 wells	K 6935	PLATE	Ready to use
Wash Buffer (ELISA wash buffer concentrate (x10)	2 x 100 mL	K 6935	WASHBUF	Dilute 1:10 before use (100 mL WASHBUF + 900 mL ultra–pure water*
Sample Buffer (Sample dilution buffer)	1 x 100 mL	K 6935	SAMPLEBUF	Ready to use
Calprotectin standards (Lyophilised: 0; 3.9; 15.6; 62.5; 250 ng/mL)	2 x 5 Vials	K 6935	STD	Reconstitute each vial with 500 µL of ultra–pure water*
Control (lyophilised: see specification for range)	2 x 1 Vial	K 6935	CTRL 1	
Control (lyophilised: see specification for range)	2 x 1 Vial	K 6935	CTRL 2	
Conjugate	15 mL	K 6935	CONJ	Ready to use
TMB Substrate (Tetramethylbenzidine)	15 mL	K 6935	SUB	Ready to use
ELISA stop solution	15 mL	K 6935	STOP	Ready to use

***ImmunodiagnostikTM AG recommends the use of Ultra–Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25°C (≥ 18.2 MΩcm).**

PREPARATION AND STORAGE OF REAGENTS

To run the assay more than once, ensure that reagents are stored at the conditions stated on the label. Prepare only the appropriate amount necessary for each run. The kit can be used up to 4 times within the expiry date stated on the label.

Reagents with volume less than 100 µL should be centrifuged before use to avoid loss of volume

Preparation of the wash buffer: The wash buffer concentrate (WASHBUF) should be diluted with ultra–pure water 1:10 before use (100 mL WASHBUF + 900 mL ultra–pure water), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals must be redissolved at room temperature or in a water bath at 37°C before dilution of the buffer solutions. The WASHBUF is stable at 2 – 8°C until the expiry date stated on the label. Wash buffer (1:10 diluted WASHBUF can be stored in a closed flask at 2 – 8°C for one months.

The lyophilised STD (standards) and CTRL (controls) are stable at 2 – 8°C until expiry date stated on the label. Before use, the STD (standards) and CTRL (controls) must be reconstituted with 500 µL ultra–pure water. Allow the vial content to dissolve for 10 minutes and mix thoroughly by gentle inversion to ensure complete reconstitution. Reconstituted standards and controls can be stored at 2 – 8°C for four (4) weeks.

All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at 2 – 8°C.

III. Immunodiagnostik™ (IDK®) S100A12 ELISA K 6938 in Serum and Plasma

Reagents	Quantity	Cat. No.	Label	Reconstitution
Microtitre Plate (one holder with precoated strips)	12 x 8 wells	K 6938	PLATE	Ready to use
Wash Buffer (ELISA wash buffer concentrate (x10)	1 x 100 mL	K 6938	WASHBUF	Dilute 1:10 before use (100 mL WASHBUF + 900 mL ultra–pure water*
Extraction Buffer Concentrate (2.5x)	1 x 100 mL	K 6938	EXBUF	Dilute 1:2.5 before use (100 mL EXBUF + 150 mL ultra–pure water*
Sample Buffer (Sample dilution buffer)	1 x 100 mL	K 6938	SAMPLEBUF	Ready to use
Calprotectin standards (Lyophilised: 0; 0.66; 2.0; 6.0; 18.0; 54 ng/mL)	2 x 6 Vials	K 6938	STD	Reconstitute each vial with 500 µL of ultra–pure water*
Control (lyophilised: see specification for range)	2 x 1 Vial	K 6938	CTRL 1	
Control (lyophilised: see specification for range)	2 x 1 Vial	K 6938	CTRL 2	
Conjugate (Peroxidase–labelled concentrate (100x)	1 x 200 µL	K 6938	CONJ	Ready to use
TMB Substrate (Tetramethylbenzidine)	1 x 15 mL	K 6938	SUB	Ready to use
ELISA stop solution	15 mL	K 6938	STOP	Ready to use

***Immunodiagnostik™ AG recommends the use of Ultra–Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25°C (≥ 18.2 MΩcm).**

STORAGE AND PREPARATION OF REAGENTS

To run the assay more than once, ensure that reagents are stored at the conditions stated on the label. Prepare only the appropriate amount necessary for each run. The kit can be used up to 2 times within the expiry date stated on the label.

Reagents with volume less than 100 µL should be centrifuged before use to avoid loss of volume

Preparation of the wash buffer: The wash buffer concentrate (WASHBUF) should be diluted with ultra–pure water 1:10 before use (100 mL WASHBUF + 900 mL ultra–pure water), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals must be redissolved at room temperature or in a water bath at 37°C before dilution of the buffer solutions. The WASHBUF is stable at 2 – 8°C until the

expiry date stated on the label. Wash buffer (1:10 diluted WASHBUF can be stored in a closed flask at 2 – 8°C for one months.

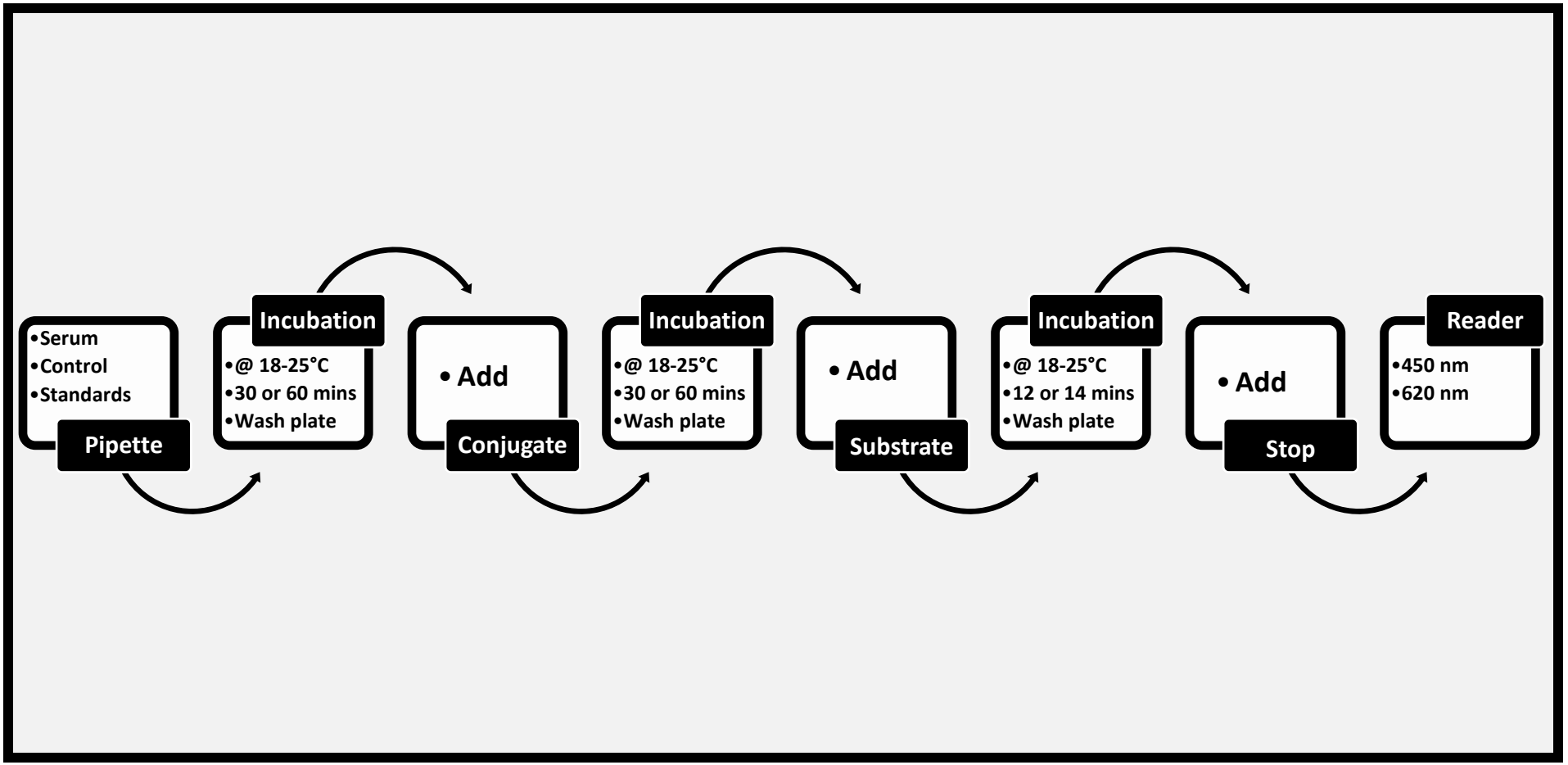
Preparation of the extraction buffer: The extraction buffer concentrate (EXBUF) must be diluted with ultra–pure water 1:2.5 before use (100 ml EXBUF + 150 mL ultra–pure water), mix well. Crystals could occur due to high salt concentration in the stock solutions. Before dilution, the crystals must be redissolved at 37°C in a water bath. The EXBUF is stable at 2 – 8°C until the expiry date stated on the label. Extraction buffer (1:2.5 diluted EXBUF can be stored in a closed flask at 2 – 8°C for three months.

