# AN INVESTIGATION OF THE PRE-ANALYTICAL VARIABILITY IN LABORATORY TESTING AND ITS INFLUENCE ON RESULT INTERPRETATION AND PATIENT MANAGEMENT

**By**

**Neil Anderson BSc (Hons) MSc FRCPath**

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

**The University of Warwick Medical School.**

**March 2018**

# Table of Contents

# Table of Figures

# Table of Tables

## Acknowledgements

I am thankful to Professor Rousseau Gama and Professor P Saravanan for their insight in spotting my abilities and, with Ms Kam Chatha, co-authored many of the papers I have submitted and Dr Phil Hudson for his guidance throughout.

I am very grateful to my supervisor Professor Dimitris Grammatopoulos for constant encouragement, patience and enthusiasm. I have learnt a great deal through working with him.

Without example set from Desmond Anderson and Richard Griffiths, I would not have had the drive to complete this work. Without the encouragement from my mother Heather, my children Patrick and Benjamin and especially my wife, Ann, I would neither have had the will or inclination to complete this work. I dedicate this work to them.

# Declaration

I, Neil Anderson, declare that the publications submitted in this thesis have not been submitted or are currently being submitted whether in published or unpublished form, for a degree, diploma or similar educational qualification at any University or Higher educational establishment.

The publications arising from the first and third studies have been submitted as part of the final FRCPath examination, held by the Royal College of Pathologists.

## Summary

Interpretation of laboratory tests in clinical practice is based on an understanding of the disease process within or between individuals. This is demonstrated by the variability of pathology results as compared to the previous result or against the reference range, made up from the intrinsic pathophysiological changes and also variation associated with the in vitro changes to the sample. My work is on identification and minimisation of the result variation in the pre-analytical phase, accounting for 60-70% of the errors associated with laboratory testing.

The first project of my thesis is based on four studies that consider the in vitro stability of parathyroid hormone (PTH) and C-reactive protein (CRP), in which significant sample degradation is observed due to sample tube type, anticoagulant used and time to separation.

The second project considers ethnic variation as a source of intra individual variation. Specifically considering intra individual ethnic variation in total cholesterol (TC) and high density lipoprotein cholesterol (HDLC), reporting significant differences were observed between Caucasian Indo-Asians in HDLC, in addition I investigated the relationship between low maternal vitamin B12 concentrations in Caucasian women and cord blood cholesterol.

The third project considered the variation in laboratory results due to pre-existing conditions causing interference in common laboratory tests. I published on the effect of lipaemia on common laboratory tests, showing lipaemia does have a significant effect on laboratory tests. The following study found that the raised prolactin seen in rheumatoid arthritis is not artefactual but due to changes in cross reactivity due of prolactin subtypes. The final paper of this project shows, through a collection of case studies falsely elevated serum calcium levels in patients with paraproteinaemia.

I conclude with two studies that demonstrate how inappropriate test selection can cause variability and therefore affect the utility of a test. My papers in this area have been cited over 140 times.

Word count 306

# Chapter 1 Background

The diagnosis and management of patients increasingly relies on the use of laboratory testing with Pathology requests in 100% of care records, 80% patient episodes (1). However, the true value of pathology testing, to the requestor, relies providing the requestor with information to aid in the assessment of the presence/absence or a condition or progression/regression of a condition. Assuming the test has value to the requestor when the test is being made how does the result inform the requestor? This can only be through result interpretation, this is the added value step that turns data into information (2).

Across Pathology the level of interpretation will vary according to speciality. The work undertaken in this thesis will deal specifically with the field of Clinical Biochemistry, however it is recognised that all specialities in Pathology interpret data to give the requestor information.

Interpretation could best be described as the contextualisation of variation of pathology results, being the difference between an expected result and observed result. In the clinical setting results have variation due to the underlying disease process, this is usually described with reference to the variation from the reference range, using a modified risk score or as variation from the previous result. More recently management of long term conditions increasingly relies on changes in results over time, which can be as part of complex algorithms that inform clinical decision making. This is seen in the guidance from the renal registry which defines the parathyroid hormone control as a marker of overall control of management of renal disease (3) or some algorithms for cardiovascular disease that use highly sensitive C-reactive protein in combination with HDL, age and smoking to define cardiovascular risk (4). Other commonly used algorithms include Well's score for assessment of deep vein thrombosis and estimated glomerular filtration rate (5). It is extremely important to stress that the use of algorithms assumes any variation in result is due to the physiological condition (6).

Clinical laboratory tests are used to provide information that will help elucidate the pathophysiology of a presenting condition, to directly manage a condition based on a series of results (give drug x if result is y) or to assess compliance to a treatment regime (1). When the requesting clinician uses the information from laboratory testing or laboratory results are used in guidance, the variability seen is often assumed to be that which is associated with the pathophysiology and hence the variability due to non-pathophysiology reasons is often overlooked. (7)

## 1.1 What is variation in the context of laboratory testing?

The variability in test results will affect the result and therefore its interpretation, so it is essential to gain an understanding of the sources of variation that may ultimately affect a given result and its interpretation.

Variation in results is made up from variation due to different factors:

a. the pathophysiological disease process
b. Variation due to non-pathophysiological disease processes that can usually be monitored and controlled.

Non-pathophysiological disease processes are split into two areas. The variation observes at an individual basis and the variation of the sample process. The former is made up from the intra and inter-individual variation and the latter is made up from the pre, post and analytical variation (8). The non-pathophysiological variation must be minimised to, to ensure correct interpretation of variation due to pathophysiological process. In order to quantify (and hence minimise) the variation, Fraser et al (9) described the Total analytical error for any test as being the sum of the two times the random error (standard deviation), plus the systematic error (bias):

*TaE = bias + 2 SD.*
TaE= Total analytical error, SD= Standard deviation

Any clinical laboratory will strive to minimise the total analytical error, which is determined by both bias and standard deviation. This has been further developed into the concept of Total allowable error, which then is a basis for setting goals for performance of any test (10), that is to minimise both the standard deviation and bias in any laboratory test.