The lyophilised standards (STD) and controls (CTRL) are stable at 2 – 8°C until expiry date stated on the label. Before use, the standards and controls must be reconstituted with 500 µL ultra–pure water. Allow the vial content to dissolve for 10 minutes and mix thoroughly by gentle inversion to ensure complete reconstitution. Reconstituted standards and controls are not stable and cannot be stored.

Preparation of the conjugate: The conjugate concentrate (CONJ) must be diluted 1:101 in wash buffer (100 µL CONJ + 10 mL wash buffer). The CONJ is stable at 2 – 8°C until expiry date stated on the label. Conjugate (1:101 diluted CONJ) is not stable and cannot be stored.

All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at 2 – 8°C.

APPENDIX C: A Schematic Representation of Serum Calprotectin and Serum S100A12 Assay Protocol on ELISA Template



A schematic representation of the principle framework on which the 96–well plate, two–site sandwich ELISA technique for serum calprotectin and serum S100A12 assay is based. Allowing for slight variations in the constituent of reagents based on the kit manufacturer's formulation, (i) Conjugate solution contains: anti-S100A12 antibody conjugated to horseradish peroxidase (HRP) (ii) Substrate Solution contains 3,3',5,5'–tetrabenzidine (TMB) in Citrate Buffer with Hydrogen Peroxide (H_2O_2) (iii) Stop Solution contains 0.25M Sulphuric Acid (H_2SO_4).

APPENDIX D: BÜHLMANN MRP8/14 CALPROTECTIN S100A8/S100A9 ELISA IN SERUM AND PLASMA

Author: Graham Johnson

Edited: 1st August 2016

Template

Dispense

100µl of Calprotectin Incubation Buffer (aspirate profile 1, dispense profile 4) to well(s) A1 – B1

Pipette Samples, Controls, Standards
Maximum post pipette delay for 5 minutes

Deep Well Dilution

Do not allow sample dilutions to be changed at run time
Do not pipette immediately after dilution
Let deep wells stand for 00.00 before pipetting

For Well Type ‘‘Test (T)’’

Dilute in a deep well 10 µL of Specimen with 990 µL of Calprotectin Incubation Buffer
Then pipette 100 µL of that mixture to the microplate
Do not require new tip for dispense of sample
Single shot for dispense of sample
Share dilution wells for microplate replicates (volumes will be scaled)
Do not share dilution wells across multiple assays
Do not force level detect before transfer
Use aspirate/dispense speed profile 3
Mix for 5 cycles.

Pipette Protocol for Standards and Controls

Pipette 100 µL of EK–Cal CAL A to ‘S1’ wells
Tip to dispense into microtitre well must be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of EK–Cal CAL B to ‘S2’ wells
Tip to dispense into microtitre well must be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of EK–Cal CAL C to ‘S3’ wells
Tip to dispense into microtitre well must be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of EK–Cal CAL D to ‘S4’ wells
Tip to dispense into microtitre well must be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of EK–Cal CAL E to ‘S5’ wells
Tip to dispense into microtitre well must be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of EK–Cal Control L to ‘LP1’ wells
Tip to dispense into microtitre well must be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of EK–Cal Control H to ‘HP1’ wells
Tip to dispense into microtitre well must be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of EK–Cal Control L to ‘LP2’ wells
Tip to dispense into microtitre well must be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of EK–Cal Control H to ‘HP2’ wells
Tip to dispense into microtitre well must be clean.
Fluid into microtitre well must be a single shot dispense.

Incubation

Incubate for 00:08:00 minutes at ambient temperature
Shake for 00:04:00 minutes at the start only at low speed
Incubate for 00:08:00 minutes at ambient temperature
Shake for 00:03:00 minutes at the start only at low speed
Incubate for 00:08:00 minutes at ambient temperature
Shake for 00:03:00 minutes at the start only at low speed
Incubate for 00:06:00 minutes at ambient temperature
Shake for the entire duration at low speed

Wash Plate

Purge the washer with 3000 µL of EK–Cal Wash Buffer
Sweep on last cycle only
Perform a 3–cycle wash, plate–wise, with constant timing
Dispense 350 µL of EK–Cal Wash Buffer
Do final aspirate cycle
Clean the washer after use with 3000 µL of Distilled (Deionised) Water

Enzyme Label

Dispense 100 µL of EK–Cal Enzyme Label (aspirate profile 1, dispense profile 4) to well(s) A1 – H12.

Incubation

Incubate for 00:08:00 minutes at ambient temperature
Shake for 00:04:00 minutes at the start only at low speed
Incubate for 00:08:00 minutes at ambient temperature
Shake for 00:03:00 minutes at the start only at low speed

Incubate for 00:08:00 minutes at ambient temperature
Shake for 00:03:00 minutes at the start only at low speed
Incubate for 00:06:00 minutes at ambient temperature
Shake for the entire duration at low speed

Wash Plate

Purge the washer with 3000 µL of EK–Cal Wash Buffer
Sweep on last cycle only
Perform a 5-cycle wash, plate–wise, with constant timing
Dispense 350 µL of EK–Cal Wash Buffer
Do final aspirate cycle
Clean the washer after use with 3000 µL of Distilled (Deionised) Water

Tetramethylbenzidine (TMB) Substrate

Dispense 100 µL of EK–Cal TMB Substrate (aspirate profile 1, dispense profile 1) to well(s) A1 – H12

Incubation

Incubate for 00:02:00 minutes at ambient temperature
Incubate for 00:12:00 minutes at ambient temperature
Shake for the entire duration at low speed

Stop Solution

Dispense 100 µL of EK–Cal Stop Solution (aspirate profile 1, dispense profile 1) to well(s) A1 – H12

Incubation

Incubate for 00:05:00 minutes at ambient temperature

Reader

Single/Dual wavelength mode
Primary test filter is 450nm
Initial shake for 5 seconds.

Curve Fit ‘’Fit 1’’

Standards
Standard ‘’S1.1’’ has concentration of 0.4
Standard ‘’S1.2’’ has concentration of 0.4
Standard ‘’S2.1’’ has concentration of 1.2
Standard ‘’S2.2’’ has concentration of 1.2
Standard ‘’S3.1’’ has concentration of 4.0
Standard ‘’S3.2’’ has concentration of 4.0
Standard ‘’S4.1’’ has concentration of 12.0

Standard ‘’S4.2’’ has concentration of 12.0
Standard ‘’S5.1’’ has concentration of 24.0
Standard ‘’S5.2’’ has concentration of 24.0

Fit Type ‘Sigmoid’

Average the standard replicates
Extrapolate the data
Sigmoid Max Iterations = 100
Produce Graph ‘Calprotectin µg/ml’
Horizontal axis ‘Concentration µ/ml’
With auto-scaling
With auto scaling
Result Flagging

If (Sample <S1) then result = ‘<0.4’
If (Sample >S5) then result = ‘>24.0’

Quality Control

Full QC Report
If (0.0 <LP1<2.0), then ‘Low control out of range’
If (0.0 <LP2<2.0), then ‘Low control out of range’
If (5.0 <HP1<6.2), then ‘High control out of range’
If (5.0 <HP2<6.2), then ‘High control out of range’

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QC-Data Sheet

Controls	Target value (Mean)	Expected Range (±2SD)	±SD
Low	1.4	1.0 – 2.0 µg/mL	0.2
High	5.6	5.0 – 6.2 µg/mL	0.3

Procedure

Test performed according to the actual instruction for use (IFU)