For the purposes of the thesis, I am not considering bias (however it is recognised that this is a significant source of error, especially when considering assay selection and calibration). Therefore, the focus is to minimise SD as a marker of reduction in variation.
What does this mean in reality? If variation is considered from the perspective of Clinical Biochemistry, there is a continuous fluctuation of biochemical markers in biological fluids, this is best described in terms of biological variation (CVi)

The CVi of analytes, in biological fluids, is of three types (11):

1. Variation over the life span of the analyte, eg half life
2. Cyclical variation, eg due to circadian rhythm
3. Random variation.

CVi can be between groups of patients, for example in comparing HbA1c in a diabetic and non diabetic population, which is often used for diagnosis, or it can within an individual where for a diabetic patient HbA1c is used serially to monitor glycaemic control. Hence it is important when looking at the purpose of the test, whether it is for monitoring progress or regression of a condition in an individual or use of a test for diagnosis, where the result will be compared against population norms.

The other component of variation in pathology testing is the analytical error, which can be minimised and controlled, using tools described in page 13. This is especially important if it is considered that the error due to CVi is difficult to minimize. The analytical variability (Cva) is therefore kept appropriately less than the biological variability for the test to be confidently used for clinical diagnosis and monitoring (12).

As discussed earlier the concept of Total allowable error provides a guideline on performance of a test. However that is of limited use when considering the purpose of test, the below equation calculates the significance of the change in results or reference change value (RCV), taking into account both the analytical and individual variation:

$$RCV = \sqrt{2} * Z * \sqrt{CVa^2 + CVi^2}$$

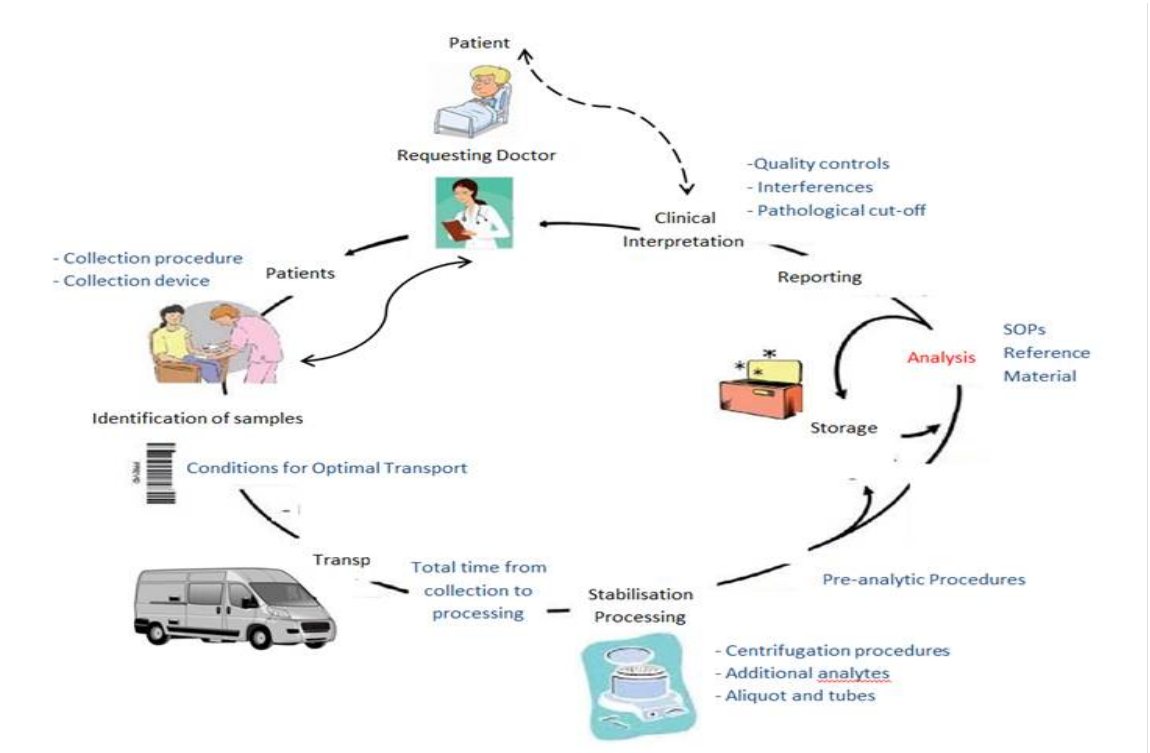
CVa = analytical imprecision, CVi = Biological variation, Z = Z value (1.96 for $p<0.05$ or 95%probablity)

In calculation of the RCV, it is acknowledged that to have a significant change in a result, one must take into account both the analytical and biological imprecision. Therefore Clinical laboratories must ensure that CVa and CVi are minimised so that any change articulated by a change in RCV is predominantly due to changes in pathophysiology. Examples of common analyte RCVs under current analytical conditions are, C-Reactive protein 206%, Creatine kinase 119%, TSH 104%. Clearly the variation due to non-physiological processes is significant and should be minimised (13). However, in order to minimise it, one has to have an understanding of the contributory sources of variation.

## 1.2 Sources of variation

The potential sources of error in the whole process of Pathology requesting to testing and interpretation is best described through the Brain-to-Brain Loop Concept for Laboratory testing in which Lundberg (14) introduced the concept of the thought of requesting an investigation being generated in the brain of the clinician caring for the patient through to the result affecting a future decision on the patient to be made by the clinician. The first step involves the selection of laboratory tests and the final step is the transmission of the test result to the ordering physician.

Figure 1.1 Adapted version of the Lundbergs brain to brain cycle



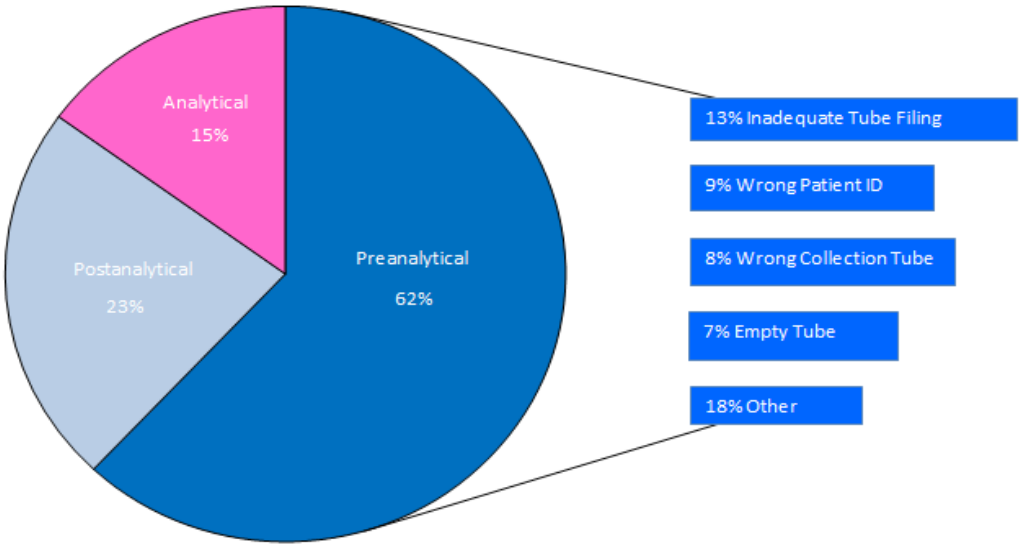

The introduction of this concept led to a system to identify and classify errors associated of laboratory test performance, namely pre-analytic, analytic, and post-analytic errors. These definitions still hold true four decades later, however more recently Plebani et al (15) have identified further modifications in consideration of the test and its eventual outcome, the addition of point of care testing and the advent of molecular medicine. I would like to add an additional source of variation that is not often considered, this is selection of an inappropriate test for that which is being investigated. This was clearly shown in my collaborative first

published paper on laboratory processes, where I described the inappropriate use of glucose meters to diagnose hypoglycaemia and the consequences of the misdiagnosis that followed (16).

It must be emphasised that, where possible, the responsibility of the laboratory professional to minimise these sources of variation, to allow for results to accurately reflect the variation due to the presence or change in disease pattern.

The proportion of each of the three main types of error is shown in figure 1.2 as described by Carraro and Plebani (17), pre-analytical being the greatest at 62% of the total errors, analytical 23% and post analytical 15%. This audit study was based on observed error rates across four hospital departments, where the clinical teams notified the laboratory of questionable findings.

Figure 1.2 Proportion of errors associated with laboratory testing.




A further source of variation is the post/post analytical phase, which is the part of the cycle that deals with the results being inadequately followed up of dealt with by the requesting physician (18)

Before discussing the component parts of variation it is important to understand that the relative contribution of each form of variation will change according to time and according to the assays used. For example in the 1980s the contribution of analytical variation was more significant as assay performance was poor with common endocrine tests having CVs of up to

25%. However, presently, pre-analytical variation is the most significant source of error as the variables associated with it are difficult to quantify and control (18). In my view it is likely that, as testing complexity increases, the significant errors will be post- analytical (19).

### 1.2.1 Analytical variation

The variability seen in the analytical phase is well documented (20). This could arise from equipment breakdown or malfunction, sample mix ups, interference or poor analytical performance as demonstrated through failure in quality control. It is summarised as variation due to analytic process variability. It is the job of the laboratory to eliminate and if that is not possible to understand each possible source of variation. There are many tools used to highlight and minimise laboratory variation:

1. Internal quality control to assess intra-laboratory analytical variation
2. Analyte External quality control (EQA) to assess lab to lab variation
3. UKAS/ISO15189 (2012). For Pre-analytical, analytical and post analytical
4. Continuing personal development of laboratory staff
5. Harmonisation of practice and protocols

Analytical variation is the component of variation that is in many ways easier to identify (through the above means) and the process by which variation is identified is tried and tested (21). The tools and principles of assessment and control have remained constant and in the last 30 years (22), although the methods have changed.

### 1.2.2 Post-analytical variation

Although variation does occur in this phase, it is likely to be based on the interpretation of results, rather than the generation of results. Such errors would include erroneous validation of analytical data, failure in reporting, excessive turnaround time, improper data entry and manual transcription errors and failure /delay in reporting critical values. So in order to interpret results adequately it is for the Clinical laboratory staff to ensure that such variation is minimised and to interpret the result in the light of the remaining variation i.e. is it related to a change in the underlying condition?

The following are routinely used to understand, control and minimise the post analytical variation (ref):

1. National EQAS for interpretative comments

2. Continuous personal development
3. Discrepancy meetings where reporting difference are discussed, these could be raised at MDT for example
4. Education in attending conferences and clinical meetings

By quantifying the post analytical variation it is possible to limit the effects by setting targets for improvement through the above processes.

### 1.2.3 Pre-analytical variation

Pre-analytical variation is the most significant component of total variation which was also the most difficult to quantify. Therefore, a categorisation of the variation leads to an improved understanding and ultimately to a mitigation of the risk associated with the variation (through a change in process or better understanding)

The constituent parts of pre-analytical variability (23), some of which have are investigated in the papers submitted in this thesis, are shown below:

1. Variability due to intrinsic physiology
    a. Age
    b. Sex
    c. Race
    d. Circadian rhythm
2. Variability due to life style
    a. Diet, including alcohol, caffeine, calorific intake
    b. Behaviour, including smoking, exercise, stress
    c. Posture
3. Variability due to inappropriate test selection
4. Variability due to disease process
5. Variability due to concurrent conditions, which cause interference with analytical process
    a. Liver disease causing raised bilirubin

b. Hyperlipidaemia

c. Haemolysis

d. Hyperproteinaemia

6. Variability due to phlebotomy affecting haemostasis

7. Variability due to in vivo drug interactions

8. Variability due to in vitro sample tube collection (including anticoagulant)

9. Variability due to sample transport and storage.

The question to be asked is what is done with the information on variation? The risk due to pre-analytical error is understood and there are the tools to detect and quantify it, however the clinical laboratory must also set standards to minimise the risk, whether that is through defining fasting/non fasting or samples taken at a specific time of the day, it could be producing clear guidance on sample tube type to use for phlebotomy or understanding complex drug interactions.