I. Controls

Controls (QC)	Absorbance (nm)		Mean Absorbance (nm)	Result (µg/mL)			CV (%)
	1	2		1	2	Mean	
Low	0.247	0.238	0.242	1.5	1.4	1.5	2.5
High	0.852	0.866	0.859	5.5	5.7	5.6	1.2

II. Standards

Standards (Calibrators)	Concentration (µg/mL)	Absorbance (nm)		Mean Absorbance (nm)	CV (%)
		1	2		
1	0.4	0.073	0.078	0.075	4.5
2	1.2	0.198	0.200	0.199	0.8
3	4.0	0.625	0.646	0.636	2.3
4	12.0	1.549	1.608	1.579	2.6
5	24.0	2.406	2.409	2.407	0.1

APPENDIX E: IMMUNODIAGNOSTIK™ (IDK®) CALPROTECTIN (MRP8/MRP14 S100A8/S100A9) ELISA K 6935 IN SERUM AND PLASMA

Author: Graham Johnson

Edited: 14th June 2016

Template

Dispense

Pipette Samples, Controls, Standards
Maximum post pipette delay for 5 minutes

Deep Well Dilution

Do not allow sample dilutions to be changed at run time
Do not pipette immediately after dilution
Let deep wells stand for 00.00 before pipetting

For Well Type ‘‘Test (T)’’

Dilute in a deep well 10 µL of Specimen with 990µl of IDK® Calprotectin K6935 Sample buffer
Then pipette 100 µL of that mixture to the microplate
Do not require new tip for dispense of sample
Single shot for dispense of sample
Share dilution wells for microplate replicates (volumes will be scaled)
Share dilution wells across multiple assays (volumes will be scaled)
Do not force level detect before transfer
Use aspirate/dispense speed profile 3
Mix for 3 cycles.

Pipette Protocol for Standards and Controls

Pipette 100 µL of IDK® K6935 STD5 to ‘S1’ wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of IDK® K6935 STD4 to ‘S2’ wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of IDK® K6935 STD3 to ‘S3’ wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of IDK® K6935 STD2 to ‘S4’ wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of IDK® K6935 STD1 to ‘S5’ wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of IDK® K6935 CTRL1 to ‘C1’ wells

Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of IDK® K6935 CTRL2 to 'C2' wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Incubation

Incubate for 30 minutes at ambient temperature

Wash Plate

Purge the washer with 9999 µL of IDK® ELISA Wash Buffer
Super sweep on last cycle only
Perform a 5–cycle wash, plate–wise, with constant timing
Dispense 350 µL of IDK® ELISA Wash Buffer
Do final aspirate cycle
Clean the washer after use with 5000 µL of Deionised Water

Conjugate

Dispense 100 µL of IDK® Calprotectin K6935 Conjugate to well(s) A1 – H12

Incubation

Incubate for 30 minutes at ambient temperature

Wash Plate

Purge the washer with 9999 µL of IDK® ELISA Wash Buffer
Super sweep on last cycle only
Perform a 5–cycle wash, plate–wise, with constant timing
Dispense 350 µL of IDK® ELISA Wash Buffer
Do final aspirate cycle
Clean the washer after use with 5000 µL of Deionised Water

Substrate

Dispense 100 µL of IDK® ELISA Substrate to well(s) A1 – H12

Incubation

Incubate for 12 minutes at ambient temperature

Stop Solution

Dispense 100 µL of IDK® ELISA Stop Solution to well(s) A1 – H12

Reader

Single/Dual wavelength mode
Primary test filter is 450nm
Primary reference filter is 620nm
Initial shake for 3 seconds.

Curve Fit ‘’Fit 1’’

Standards

Standard ‘’S1.1’’ has concentration of 250
Standard ‘’S1.2’’ has concentration of 250
Standard ‘’S2.1’’ has concentration of 62.5
Standard ‘’S2.2’’ has concentration of 62.5
Standard ‘’S3.1’’ has concentration of 15.6
Standard ‘’S3.2’’ has concentration of 15.6
Standard ‘’S4.1’’ has concentration of 3.9
Standard ‘’S4.2’’ has concentration of 3.9
Standard ‘’S5.1’’ has concentration of 0.001
Standard ‘’S5.2’’ has concentration of 0.001
Standard ‘’C1.1’’ has concentration of 210
Standard ‘’C1.2’’ has concentration of 210
Standard ‘’C2.1’’ has concentration of 840
Standard ‘’C2.2’’ has concentration of 840

Quality Control

Full QC Report
If ([Low LP] < = C1 < = [High LP]), the ‘’C1 is out of range’’
If ([Low HP] < = C2 < = [High HP]), then ‘’C2 is out of range’’

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QC - Data Sheet

Controls	Measured	Target value (Mean)	Expected Range (±2SD)	±SD
Ctrl. 1	15.15 ng/mL	15.40 ng/mL	7.40 – 23.40 ng/mL	4.00
Ctrl. 2	52.65 ng/mL	49.00 ng/mL	31.00 – 67.00 ng/mL	9.00

Procedure

Controls and Standards

Re–suspend the lyophilized contents (controls and standards) in 500 µL of ultrapure water. Allow the vial content to dissolve for 10 minutes and mix thoroughly by gentle inversion to ensure complete reconstitution. Reconstituted controls are not stable.

The given ranges were measured with reagents of the Calprotectin ELISA Kit Catalogue K 6935. The use of other reagents or measuring systems could give deviant results.

Standards (Calibrators)	Concentration (ng/mL)	Absorbance [nm]		Mean Absorbance (nm)	CV (%)
		1	2		
1	0.00	0.047	0.049	0.048	2.95
2	3.90	0.174	0.169	0.172	3.90
3	15.60	0.472	0.485	0.479	1.92
4	62.50	1.188	1.200	1.194	0.71
5	250.00	2.037	2.085	2.061	1.65

The data shown is for demonstration only. The quality of the results is not affected when the measured extinctions (optical density or absorbance) are not identical to the example. Deviations of the measured values are influenced by specific laboratory work conditions, e.g., temperature, shaking, washing, dilution of the conjugate and stopping of the colour development reaction.

APPENDIX F: IMMUNODIAGNOSTIK™ (IDK®) S100A12[a] ELISA K 6938 IN SERUM AND PLASMA

Author: Graham Johnson

Edited: 14th June 2016

Template

Wash Plate

Purge the washer with 9999 µL of IDK® ELISA Wash Buffer
Super sweep on last cycle only
Perform a 5–cycle wash, plate–wise, with constant timing
Dispense 350 µL of IDK® ELISA Wash Buffer
Do final aspirate cycle
Clean the washer after use with 5000 µL of Deionised Water

Pipette Samples, Controls, Standards
Maximum post pipette delay for 5 minutes

Deep Well Dilution

Do not allow sample dilutions to be changed at run time
Do not pipette immediately after dilution
Let deep wells stand for 00.00 before pipetting

For Well Type ‘‘Test (T)’’

Dilute in a deep well 10µl of Specimen with 90 µL of IDK® S100A12 K6938 Sample buffer
Then pipette 100 µL of that mixture to the microplate
Do not require new tip for dispense of sample
Single shot for dispense of sample
Share dilution wells for microplate replicates (volumes will be scaled)
Share dilution wells across multiple assays (volumes will be scaled)
Do not force level detect before transfer
Use aspirate/dispense speed profile 3
Mix for 3 cycles.

Pipette Protocol for Standards and Controls

Pipette 100 µL of IDK® S100A12 STD6 to ‘S1’ wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of IDK® S100A12 STD5 to ‘S2’ wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of IDK® S100A12 STD4 to ‘S3’ wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of IDK® S100A12 STD3 to ‘S4’ wells

Tip to dispense into microtitre well does not have to be clean.

Fluid into microtitre well must be a single shot dispense.
Pipette 100 µL of IDK® S100A12 STD2 to 'S5' wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of IDK® S100A12 STD1 to 'S6' wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of IDK® S100A12 CTRL1 to 'C1' wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Pipette 100 µL of IDK® S100A12 CTRL2 to 'C2' wells
Tip to dispense into microtitre well does not have to be clean.
Fluid into microtitre well must be a single shot dispense.