We must set standards for reduction in errors and key performance indicators to reflect the complexity of error, only then can the error be reduced through standardisation and education. Westgard has established a data base on desirable specifications for Total Error, Imprecision, and Bias, derived from intra- and inter-individual biologic variation, this is an invaluable tool when considering variation in the pre-analytical phase (24).

The work I have carried out in this thesis has been to elucidate the variability due to pre-analytical sources, which include elements of the intra and inter individual variability. The purpose was to identify the sources of variability that may affect the interpretation of certain analytes that were in use at the time. The analytes were selected due to their potential influence on medical practice and therefore at the time the work was both timey and relevant. However the scientific principles behind the hypothesis was and is of importance, as all methods must be assessed for their variabilities (25). Much of my research has focused on the identification of and mitigation of the pre-analytical variability in common Clinical Biochemistry tests. The propose of this is to quantify some of the components of variation inherent in laboratory testing, in order to minimise where possible and educate the individuals the interpret the laboratory results for the requesting clinicians, the papers I have written have

been cited 147 times detailed in section 3), demonstrating a dissemination of knowledge and good working practices.

Specifically I have published in relation to the effect of patients ethnicity (26-27), underlying concurrent conditions (28-30), patient preparation prior to blood sampling, venesection, the sample tube the blood is drawn into and effects of anticoagulants and clot separators (31-35), physiological interferants in the sample that affect the result, i.e. lipaemia, haemolysis and icteric samples (36) and the effect of the point of care testing on result reliability for interpretation (16). My work provides clear evidence that there is considerable variation due to the above factors and these must be understood if one is to make an adequate assessment of Clinical Biochemistry results in relation to the presenting clinical information.

The work I have completed has lent significant weight to the understanding of pre-analytical variability. Unfortunately there is no substitution for an in depth understanding of the sources of variability, even if one was able to quantify the extent of the variation, the range and type of pre-analytical variability are so numerous that this would be a meaningless exercise.

It is important to recognise that, although these papers were published some time ago, the techniques, the mathematics and the principles of investigation are as relevant now as they were at the time of my earliest publication

## 1.3 Research questions

There are three main areas of study that form the basis of the investigations supporting this thesis, in addition there are two further papers that describe variation outside the three main areas, which none the less also describe important sources of variation.

**Project 1:** This project studies into the *in vitro* stability of analytes post venesection.

This is an extremely important area of investigation, as the laboratories I currently work in receive approximately 10 000 samples per day. Those analytes requested may be subject to degradation due to metabolic process in the sample, which can be inhibited by various stabilisation agents in the tube or by variation in the conditions in which the tube is transported such as time, temperature and atmospheric pressure. Specifically I looked at two analytes parathyroid hormone (PTH) and C-Reactive protein (CRP). PTH was selected for study as this is a labile protein used in the assessment of bone disease. It used as a marker in the

differential diagnosis of calcium metabolism, this was an important area of research activity as PTH was being used as a marker of calcium homeostasis by the Renal Registry (37) with increased variation, against prescribed limits, being attributed to poor management CRP was selected as it was proposed as a novel marker of cardiovascular disease, however the measurement was both technically difficult and thus challenging to interpret (38). It was important to assess the contribution of pre-analytical variation to the small changes seen in CRP concentrations and to assess the effectiveness of the marker in that light.

**Project 2:** This project studied the intra individual variation in laboratory analytes due to ethnicity.

Specifically there were three areas studied. I investigated whether there was ethnic variation in total cholesterol and HDL. Ethnicity as a component of any population varies, therefore with the use of algorithms such as The Sheffield tables (39), whether such variation described could be due to ethnicity rather than pathophysiological reasons. I also studied the ethnic variation in CRP, as CRP was proposed as a marker in cardiovascular risk assessment. Finally I wanted to assess whether there was maternal variation of B12 in white Caucasians living in the UK

**Project 3:** Variation in laboratory results due to a pre-existing condition causing an elevation in interfants.

This work assessed three areas, initially I investigated the effect of lipaemia on other commonly requested tests. Lipaemia is a potential interference in many photometric and colorimetric assays commonly used in the laboratory. I wished to test whether the potential interference that is frequently reported, does in effect cause a variation in results. Secondly was the hyperprolactinaemia observed in rheumatoid arthritis genuine or laboratory artefact and finally a series of case histories investigated pseudo-pseudo hypercalcaemia observed in Waldenstroms macroglobulinaemia, as a significant cause of variation that could lead to over investigation.

**Additional studies**: Investigated how the high variability in CRP would render it ineffective in its incorporation into coronary heart disease risk assessment, this bought together previous observations around the pre-analytical variation and variation due to ethnicity. My final study for inclusion was the effect of inappropriate equipment selection for the diagnosis of hypoglycaemia and how it can lead to inaccurate results and therefore unnecessary investigations.

## 1.4 Relevance of studies and findings to current approaches to minimising pre-analytical variation

Pre-analytical variation has and always will be a component of non-pathological variation, which is in turn part the total variation seen in pathology testing, as articulated by Compton in Garbage in, Garbage out (40). The mathematics underpinning the quantification of variation are constant and are articulated in chapter 1.1, these have not varied over the last 40 years. However Simundic et al (41) have recently refined definitions and nomenclature to standardise the laboratory approach to quantification of variation.

The constituent parts of non-pathological variation are detailed in chapter 1.2, as demonstrated in Lundberg's brain to brain cycle first put forward in the 1970s (14). These have been modified by Plebani et al in 2012 (15), to include new development in diagnostics such as point of care testing and molecular testing. These novel areas are of increasing relevance to clinical pathology testing as evidenced by the rise in one stop clinics and personalised medicine. Plebani's work also demonstrates the enduring nature of pre-analytical variability in total variation, however it is recognised that over time as the emphasis on testing and technologies changes, the significance of the various constituent parts may, in it self, vary. For example, a significant source of pre-analytical variation in glucose measurement by laboratory is the time to stabilisation of the sample, whereas with point of care glucose testing, time to analysis is minimal, however method variation is.

The work I have carried out was relevant for those assays and the application of those assays at the time and still have relevance as long as those assays are in use. However the principles still hold relevance as demonstrated through the UKAS ISO 15189 standards for laboratory testing, specifically clause 5.4, pre examination processes, which articulates the need for the laboratory to understand the pre-analytical variables associated with an assay (25). Therefore the use of the techniques I have used in this work are relevant to current practice and must still be used in the assessment of new assays and, most importantly, the variation associated with the assays must be quantified and used in the interpretation of the total variation in any result.

Another example of the importance of an understanding of preanalytical variation is to provide evidence to support the change in delivery of pathology. NHSi have recently published recommendations around optimal configuration of pathology laboratories (42), suggesting that

large networks should be formed, with the majority of work being undertaken in factory laboratories. In order to facilitate that, pathology samples will have to be transported long distances. Therefore pathology laboratories must understand the effect of transport, temperature and time to stabilisation on the assays it offers, if they are to offer those tests from the factory laboratory. This demonstrates the value of the investigations I have carried out on PTH and CRP stability and highlights possible areas for future research.

Further areas of research would be in the emerging technologies being used in the laboratory such as point of care testing and molecular testing, where an understand of the non-pathological variation is essential to the interpretation of any test. Another interesting area of work is in algorithm development, where groups of tests are used to determine change in a condition, however, often the role of pre-analytical variation is ignored or underestimated.

In summary the work undertaken in this thesis is relevant for those assays studied, as those assays are still in use. The principles of investigation are relevant for all assays, given the UKAS guidance and there is significant research to do looking at novel techniques and applications in the laboratory. The purpose of any work undertaken must support the purpose of this work, to identify and reduce non-pathological variation to inform result interpretation.

# Chapter 2 Commentary linking the published work to the studies outlined in the previous chapter

## 2.1 Project1. Studies into the *in vitro* stability of analytes post venesection.

Analytes may be subject to degradation due to metabolic process in the sample, which can be inhibited by various stabilisation agents in the tube or by variation in the conditions in which the tube is transported such as time, temperature and atmospheric pressure. The research questions I studied were, whether there are significant pre-analytical variations observed in the laboratory testing process, and could this affect the utility of the test. Specifically I looked at two analytes parathyroid hormone (PTH) and C-Reactive protein (CRP).

### 2.1.1 Study 1.

My first investigation was into the effect of a protease inhibitor on in vitro stability of intact parathyroid hormone. Previous studies on intact parathyroid hormone (iPTH) have reported that any in vitro degradation of iPTH in unseparated blood samples may be sample tube- and time-dependent (43-44). The greater degradation of iPTH observed in serum compared to EDTA plasma (45) remains unexplained. Increased in vitro degradation of iPTH has been reported in patients who have high concentrations of circulating proteases (46). Additionally, it has been demonstrated that the addition of protease inhibitors may arrest the in vitro decline in iPTH over 24h (47), but this may be assay- and sample population dependent. Thrombin plays a significant in vivo role in platelet aggregation as part of the clotting process. Initiation of platelet aggregation requires protease-dependent binding of thrombin to platelet thrombin receptors (48). This increase in protease activity may be responsible for the in vitro degradation of PTH. I therefore investigated whether the addition of aprotinin (a potent protease inhibitor) influenced the in vitro stability of serum iPTH in unseparated samples from patients with chronic renal disease.

#### 2.1.1.1 Materials and methods

We venesected 11 patients with chronic renal failure prior to them receiving dialysis. Blood samples were collected into 10-mL plain glass tubes (Z10/GN, LIP Equipment and Services Ltd, Shipley, UK), with and without 2000 KIU of aprotinin (Bayer AG, Germany), and 2.7-mL EDTA tubes (Sarstead Monovet 2.7 mL KE, Aktiengesellschaft & Co, Germany). Samples were then transported to the laboratory on ice and remained unseparated at room temperature (17–23°C) until centrifugation. At 20 min (baseline), 1h, 2 h, 4 h, 8 h and 24 h, a 1-mL aliquot was taken from each tube and centrifuged. The resultant supernatant was frozen at – 20°C until analysis, which was carried out in one batch using the DPC Immulite iPTH assay (intra-assay coefficient of variation 4.8) (LKPH, Diagnostic Products Corporation, Los Angeles, CA, USA) immediately following thawing of the aliquot. Following logarithmic transformation, the data were normally distributed. Statistical analysis of the transformed data was by parametric repeat measures ANOVA with Tukey–Kramer post-test comparison. Results are given as pre-transformed data expressed as median (95% confidence intervals).

#### 2.1.1.2 Results

Concentrations of iPTH in each tube type over time are shown in Table 2.1. The iPTH concentration in the plain sample separated at 24 h was significantly lower than in those separated at baseline ($P<0.001$), 1.h ($P<0.001$), 2 h ($P<0.001$), 4 h ($P<0.001$) and 8 h ($P<0.001$). There were no significant differences between any other time points. Between baseline and 24 h the decline in iPTH in plain tubes was 24.7%. The iPTH value in the aprotinin sample separated at 24h was significantly lower than those separated at baseline ($P<0.01$), 1 h ($P<0.001$), 2 h ($P<0.001$), 4 h ($P<0.001$) and 8 h ($P<0.001$). There were no significant differences between any other time points. Between baseline and 24 h the decline in iPTH in aprotinin

tubes was 9.6%. There were no differences in iPTH concentrations between the EDTA samples separated at any time point. iPTH concentration was significantly lower in plain tubes when compared with aprotinin tubes (P<0.05) at 24 h. iPTH concentration was also significantly lower in plain tubes (P<0.001) and aprotinin tubes (P<0.01) when compared with EDTA tubes at 24 h. There were no other significant between-tube differences at any other time points.

| | | Tube Type | | |
|---|---|---|---|---|
| Time (h) | Number of Samples | Plain | Aprotinin | EDTA |
| 0.3 | 11 | 46.5 (20.2-62.4) | 43.7 (19.1-61.8) | 51.3 (20.7-65.6) |
| 1 | 11 | 51.7 (20.6-61.4) | 45.8 (20.4-60.0) | 45.8 (20.6-62.5) |
| 2 | 11 | 47.3 (19.9-61.7) | 48.9 (23.3-62.5) | 52.4 (21.6-63.4) |
| 4 | 11 | 46.6 (20.9-65.3) | 47.4 (20.7-64.2) | 50.8 (20.9-67.1) |
| 8 | 11 | 46.6 (20.2-62.9) | 45.0 (20.6-64.4) | 53.3 (22.2-67.8) |
| 27 | 11 | 30.7ǂǂ (13.9-48.5) | 34.3ǂ, C* (16.9-56.4) | 49.2 (A**, b** (19.9-63.9) |

Table 2.1 Intact parathyroid hormone concentrations (pmol/L) in different sample tubes against time.