Incubation

Incubate for 60 minutes at ambient temperature
Shake for the entire duration at medium speed

Wash Plate

Purge the washer with 9999 µL of IDK® ELISA Wash Buffer
Super sweep on last cycle only
Perform a 5–cycle wash, plate–wise, with constant timing
Dispense 350 µL of IDK® ELISA Wash Buffer
Do final aspirate cycle
Clean the washer after use with 5000 µL of Deionised Water

Conjugate

Dispense 100 µL of IDK® S100A12 K6938 Conjugate to well(s) A1 – H12

Incubation

Incubate for 60 minutes at ambient temperature
Shake for the entire duration at medium speed

Wash Plate

Purge the washer with 9999 µL of IDK® ELISA Wash Buffer
Super sweep on last cycle only
Perform a 5–cycle wash, plate–wise, with constant timing
Dispense 350 µL of IDK® ELISA Wash Buffer
Do final aspirate cycle
Clean the washer after use with 5000 µL of Deionised Water

Substrate

Dispense 100 µL of IDK® ELISA Substrate to well(s) A1 – H12

Incubation

Incubate for 12 minutes at ambient temperature

Stop Solution

Dispense 100 µL of IDK® ELISA Stop Solution to well(s) A1 – H12

Reader

Single/Dual wavelength mode
Primary test filter is 450nm
Primary reference filter is 620nm
Initial shake for 3 seconds.

Curve Fit ‘’Fit 1’’

Standards
Standard ‘’S1.1’’ has concentration of 54
Standard ‘’S1.2’’ has concentration of 54
Standard ‘’S2.1’’ has concentration of 18
Standard ‘’S2.2’’ has concentration of 18
Standard ‘’S3.1’’ has concentration of 6
Standard ‘’S3.2’’ has concentration of 6
Standard ‘’S4.1’’ has concentration of 2
Standard ‘’S4.2’’ has concentration of 2
Standard ‘’S5.1’’ has concentration of 0.66
Standard ‘’S5.2’’ has concentration of 0.66
Standard ‘’S6.1’’ has concentration of 0.001
Standard ‘’S6.2’’ has concentration of 0.001
Standard ‘’C1.1’’ has concentration of 840
Standard ‘’C1.2’’ has concentration of 840
Standard ‘’C2.1’’ has concentration of
Standard ‘’C2.2’’ has concentration of

Quality Control

Full QC Report
If ([Low LP] < = C1 < = [High LP]), the ‘’C1 is out of range’’
If ([Low HP] < = C2 < = [High HP]), then ‘’C2 is out of range’’

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[a]Common aliases of S100A12

- S100 Calcium–Binding Protein A12
- Calgranulin C (CAGC)
- Cytosolic Protein, p6
- Calcium–Binding Protein in Amniotic Fluid 1 (CAAF1)
- Neutrophil S100 protein
- Calcitonin Gene–Related Peptide (CGRP)
- Extracellular Newly identified RAGE Binding Protein (EN–RAGE)
- Myeloid–Related Protein 6 (MRP6)
- Corneal or Cornea–Associated Antigen (CO–Ag)

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QC-Data Sheet

Controls	Measured	Target value (Mean)	Expected Range (±2SD)	±SD
Ctrl. 1	0.25 ng/mL	0.22 ng/mL	0.02 – 0.42 ng/mL	0.10
Ctrl. 2	5.53 ng/mL	6.50 ng/mL	3.50 – 9.50 ng/mL	1.50

Procedure

Controls and Standards

Re–suspend the lyophilized contents (controls and standards) in 500 µL of ultrapure water. Allow the vial content to dissolve for 10 minutes and mix thoroughly by gentle inversion to ensure complete reconstitution. Reconstituted controls are not stable.

The given ranges were measured with reagents of the calprotectin ELISA Kit Catalogue K 6938. The use of other reagents or measuring systems could give deviant results.

Standards (Calibrators)	Concentration (ng/mL)	Absorbance [nm]		Mean Absorbance (nm)	CV (%)
		1	2		
1	0.00	0.007	0.006	0.007	10.88
2	0.66	0.054	0.057	0.056	3.82
3	2.00	0.143	0.144	0.144	0.49
4	6.00	0.449	0.472	0.461	3.53
5	18.00	1.251	1.279	1.265	1.57
6	54.00	2.730	2.780	2.755	1.28

The data shown is for demonstration only. The quality of the results is not affected when the measured extinctions (optical density or absorbance) are not identical to the example. Deviations of the measured values are influenced by specific laboratory work conditions, e.g., temperature, shaking, washing, dilution of the conjugate and stopping of the colour development reaction.

APPENDIX G: RESULTS OF ROC ANALYSES AND CUT–OFF VALUES WITH THE HIGHEST SENSITIVITY AND SPECIFICITY

I. Coordinates of the ROC Curve for Bühlmann MRP8/14 Calprotectin S100A8/S100A9

Test Result Variable(s)	Positive (+) if Greater than or Equal To[a] (≥)	Sensitivity	1 – Specificity
Bühlmann MRP8/ MRP14 Calprotectin S100A8/S100A9 (µg/mL)	0.29000	1.000	1.000
	1.85550	1.000	0.929
	2.72450	0.950	0.929
	3.04750	0.950	0.857
	3.22650	0.900	0.857
	3.42500	0.900	0.786
	3.47650	0.900	0.714
	3.57000	0.900	0.643
	3.70250	0.900	0.571
	3.81800	0.900	0.500
	3.92750	0.850	0.500
	4.01050	0.800	0.500
	4.21150	0.800	0.429
	4.47200	0.800	0.357
	4.79000	0.800	0.286
	5.02650	0.800	0.214
	5.22300	0.750	0.214
	5.42750	0.700	0.214
	5.47950	0.650	0.214
	5.63350	0.600	0.214
	5.85900	0.550	0.214
	6.09400	0.500	0.214
	6.30500	0.450	0.214
	6.42350	0.400	0.214
	6.72600	0.400	0.143
	7.17050	0.400	0.071
	7.95250	0.350	0.071
	8.65550	0.350	0.000
	8.93900	0.300	0.000
	9.12700	0.250	0.000
	10.14000	0.200	0.000
	11.40550	0.150	0.000
	12.32450	0.100	0.000
	14.18050	0.050	0.000
	16.38000	0.000	0.000

[a] = The smallest cut-off value is the minimum observed test value minus 1, and the largest cut–off value is the maximum observed test value plus 1. All the other cut–off values are the averages of two consecutive ordered observed test values.

II. Coordinates of the ROC Curve for Immunodiagnostik™ K6935 Serum Calprotectin

Test Result Variable(s)	Positive if Greater Than or Equal To[a] (≥)	Sensitivity	1 – Specificity
Immunodiagnostik™ K 6935 Calprotectin (ng/mL)	674.07	1.000	1.000
	1030.55	1.000	0.929
	1511.95	0.950	0.929
	1745.06	0.950	0.857
	1871.47	0.900	0.857
	1901.26	0.900	0.786
	1919.32	0.900	0.714
	1981.21	0.900	0.643
	2068.50	0.900	0.571
	2141.37	0.900	0.500
	2198.08	0.850	0.500
	2254.23	0.850	0.429
	2317.23	0.800	0.429
	2522.58	0.800	0.357
	2738.55	0.800	0.286
	2940.07	0.800	0.214
	3139.29	0.750	0.214
	3254.20	0.700	0.214
	3380.62	0.650	0.214
	3499.94	0.600	0.214
	3612.06	0.600	0.143
	3723.62	0.550	0.143
	3821.25	0.500	0.143
	3853.07	0.450	0.143
	3942.38	0.400	0.143
	4217.99	0.400	0.071
	4523.80	0.400	0.000
	4844.42	0.350	0.000
	5119.54	0.300	0.000
	5221.11	0.250	0.000
	6128.61	0.200	0.000
	7029.63	0.150	0.000
	7296.07	0.100	0.000
	8043.61	0.050	0.000
	8556.33	0.000	0.000

[a] = The smallest cut-off value is the minimum observed test value minus 1, and the largest cut–off value is the maximum observed test value plus 1. All the other cut–off values are the averages of two consecutive ordered observed test values.

III. Coordinates of the ROC Curve for Immunodiagnostik™ K6938 Serum S100A12

Test Result Variable(s)	Positive if Greater Than or Equal To[a] (≥)	Sensitivity	1 – Specificity
Immunodiagnostik™ K 6938 S100A12 (ng/mL)	76.18	1.000	1.000
	139.68	1.000	0.929
	223.29	0.950	0.929
	251.90	0.900	0.929
	267.02	0.850	0.929
	286.10	0.850	0.857
	304.97	0.850	0.786
	314.17	0.850	0.714
	318.85	0.800	0.714
	321.77	0.800	0.643
	335.18	0.800	0.571
	351.23	0.800	0.500
	359.23	0.800	0.429
	371.11	0.800	0.357
	383.06	0.750	0.357
	389.64	0.700	0.357
	395.67	0.700	0.286
	400.68	0.650	0.286
	411.56	0.600	0.286
	427.03	0.550	0.286
	437.69	0.500	0.286
	444.68	0.450	0.286
	467.18	0.450	0.214
	494.62	0.400	0.214
	501.62	0.400	0.143
	519.83	0.350	0.143
	548.91	0.350	0.071
	568.74	0.350	0.000
	598.33	0.300	0.000
	642.20	0.250	0.000
	670.72	0.200	0.000
	720.90	0.150	0.000
	776.12	0.100	0.000
	812.91	0.050	0.000
	839.46	0.000	0.000

[a] = The smallest cut-off value is the minimum observed test value minus 1, and the largest cut–off value is the maximum observed test value plus 1. All the other cut–off values are the averages of two consecutive ordered observed test values.

APPENDIX H

C-REACTIVE PROTEIN

REF 8G65-21

30-4143/R1

ARCHITECT® / AEROSET®

C-REACTIVE PROTEIN

This package insert contains information to run the C-Reactive Protein assay on the ARCHITECT cSystems™ andthe AEROSET System.

NOTE: Changes Highlighted

NOTE: This package insert must be read carefully prior to product use. Package insert instructions must befollowed accordingly. Reliability of assay results cannot be guaranteed if there are any deviations from the instructions in this package insert.

Customer Support

United States: 1-877-4ABBOTT

Canada: 1-800-387-8378 (English speaking customers)1-800-465-2675 (French speaking customers)

International: Call your local Abbott representative

Symbols in Product Labelling

CAL 1-6	Calibrators 1 through 6	REF	Catalog number/List number
CONC	Concentration	SN	Serial number
EC REP	Authorized Representative in the European Community		Consult instructions for use
INGRED	Ingredients		Manufacturer
IVD	In vitro diagnostic medical device		Temperature limitation
LOT	Batch code/Lot number		Use by/Expiration date
R1	Reagent 1		
R2			

CE
ABBOTT LABORATORIES
Abbott Park, IL 60064, USA

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Abbott Diagnostics

December 2008

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NAME

C-REACTIVE PROTEIN

INTENDED USE

The C-Reactive Protein (CRP) assay is used for the quantitative analysis of C-reactive protein in human serum or plasma.

SUMMARY AND EXPLANATION OF TEST

C-reactive protein was discovered in 1930 and named for its reaction with the C-polysaccharide of the pneumococcal cell wall. CRP is a cyclic pentamer with an approximate molecular mass of 118 kDa. Each subunit is composed of 206 amino acids. CRP is synthesized in the liver and released into the circulatory system in response to proinflammatory stimuli, the strongest of which is interleukin-6 (IL-6). It is this positive response to inflammation from which CRP derives its diagnostic utility and categorizes it with "acute phase reactants". CRP has been called the archetype of acute phase proteins due to its rapid (24 to 28 hours) and marked response to a wide variety of inflammatory conditions and diseases. These stimuli include bacterial and fungal components, as well as damaged cell membranes from injured tissue arising from trauma, arthritis, vasculitides, and a spectrum of autoimmune processes. A reactant common to these stimuli is phosphocholine, which shows calcium-dependent binding to CRP and is the major ligand. CRP shares some of the immunological functions of IgG. It activates the classical complement pathway, binds to Fc receptors, and acts as an opsonin. These CRP activities make it a critical component of the innate immunological system that provides early defense against infectious agents. As stated by Volanakis, the main biological function of CRP appears to be host defense against bacterial pathogens and clearance of apoptotic and necrotic cells.[1] CRP is clinically useful due to its 1,000-fold rise during an inflammatory response, which provides a nonspecific indication that an inflammatory process is present. Its concentration falls rapidly when the condition resolves, due to a short half-life (19 hours). The biology and chemistry of CRP have been reviewed by Bienvenu et al.,[2] Du Clos,[3] and Volanakis.[1]

PRINCIPLES OF PROCEDURE

C-Reactive Protein is an in vitro diagnostic assay for the quantitative determination of CRP in human serum and plasma. When an antigen-antibody reaction occurs between CRP in a sample and polyclonal anti-C-reactive protein antibody, which has been adsorbed to latex particles, agglutination results. This agglutination is detected as an absorbance change, with the magnitude of the change being proportional to the quantity of CRP in the sample. The actual concentration is then determined by interpolation from a calibration curve prepared from calibrators of known concentration. The increase in absorbance at 572 nm is proportional to the CRP concentration.

Methodology: Immunoturbidimetric

REAGENTS

Reagent Kit

REF 8G65 C-Reactive Protein is supplied as a liquid, ready-to-use, two-reagent kit which contains:

- R1 3 x 38 mL
- R2 3 x 43 mL

Estimated tests per kit: 900
Calculation is based on the minimum reagent fill volume per kit.

Reactive Ingredients		Concentration
R1		
	Ethylenediaminetetraacetic Acid Disodium Salt Dihydrate	1.86%
	Sodium Azide	0.09%
R2	Latex particle adsorbed anti-human CRP	0.15%
	Sodium Azide	0.09%

REAGENT HANDLING AND STORAGE

Reagent Handling

Mix reagent cartridges by gentle inversion prior to placing on the instrument.

Remove air bubbles, if present in the reagent cartridge, with a new applicator stick. Alternatively, allow the reagent to sit at the appropriate storage temperature to allow the bubbles to dissipate. To minimize volume depletion, do not use a transfer pipette to remove the bubbles.

CAUTION: Reagent bubbles may interfere with proper detection of reagent level in the cartridge, causing insufficient reagent aspiration which could impact results.

Reagent Storage

Unopened reagents are stable until the expiration date when stored at 2 to 8°C. R1 should be clear. R2 should appear milky.

Reagent stability is 54 days if the reagent is uncapped and onboard.

WARNINGS AND PRECAUTIONS

Precautions for Users

1. For in vitro diagnostic use.
2. Do not use components beyond the expiration date.
3. Do not mix materials from different kit lot numbers.
4. **CAUTION:** This product requires the handling of human specimens.It is recommended that all human sourced materials be considered potentially infectious and be handled in accordance with the OSHA Standard on Bloodborne Pathogens.[4] Biosafety Level 2[5] or other appropriate biosafety practices[6,7] should be used for materials that contain or are suspected of containing infectious agents.
5. This product contains sodium azide; for a specific listing, refer to the REAGENTS section of this package insert. Contact with acid liberates very toxic gas. This material and its container must be disposed of in a safe way.

NOTE: Refer to *Section 8* of the instrument-specific operations manual for proper handling and disposal of reagents containing sodium azide.

SPECIMEN COLLECTION AND HANDLING

Suitable Specimens

Serum and plasma are acceptable specimens.

- **Serum:** Use serum collected by standard venepuncture techniquesinto glass or plastic tubes with or without gel barriers. Ensure complete clot formation has taken place prior to centrifugation. When processing samples, separate serum from blood cells or gel according to the specimen collection tube manufacturer's instructions.

  Some specimens, especially those from patients receiving anticoagulant or thrombolytic therapy, may take longer to complete their clotting processes. Fibrin clots may subsequently form in these sera and the clots could cause erroneous test results.
- **Plasma:** Use plasma collected by standard venepuncture techniques into glass or plastic tubes. Acceptable anticoagulants are lithium heparin (with or without gel barrier), sodium heparin, and EDTA. Ensure centrifugation is adequate to remove platelets.When processing samples, separate plasma from blood cells or gel according to the specimen collection tube manufacturer's instructions.

For total sample volume requirements, refer to the instrument-specific ASSAY PARAMETERS section of this package insert and *Section 5* of the instrument-specific operations manual.

SPECIMEN COLLECTION AND HANDLING (Continued)

Specimen Storage

Serum and plasma

Temperature	Maximum Storage	Bibliographic Reference
20 to 25°C	15 days	8
2 to 8°C	2 months	8, 9
-20°C	3 years	8
-70°C	indefinitely	10

Guder et al.[8] suggest storage of frozen specimens at -20°C for no longer than the time interval cited above. However, limitations of laboratory equipment make it necessary in practice for clinical laboratories to establish a range around -20°C for specimen storage. This temperature range may be established from either the freezer manufacturer's specifications or your laboratory standard operating procedure(s) for specimen storage.