Results are median (95% confidence limits). Within-tubes, the only significant differences observed compared with baseline (0.3 h) were at 24 h:ǂ*P<0.01,* ǂǂ*P<0.001*. Between-tubes, the only significant differences were observed at 24 h: a, EDTA compared to plain tube, ******P<0.001; b,*** EDTA compared to aprotinin tube, *****P<0.01; c,*** aprotinin tube compared to plain tube, ****P<0.05***.

#### 2.1.1.3 Summary and significance

The unchanged plasma iPTH concentrations in EDTA tubes, left unseparated for up to 24 h in pre-dialysis samples collected from patients with chronic renal failure, confirms that iPTH is stable for up to 24 h in unseparated EDTA samples (49). Although I found no significant difference in baseline results between any of the tubes, Omar et al. reported significantly higher baseline iPTH values in EDTA tubes compared with plain tubes in samples collected from patients attending a renal stone clinic (50). These differences could be explained by the different mixture of PTH fragments in each of the different sample populations, which may have different in vitro stability.

In this study, serum iPTH concentrations were stable when left unseparated for up to 8 h in plain and aprotinin-containing tubes. The addition of aprotinin to plain tubes significantly reduced the decline in iPTH at 24 h. However, there was still significant difference in iPTH in aprotinin tubes when compared with EDTA tubes at 24 h. Levin and Nesbit(44) using the Nichols Institute, Allegro iPTH assay, found that addition of two protease inhibitors (aprotinin and leupeptin) eliminated the decline in iPTH that I observed at 24 h when compared to EDTA tubes. It is possible that the combination of protease inhibitors confers greater protection against in vitro iPTH instability. However, as both studies used different iPTH assays and different study populations, this is not clear.

My findings suggest that increased protease activity could explain, in part, the decline in serum iPTH in blood samples left unseparated for greater than 8 h. Aprotinin is a serine protease inhibitor and probably acts in vivo by inhibiting the thrombin induced proteolytic activation of platelets (47). This mechanism may explain the in vitro observation that iPTH decline is observed in serum and not in EDTA plasma, suggesting that the increased protease activity may be due to the clotting process. Plain and EDTA tubes are appropriate sample tubes for

collection of iPTH in patients with renal disease, if left unseparated for up to 8 h. Increased protease activity may solely or partially contribute to the decline in serum iPTH in blood samples left unseparated for longer than 8 h.

### 2.1.2 Study 2.

I compared the stability of IPTH in blood samples taken into gel tubes (Sarstead Monovet 4.7 mL, Z GEL) and plain glass tubes (Lip Z10/GN).

#### 2.1.2.1 Materials and methods

Blood was drawn from nine patients with chronic renal failure before commencement of haemodialysis. Immediately after venesection, samples were split between the plain and the gel tubes. Samples were then separated after 20 min at room temperature and serum frozen at -20$^{o}$C until assayed for iPTH using the DPC Intact PTH assay on the Immunlite automated immunoassay analyser.

#### 2.1.2.2 Results

Intact PTH values were 13% higher ($P<0.01$, two-tailed paired *t*-test) in plain serum tubes [mean (SD): 7.6 (2.57) pmol/L] than in gel serum tubes [6.7 (2.19) pmol/L], as shown in figure 2.1.

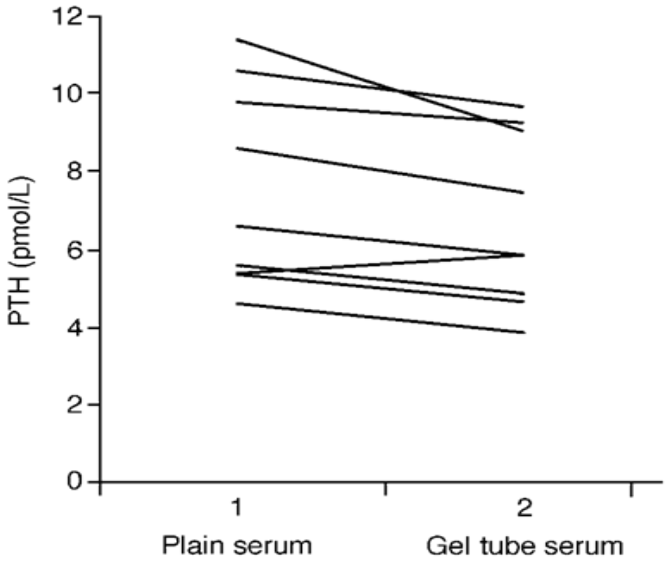



Figure 2.1 Stability of intact PTH at room temperature in plain serum tubes (1) and gel tubes (2). All samples separated by 20 min and serum stored at – 20 until analysis

#### 2.1.2.3 Summary and significance

I suggest that these differences in iPTH values are attributable to the gel barrier or clot activator or both, especially since these results are consistent with other studies reporting a similar 'gel effect' on other immunoassays (49).

DPC also recommended that blood samples when collected into EDTA should fill the tube. Otherwise, the EDTA concentration in the sample will proportionately increase, affecting the Immulite substrate alkaline phosphatase reaction and thus lowering the iPTH result.

These factors may help explain both the variation between EDTA plasma and (gel) serum as reported (50) and the differences between this study, which reports that 'serum' iPTH collected in gel tubes in unstable at 3h, and other studies which have reported that iPTH collected into plain serum tubes is stable for up to 8 h (51)

### 2.1.3 Study 3.

In this study, I consider highly sensitive C-reactive protein (hs-CRP) sample collection conditions. hs-CRP was assuming increased importance in the evaluation of patients with coronary artery disease (CAD). It is a prognostic indicator in acute coronary syndromes (52-54) and a predictor of future coronary events in those with and without overt CAD (55-56). As small changes in hs-CRP concentration potentially have considerable clinical impact, I wished to evaluate all aspects of the analytical process.