NOTE: Stored specimens must be inspected for particulates. If present, mix and centrifuge the specimen to remove particulates prior to testing.

PROCEDURE

Materials Provided

REF 8G65 C-Reactive Protein Reagent Kit

Materials Required but not Provided

- REF 8G68 C-Reactive Protein Calibrator, CAL 1-6 1 x 2 mL
- Control Material
- Saline (0.85% to 0.90% NaCl) for specimens that require dilution

Assay Procedure

For a detailed description of how to run an assay, refer to *Section 5* of the instrument-specific operations manual.

Specimen Dilution Procedures

The ARCHITECT *c* Systems and the AEROSET System have automatic dilution features; refer to *Section 2* of the instrument-specific operations manual for additional information.

Serum and plasma: Specimens with CRP values exceeding

30.00 mg/dL (300.0 mg/L) are flagged and may be diluted using the Automated Dilution Protocol or the Manual Dilution Procedure.

Automated Dilution Protocol

If using the Automated Dilution Protocol, the system performs a 1:10 or 1:25 dilution of the specimen and automatically corrects the concentration by multiplying the result by the appropriate dilution factor.

Manual Dilution Procedure

Manual dilutions should be performed as follows:

- Use saline (0.85% to 0.90% NaCl) to dilute the sample.
- The operator must enter the dilution factor in the patient or control order screen. The system uses this dilution factor to automatically correct the concentration by multiplying the result by the entered factor.
- If the operator does not enter the dilution factor, the result must be multiplied by the appropriate dilution factor before reporting the result.

NOTE: If a diluted sample result is flagged indicating it is less than the linear low limit, do not report the result. Rerun using an appropriate dilution.

For detailed information on ordering dilutions, refer to *Section 5* of the instrument-specific operations manual.

The patient result flag ">" (ARCHITECT *c* Systems) and the EXT and LH result error codes (AEROSET) may indicate antigen excess. Dilute specimens and rerun. Specimens with CRP values greater than 30.00 mg/dL (300.0 mg/L) up to 149.70 mg/dL (1,497.0 mg/L) were tested, and the results were flagged appropriately.

CALIBRATION

Calibration is stable for approximately 30 days (720 hours) and is required with each change in reagent lot number. Verify calibration with at least two levels of controls according to the established quality control requirements for your laboratory. If control results fall outside

acceptable ranges, recalibration may be necessary.

For a detailed description of how to calibrate an assay, refer to

Section 6 of the instrument-specific operations manual.

For information on calibrator standardization, refer to the C-Reactive Protein Calibrator package insert.

QUALITY CONTROL

The following is the recommendation of Abbott Laboratories for quality control. As appropriate, refer to your laboratory standard operating procedure(s) and/or quality assurance plan for additional quality control requirements and potential corrective actions.

- Two levels of controls (normal and abnormal) are to be run every24 hours or each day of use.
- If more frequent control monitoring is required, follow the established quality control procedures for your laboratory.
- If quality control results do not meet the acceptance criteria defined by your laboratory, patient values may be suspect. Followthe established quality control procedures for your laboratory. Recalibration may be necessary.
- Review quality control results and acceptance criteria following a change of reagent or calibrator lot.

RESULTS

Refer to the instrument-specific operations manual for information on results calculations.

- **ARCHITECT System Operations Manual**—*Appendix C*
- **AEROSET System Operations Manual**—*Appendix A*

Representative performance data are given in the EXPECTED VALUES and SPECIFIC PERFORMANCE CHARACTERISTICS sections of this package insert. Results obtained in individual laboratories may vary.

LIMITATIONS OF THE PROCEDURE

Refer to the SPECIMEN COLLECTION AND HANDLING and SPECIFIC PERFORMANCE CHARACTERISTICS sections of this package insert.

EXPECTED VALUES

Reference Range

Serum[11]

	Range (mg/dL)	Range (mg/L)
Adult	0.01 to 0.82	0.1 to 8.2

To convert results from mg/dL to mg/L, multiply mg/dL by 10.

A study was conducted using 120 serum samples from adult volunteers. Data were analyzed as described by Clinical and Laboratory Standards Institute (CLSI) protocol NCCLS C28-A2.[12] From this study, results in the 95th percentile were less than 0.58 mg/dL, with samples ranging up to 0.70 mg/dL.

CRP is a nonspecific indicator for a wide range of disease processes. It is recommended that each laboratory determine its own appropriate upper limit for the reference interval, as reference intervals are affected by many factors that may differ for each population studied.

SPECIFIC PERFORMANCE CHARACTERISTICS

Reportable Range

The reportable range for C-Reactive Protein is 0.50 to 30.00 mg/dL (5.0 to 300.0 mg/L). Linearity was verified using a modified CLSI protocol NCCLS EP6-P.[13] A study performed on an ARCHITECT *c* System and the AEROSET System demonstrated linearity within ± 10% or 0.20 mg/dL (2.0 mg/L) across the reportable range of the assay.

SPECIFIC PERFORMANCE CHARACTERISTICS (Continued)

Recovery

Known concentrations of C-reactive protein were added to human serum. Each sample was analyzed in replicates of four using the CRP assay. The resulting %Recovery was calculated according to the following equation:

%Recovery = (Mean ÷ Target Concentration) × 100

Target Concentration (mg/dL)	Mean (mg/dL)	Delta (mg/dL)	Percent (%) Recovery
0.190	0.2013	0.011	106.02
0.445	0.4003	-0.045	89.87
0.891	0.7955	-0.096	89.31
1.781	1.6385	-0.143	91.98
3.563	3.4115	-0.152	95.75
7.126	7.0438	-0.082	98.85
14.251	14.4508	0.200	101.40
18.002	18.2613	0.259	101.44
24.002	25.0260	1.024	104.27
28.502	29.1440	0.642	102.25
Overall,			98.11

The overall average recovery for the CRP assay was 98.11%, with individual spiked recoveries ranging from 89.31% to 106.02% of target concentrations. Acceptable recovery is considered to be ± 10% or within 0.2 mg/dL of target concentration.

Limit of Quantitation (LOQ)

The LOQ for C-Reactive Protein is 0.50 mg/dL (5.0 mg/L). The LOQ is the analyte concentration at which the CV = 20%. Performance studies produced an LOQ of 0.5 mg/dL (5.0 mg/L).

Interfering Substances

Interference studies were conducted using a modified CLSI protocol NCCLS EP7-P.[14] Interference effects were assessed by Dose Response and Paired Difference methods, at the medical decision level of the analyte.

Interfering Substance	Interferent Concentration	N	Target (mg/dL)	Observed (% of Target)
Bilirubin	30 mg/dL (0.30 g/L)	5	1.57	100.6
	60 mg/dL (0.60 g/L)	5	1.57	100.4
Hemoglobin	1,000 mg/dL (10.0 g/L)	4	1.40	99.2
	2,000 mg/dL (20.0 g/L)	4	1.40	96.3
Intralipid	1,000 mg/dL (10.0 g/L)	5	1.52	99.3
	2,000 mg/dL (20.0 g/L)	5	1.52	99.4
Rheumatoid factor	160 U/mL (160 kU/L)	4	1.62	105.2
	302 U/mL (302 kU/L)	4	1.62	111.0

Bilirubin solutions at the above concentrations were prepared by addition of an unconjugated bilirubin solution to human serum pools. Hemoglobin solutions at the above concentrations were prepared by addition of hemolysate to human serum pools. Intralipid solutions at the above concentrations were prepared by addition of Intralipid to human serum pools. Rheumatoid factor solutions at the above concentrations were prepared by addition of a concentrated rheumatoid factor solution to human serum pools.

Pharmaceuticals listed below may affect CRP concentration.[15]

1. Oral contraceptives (progestogen effect), antimicrobial therapy, aurothiomalate, methotrexate, penicillamine, pentopril, and sulfasalazine may cause a physiological decrease in serum CRP levels.
2. Nonsteroidal anti-inflammatory drugs may cause a physiological decrease or may have no significant effect on serum CRP levels.
3. Oral contraceptives (oestrogen effect) and tumor necrosis factor-α may cause a physiological increase in serum CRP levels.

Precision

The imprecision of the CRP assay is ≤ 5% Total CV. Representative data from studies using CLSI protocol NCCLS EP5-A[16] are summarized below.

Control		Level 1	Level 2	Level 3
N		80	80	80
Mean (mg/dL)		1.72	2.72	3.72
Within Run	SD	0.03	0.05	0.02
	%CV	1.6	2.0	0.6
Between Run	SD	0.00	0.00	0.02
	%CV	0.0	0.0	0.4
Between Day	SD	0.02	0.03	0.04
	%CV	1.2	1.2	1.1
Total	SD	0.03	0.06	0.05
	%CV	2.0	2.3	1.3

Method Comparison

Correlation studies were performed using a modified CLSI protocol NCCLS EP9-A.[17]

Serum results from the CRP assay on the AEROSET System were compared with those from a commercially available nephelometry methodology.

Serum and plasma results from the CRP assay on the AEROSET System were compared with those from a commercially available turbimetric methodology.

Serum and plasma results from the CRP assay on an ARCHITECT *c* System were compared with the CRP assay on the AEROSET System.

Serum	AEROSET vs. Nephelometry	AEROSET vs. Turbimetric	ARCHITECT vs. AEROSET
N	96	124	123
Y - Intercept	0.05	0.25	0.051
Correlation Coefficient	0.995	0.998	0.999
Slope	0.97	0.89	0.99
Range (mg/dL)*	0.70 to 26.76	0.60 to 28.00	0.50 to 26.29

*AEROSET Range

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TRADEMARKS

The ARCHITECT *c* System family of instruments consists of *c* 4000, *c* 8000, and *c* 16000 instruments.

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ARCHITECT®

C-Reactive Protein Serum/Plasma—Conventional and SI Units

Configure assay parameters — General

● General O Calibration O SmartWash O Results O Interpretation

Assay: **CRP** Type: **Photometric** Version: †
Number: **1061**

● Reaction definition O Reagent / Sample O Validity checks

Reaction mode: **Rate up**
Primary Secondary Read times
Wavelength: **572 / None** Main: **20 – 26**
Last required read: **26** Flex: **–**
Absorbance range: **__ –** Color correction: **–**
Sample blank type: **None**

O Reaction definition ● Reagent / Sample O Validity checks

						R1	R2
Reagent: **CRP00**					Reagent volume:	**100**	**113**
Diluent: **Saline**					Water volume:		
Diluent dispense mode: **Type 0**					Dispense mode:	**Type 0**	**Type 0**

Dilution name	Sample	Diluted sample	Diluent	Water	Dilution factor	Default dilution
STANDARD	: **2.0**			=	**1:1.00**	●
1:10	: **10.0**	**2.0**	**90**	=	**1:10.00**	O
1:25	: **5.0**	**2.0**	**120**	=	**1:25.00**	O

O Reaction definition O Reagent / Sample ● Validity checks

Reaction check: **None**

Rate linearity %:

Configure assay parameters — Calibration

O General ● Calibration O SmartWash O Results O Interpretation

Assay: **CRP** Calibration method: **Spline**

● Calibrators O Volumes O Intervals O Validity checks

Calibrator set: **CRP**
Replicates: **3** [Range 1 – 3]

	Calibrator level:	Concentration:
Blank:	**Water**	**0**††
Cal 1:	**CRP1**	‡
Cal 2:	**CRP2**	‡
Cal 3:	**CRP3**	‡
Cal 4:	**CRP4**	‡
Cal 5:	**CRP5**	‡
Cal 6:	**CRP6**	‡

O Calibrators ● Volumes O Intervals O Validity checks

Calibrator: **CRP**

	Calibrator level	Sample	Diluted sample	Diluent	Water
Blank:	**Water**	**2.0**			
Cal 1:	**CRP1**	**2.0**			
Cal 2:	**CRP2**	**2.0**			
Cal 3:	**CRP3**	**2.0**			
Cal 4:	**CRP4**	**2.0**			
Cal 5:	**CRP5**	**2.0**			
Cal 6:	**CRP6**	**2.0**			

O Calibrators O Volumes ● Intervals O Validity checks

Calibration intervals:
Full interval: **720** (hours)
Calibration type:
Adjust type: **None**

Configure assay parameters — SmartWash

O General O Calibration ● SmartWash O Results O Interpretation

Assay: **CRP**

COMPONENT	REAGENT / ASSAY	WASH	Volume	Replicates
Cuvette	**Trig**	**10% Detergent B**	**345**	

C-Reactive Protein Serum/Plasma—Conventional Units

Configure assay parameters — Results

O General O Calibration O SmartWash ● Results O Interpretation

Assay: **CRP** Assay number: **1061**
Dilution default range: Result units: **mg/dL**
Low-Linearity: **0.50**‡‡
High-Linearity: **30.00**
Gender and age specific ranges:*

GENDER	AGE (UNITS)	NORMAL	EXTREME

Configure result units

Assay: **CRP**
Version: †

Result units: **mg/dL**
Decimal places: **2** [Range 0 – 4]

Correlation factor: **1.0000**
Intercept: **0.0000**

C-Reactive Protein Serum/Plasma—SI Units

Configure assay parameters — Results

O General O Calibration O SmartWash ● Results O Interpretation

Assay: **CRP** Assay number: **1061**
Dilution default range: Result units: **mg/L**
Low-Linearity: **5.0**‡‡
High-Linearity: **300.0**
Gender and age specific ranges:*

GENDER	AGE (UNITS)	NORMAL	EXTREME

Configure result units

Assay: **CRP**
Version: †

Result units: **mg/L**
Decimal places: **1** [Range 0 – 4]

Correlation factor: **1.0000**
Intercept: **0.0000**

O Calibrators O Volumes O Intervals ● Validity checks

Blank absorbance range: **____ – ____**
Span: **Blank – Blank**
Span absorbance range: **____ – ____**
Expected cal factor: **0.00**
Expected cal factor tolerance %: **0**

† Due to differences in instrument systems and unit configurations, version numbers may vary.
†† Displays the number of decimal places defined in the decimal places parameter field.
‡ Refer to the concentration specified on calibrator labeling or value sheet. In ARCHITECT software version 5.00 and above, these values are defined on the Configure calibrator screen. ‡‡ The linear low value (Low-Linearity) is the LOQ. * User defined.

AEROSET®

C-Reactive Protein Serum/Plasma—Conventional Units

Assay Configuration: Outline Page

Assay Name	Assay #	Line
CRP	61	A-Line

Quantitative Ranges

Min Text	Min	Panic-L	L-Reference-H	Panic-H	Max	Max Text
*	0.0*	0.0	0.0*	0.0*	0.0	*
	0.50**	L-Linear Range-H		30.00		

Reference Ranges

Age	Male	Female
0 Year	0.0 – 0.0	0.0 – 0.0
0 Year	0.0 – 0.0	0.0 – 0.0
0 Year	0.0 – 0.0	0.0 – 0.0
0 Year	0.0 – 0.0	0.0 – 0.0

Assay Configuration: Base Page

Reaction Mode	Wavelength-Prim/Sec	Read time-Main/Flex	Linearity%
	N/A	20 – 26 / 0 – 0	0

Qualitative Ranges

RATE UP 572 /

	S.Vol	DS.Vol	D.Vol	Abs Window	Rgt Name/Pos
Standard	2.0	0.0	0	0 – 0	Diluent: DILUENT C–10*0
					Type#

	Rgt Name/Pos	R.Vol	W.Vol	Type#
Reagent 1	CRP0051 – ___ *	100	0	0
Reagent 2	CRP0052 – ___ *	113	0	0

Reaction Check	Read Time – A/B	Range	Minimum
	1 – 1 / 1 – 1	0.0 – 0.0	0.0

Factor/Intercept	Decimal Places	Units
1.0 / 0.0	2	mg/dL

Assay Configuration: Calibration Page

Calib Mode	Interval (H)
Spline	720

Blank/Calib Replicates	Extrapolation %	Span	Span Abs Range
3 / 3	1	BLK – 1	0.0 – 0.0

	Sample	S.Vol	DS.Vol	D.Vol	W.Vol	Blk Abs Range
BLK	Water	2.0	0.0	0	0	0.0 – 0.0
C1	CRP 1	2.0	0.0	0	0	Cal Deviation
C2	CRP 2	2.0	0.0	0	0	0.0
C3	CRP 3	2.0	0.0	0	0	FAC Limit (%)
C4	CRP 4	2.0	0.0	0	0	10
C5	CRP 5	2.0	0.0	0	0	
C6	CRP 6	2.0	0.0	0	0	