Highly sensitive CRP has undergone numerous evaluations on pre-analytical and analytical variability. In healthy individuals, hs-CRP exhibits no diurnal variation (57), and hs-CRP shows little variation over a 12-month period, with a similar stability of measurement to total cholesterol (58). Analytical variability of hs-CRP assays has been assessed recently by Roberts and colleagues, who reported coefficients of variation (CV) <10% at 0.15 mg/L (59).

It is widely recognised that sample collection tube type may affect analyte concentrations. In particular, 'gel' tube effects have been described for anticonvulsant drugs (60) and intact parathyroid hormone (61). However, the effect of sample collection tube type on hs-CRP concentration has not been studied.

This study aims to assess the stability of serum and plasma hs-CRP in different collection tubes over a 24-hour period, using the DPC Immulite hs CRP assay.

#### 2.1.3.1 Methods

Blood samples were collected from seven patients with chronic renal failure (on dialysis) into a 10 mL plain glass tube (tube A; Lip Z10/GN), a 2.7 mL EDTA tube (tube B [2.7 ml KE]; Sarstedt Monovet, Germany) and a 4.2 mL gel tube (tube C [4.2 ml, Z GEL]; Sarstedt Monovet).
Samples were transported to the laboratory on ice and remained unseparated at room temperature (17-23°C) until centrifuged. At 20 min (baseline), 1, 2, 4, 8 and h, a 1 mL sample was taken from each tube and centrifuged. The resultant supernatant was frozen at -20°C until analysis, which was carried out in one batch using a DPC Immulite high sensitivity CRP assay (LKCR, Diagnostic Products, Los Angeles; intra-assay CV = 4.1%), immediately after thawing. Statistical analysis was by repeat measures ANOVA and, where significant, was followed by Tukey-Kramer post-test comparison.

#### 2.1.3.2 Summary and significance

Significant differences were observed in samples separated at 0.3 h (ANOVA $P$=0.0192), and hs-CRP values were significantly higher ($P$<0.05) in gel tubes than in plain and EDTA tubes. Otherwise, there were no significant between-tube differences in hs-CRP concentration.
Within the tube type, there were no significant differences in hs-CRP concentration in samples taken into plain tubes and EDTA tubes over the 24-h period. In gel tubes, however, there were significant differences over time (*ANOVA* $P$=0.03), with hs-CRP concentration significantly lower ($P$<0.05) in the samples separated at 8 and 24 h, compared with that in the basal sample separated at 0.3 h. In gel tubes, hs-CRP concentration decreased by 9.7% over 24 h.

These results demonstrate a significant decline in hs-CRP concentration over time, when blood is collected into a gel tube, and support previous studies reporting the effect of gel tubes on other analytes such as therapeutic drugs (60) and intact parathyroid hormone (61).

Several studies have proposed that hs-CRP be used as a prognostic indicator in acute coronary syndromes (54-56) or a predictor of future coronary events (57-58). However, the significant decline in hs-CRP in gel collection tubes observed over 24 h in this study could lead to the misclassification of patients in samples left unseparated for eight or more hours. This may be particularly important for samples collected in the community for evaluation of CAD risk.

In conclusion, results from this study suggest that caution should be exercised in interpreting hs-CRP results when the sample is collected into a gel tube, as I report a significant decline in hs-CRP concentration over time.

### 2.1.4 Study 4.

In this study, I considered the stability of CRP, using the DPC Immulite high-sensitivity CRP assay, in samples collected into plain glass tubes.

#### 2.1.4.1 Materials and methods

EDTA tubes gel tubes and separated at 20 min (baseline) and 1h,2h, 8h and 24h.

#### 2.1.4.2 Results

In samples separated at baseline the measured CRP concentrations were significantly higher in gel tubes than in plain and EDTA tubes. Otherwise, there were no significant differences between tube CRP concentrations at any time point. In gel tube CRP concentrations were significantly lower at 8h and 24h when compared with the sample separated at baseline. There were no significant differences in CRP concentrations in samples taken into plain or EDTA tubes over 24h.

#### 2.1.4.3 Summary and significance

These findings of higher baseline serum CRP concentrations in gel tubes compared with plain tubes are consistent with those of Chang *et al* (63). Additionally, I reported a significant decline in CRP concentrations in gel tubes by 8h post sampling (62). These findings of discrepant results from different collection tubes are similar to previous studies reporting the effect of gel tubes on other analytes, including therapeutic drugs (64) and intact parathyroid hormone (65). Serum CRP concentrations often in the 'conventional' reference range, have an important role in the evaluation of cardiovascular risk and are of prognostic value in acute coronary syndromes (66).

The between-tube differences are more likely to influence clinical interpretation of CRP at lower concentrations especially where CRP is used as an aid to assess cardiovascular risk evaluation and prognosis of acute coronary syndromes.

I reported an intra-assay coefficient of variation of 3.36% at a CRP concentration of 10.7 mg/L using the Immulite high-sensitivity CRP assay. However, there is an additional 8.8-10.1% variation (according to the sample tube type) and a 10.7% decline in CRP concentrations if gel tubes are used for sample collection. Such variability due to sample tube type could lead to potential misclassification of patients under evaluation for cardiovascular risk and acute coronary syndrome.

### 2.1.5 A summary of Project 1

This project considered the *in vitro* stability of PTH and CRP assays. The study demonstrates the importance of sample tube type and time to separation as determinants of sample stability. These findings are of significance as the correct and optimal pre-analytical conditions minimise the (CVa) ensuring appropriate interpretation of the Biological variation (CVi). These papers have been cited 18 times including reviews on sample stability, appropriate testing conditions and renal journals

## 2.2 Project 2. Intra individual variation in laboratory analytes due to ethnicity

This project contained three studies, which considered the intra-individual variation due to ethnicity, in analytes commonly used or associated with cardiovascular disease. Specifically I assessed the ethnic variation in total cholesterol and HDL, ethnic variation in CRP and maternal variation of B12 in white Caucasians living in the UK. This is an important area of research as the incidence of cardiovascular disease is observed to be higher in some ethnic populations, I assessed whether some of the variation in analyte results was due to ethnic variation and thus could determine the non-pathophysiological component of the total variation.