Assay Configuration: SmartWash Page

Rgt Probe	Reagent	Wash	Vol
	—	—	—

Cuvette	Assay Name	Wash	Vol
	—	—	—

Sample Probe	Wash
	—

C-Reactive Protein Serum/Plasma—SI Units

Assay Configuration: Outline Page

Assay Name	Assay #	Line
CRP	61	A-Line

Quantitative Ranges

Min Text	Min	Panic-L	L-Reference-H	Panic-H	Max	Max Text
*	0.0*	0.0	0.0*	0.0*	0.0	*
	5.0**	L-Linear Range-H		300.0		

Reference Ranges

Age	Male	Female
0 Year	0.0 – 0.0	0.0 – 0.0
0 Year	0.0 – 0.0	0.0 – 0.0
0 Year	0.0 – 0.0	0.0 – 0.0
0 Year	0.0 – 0.0	0.0 – 0.0

Assay Configuration: Base Page

Reaction Mode	Wavelength-Prim/Sec	Read time-Main/Flex	Linearity%
	N/A	20 – 26 / 0 – 0	0

Qualitative Ranges

RATE UP 572 /

	S.Vol	DS.Vol	D.Vol	Abs Window	Rgt Name/Pos
Standard	2.0	0.0	0	0 – 0	Diluent: DILUENT C–10*0
					Type#

	Rgt Name/Pos	R.Vol	W.Vol	Type#
Reagent 1	CRP0051 – ___ *	100	0	0
Reagent 2	CRP0052 – ___ *	113	0	0

Reaction Check	Read Time – A/B	Range	Minimum
	1 – 1 / 1 – 1	0.0 – 0.0	0.0

Factor/Intercept	Decimal Places	Units
1.0 / 0.0	1	mg/L

Assay Configuration: Calibration Page

Calib Mode	Interval (H)
Spline	720

Blank/Calib Replicates	Extrapolation %	Span	Span Abs Range
3 / 3	1	BLK – 1	0.0 – 0.0

	Sample	S.Vol	DS.Vol	D.Vc	W.Vol	Blk Abs Range
BLK	Water	2.0	0.0	0	0	0.0 – 0.0
C1	CRP 1	2.0	0.0	0	0	Cal Deviation
C2	CRP 2	2.0	0.0	0	0	0.0
C3	CRP 3	2.0	0.0	0	0	FAC Limit (%)
C4	CRP 4	2.0	0.0	0	0	10
C5	CRP 5	2.0	0.0	0	0	
C6	CRP 6	2.0	0.0	0	0	

Assay Configuration: SmartWash Page

Rgt Probe	Reagent	Wash	Vol
	—	—	—

Cuvette	Assay Name	Wash	Vol
	—	—	—

Sample Probe	Wash
	—

Refer to **Assay Configuration** in *Section 2* of the **AEROSET System Operations Manual** for information regarding assay parameters.

* User defined or instrument defined.
** The linear low value (L-Linear Range) is the LOQ.

APPENDIX I: DEFINITION OF TERMS; THE VALIDITY OF A DIAGNOSTIC TEST

The Diagnostic Value of Serum Calprotectin Data in IBD Study

[a]True Positive (TP): 'those that the test has correctly defined as having IBD'

[b]False Positives (FP): 'those that the test has incorrectly defined as not having IBD';

[c]True Negatives (TN): 'those controls that the test has correctly defined as not having IBD'

[d]False Negatives (FN): 'those cases that the test has incorrectly defined as not having IBD'

[e]Sensitivity: 'proportion of patients with the disease (IBD) who are correctly identified by the test'. Specificity is calculated as: TP / (TP+FN)

[f]Specificity: 'proportion of patients without the disease (IBD) who are correctly identified by the test'. Specificity is calculated as: TN / (FP+TN).

[g]Positive Predictive Value (PPV): 'the proportion of patients with positive test who are correctly diagnosed as disease (IBD) positive'. PPV is calculated as: TP / (TP+FP).

[h]Negative Predictive Value (NPV): 'the proportion of patients with negative test who are correctly diagnosed as being disease (IBD) negative'. NPV is calculated as: TN / (FN+TN).

[i]Efficiency; 'the proportion of those correctly categorised as IBD patients'. Efficiency is calculated as: ([TP+TN] / n); where n = total number of patients tested.

[j]Positive Likelihood Ratio (PLR); is the ‘probability that a positive test would be expected in a patient divided by the probability that a positive test would be expected in a patient without a disease’. PLR is calculated as: Sensitivity / (1 – Specificity).

[k]Negative Likelihood Ratio (NLR); is the ‘probability of a patient testing negative who has a disease divided by the probability of a patient testing negative who does not have a disease’. NLR is calculated as: (1 – Sensitivity) / Specificity.

[l]Accuracy or effectiveness; is expressed as a proportion of correctly classified subjects among all subjects, or ability to measure the true amount or concentration of a substance in a sample. Overall accuracy is calculated as: (TP+TN) / (TP+FP+TN+FN).

[m]Precision is achieved when repeated determinations (analyses) on the same sample give similar results, or the chance that a subject testing positive actually has the condition being tested for. Precision, also known as PPV, is calculated as: TP / (TP+FP).

[n]Incidence is a measure of the number of newly diagnosed cases within a particular time period. Incidence is calculated as: (TP+FP) / (TP+FP+TN+FN).

[o]Prevalence is a measure of the frequency of a disease or health condition in a population at a particular point in time. Prevalence is calculated as: (TP+FN) / (TP+FP+TN+FN).

PTH-130

THE EFFICACY OF TWO COMMERCIALLY AVAILABLE SERUM CALPROTECTIN ASSAYS TO ASSESS DISEASE ACTIVITY IN IBD

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Background Biomarkers of disease activity in IBD have variable performance when it comes to sensitivity and specificity. The clinical use of serum calprotectin remains unclear, but commercially available assays are now available. Previous studies have suggested that serum calprotectin (s-Cp) may have a role in managing IBD. Studies have shown an association between s-CP and the recurrence of Crohn's disease in the STORI cohort.[1] These studies did however show that s-Cp has a similar profile in CD to that offered by C-reactive protein. Others have investigated its role in the unselected evaluation of Gastroenterology patients and found that its performance in these cases is disappointing and unlikely to be of value.[2] There remains uncertainty about the role of s-Cp in the management of patients with IBD.

Design Patients attending the IBD clinic between July 2017 and December 2017 were assessed and standard blood tests and a faecal calprotectin were collected. Serum was analysed using two commercially available calprotectin assays (Bühlmann and Immunodiagnostics). Additional data recorded included demographics, disease classification and an assessment of the clinical interpretation of their disease activity.

Results There was no difference seen in s-Cp in UC patients comparing active vs quiescent UC, except in those with pancolitis (Montreal E3) using the Bühlmann assay (median 5098 vs 3502 ng/mL; $p = 0.0362$).

Median s-Cp was, however, significantly higher in patients with active CD than in those with quiescent CD for both assays (5507 vs 3830 ng/mL; $p = 0.0001$ (Bühlmann assay) and 5131 vs 2994 ng/mL; $p = 0.0003$ (Immunodiagnostics)). The difference in s-Cp remained significant in patients with both small ($p = 0.008$) and large bowel CD ($p = 0.005$), with similar results for each serological test.

Serum calprotectin correlated with faecal calprotectin in all patients with IBD ($r = 0.2362$; $p = 0.0051$ (Bühlmann) and $r = 0.2183$; $p = 0.0098$ (Immunodiagnostics)).

Similarly, s-Cp correlated with serum CRP in individuals with IBD and specifically CD ($r = 0.4865$; $p < 0.0001$ (Bühlmann) and $r = 0.5016$; $p < 0.0001$ (Immunodiagnostics)).

Conclusions There does not appear to be any significant association with the confirmation of ulcerative colitis disease activity. In patients with CD there appears to be a significant association between s-Cp, for both commercially available assays, and the presence of active disease irrespective of the disease location.

S-Cp also appears to significantly correlate positively with other markers of disease activity in patients with active and inactive IBD. Additional data analysis suggests that s-Cp offers a potential benefit over currently available biomarkers, specifically in patients with CD.

REFERENCE

1. http://dx.doi.org/10.1016/j.crohns.2013.06.008
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