### 2.2.1 Study 1

For this study, I investigated the ethnic differences in total and HDL cholesterol concentrations between Caucasians and predominately Punjab Sikh Indo-Asians. This is as there is increased prevalence of coronary heart disease (CHD), in Indo-Asians resident in the UK is unexplained by differences in cardiovascular risk factors of hypercholesterolaemia, hypertension, smoking or a family history of CHD (67-68). Although diabetes mellitus is more common in Indo-Asians, it does not fully explain the increased prevalence of CHD.

Low serum HDL cholesterol (HDLC) concentrations, widely reported in Indo-Asians, may contribute to the increased prevalence of CHD amongst this group. However, it has been suggested that there may be a difference in cardiovascular risk factors between culturally different Indo-Asian groups (69). I therefore compared serum total cholesterol (TC) and HDLC in Indio-Asian and Caucasian subjects residing in Wolverhampton, UK, for whom a laboratory CHD risk assessment had been requested.

#### 2.2.1.1 Subjects and methods

General practitioners (GPs) were provided with a laboratory-based 10 year CHD risk score calculation based on the Framingham equation (59). This calculation uses the individual's age, sex, systolic blood pressure (BP), TC concentration, HDLC concentration, and the presence or absence of smoking, diabetes mellitus and left ventricular hypertrophy. Clinical information was collected from a sticker fixed on the pathology request form, Afro/Caribbean, Asian, Chinese and 'other'). Patients were selected at the discretion of their GPs. Since most laboratory CHD risk assessment requests in Indo-Asians were on subjects aged 40-60 years, I restricted the study to this age group. Other exclusion criteria included overt macrovascular

disease and lipid-lowering medication. Serum TC and HDLC concentrations were measured automatically (DiaSys Diagnostic Systems Gmbh & Co, Holzheim, Germany and Bio-Stat Diagnostic Systems, Stockport, UK, respectively). Inter-assay and intra-assay coefficients of variation for TC were respectively 1.22% and 0.61% and for HDLC were respectively 1.93% and 0.75%.

Data were non-parametric. The Mann-Whitney *U-test* was therefore used to assess the differences between Indo-Asians and Caucasians. The Kruskal-Wallis test with Dunn post-test comparison was the used to assess the differences between ethnic male and female subgroups. Results are expressed as medians with 95% confidence intervals in parentheses.

#### 2.2.1.2 Results

Complete demographic, clinical and biochemical data sets were collected in 94 Indo-Asian women. 129 Indo-Asian men, 366 Caucasian women and 421 Caucasian men. Table 2.2 shows serum TC concentration was similar in Caucasians and Indo-Asians but Indo-Asians had a lower average HDLC concentration (*P<0.001*) and therefore a higher TC:HDLC ratio (*P<0.005*).

| | Caucasian | Indo-Asian | P value |
|---|---|---|---|
| n | 787 | 223 | |
| Age (years) | 52 (51.2-52.0) | 49(48.9-50-5) | <0.0001 |
| Diabetics (%) | 16.9 | 24.8 | 0.3055 |
| Current smokers (%) | 40.7 | 12-2 | <0.0001 |
| Total cholesterol (mmol/L) | 5.8 (5.7-5-9) | 5.7 (5.6-5.9) | 0.0769 |
| HDL cholesterol (mmol/L) | 1.4 (1.3-1.4) | 1.3 (1.2-1.3) | <0.0001 |
| Total:HDL cholesterol ratio | 4.6 (4.5-4.7) | 4.8 (4.6-5.0) | 0.004 |
| Systolic BP (mmHg) | 140 (139-142) | 136 (133-138) | |
| 10-year CHD risk (%) | 10.6 (10.1-11-2) | 9.4 (8.6-10.2) | 0.0714 |

Table 2.2 Cardiovascular risk factors in Indo-Asian and Caucasian subjects. Results are medians (95% confidence intervals)

Men and women in the two ethnic groups had similar TC concentrations. Indo-Asian women, Indo-Asian men and Caucasian men had similar HDLC concentrations but these were on average lower (*P<0.001*) than those in Caucasian women. The TC:LHDLC ratio was similar within each gender group. Indo-Asian and Caucasian men had a higher TC:HDLC ratio than did Caucasian women (*P<0.001*) but a similar TC:HDLC ratio compared to Indo-Asian women.

**2.2.1.3 Summary and significance**

I found that TC concentration in Indo-Asians and Caucasians was similar and this is consistent with previous studies (67). The lower HDLC concentration found in Indo-Asians when compared with Caucasians was consistent with previous studies (68-69). I suggest this is due to higher HDLC concentrations in Caucasian women, since HDLC concentrations were similar in Indo-Asian women, Indo-Asian men and Caucasian men.

Previous studies have found higher HDLC concentrations in Caucasian and Indo-Asian women compared with men (70-71). The absence of a sex difference in HDLC concentration in Indo-Asians in this study, however, has been previously reported in some indigenous Indian communities (72) but this finding remains unexplained. In women, the lower average HDLC concentration in Indo-Asian men and Caucasian men had similar HDLC concentrations. The lower HDLC concentration. The lower HDLC concentrations in Indo-Asian women may be related to lifestyle (for example, exercise, menopausal status and alcohol intake) or medication (e.g. oral contraceptives pill or hormone replacement therapy), both of which affect HDLC concentrations. It is also possible that the higher than 'expected' HDLC in Indo-Asian men may be likewise influenced by lifestyle factors.

It has been reported that there may be a variation of CHD risk factors within culturally diverse Indo-Asians (68). In this study the Indo-Asian subjects were predominately Punjabi Sikhs, whereas other studies have often included culturally diverse Indo-Asian groups. The results in this study are consistent with a previous study which observed similar HDLC concentrations in Sikh Indo-Asian men and Caucasian men but lower HDL concentrations in Muslim and Hindu Indo-Asian men (73, supporting the notion of diversity of CHD risk factors amongst Indo-Asians (68).

HDLC concentrations are lower in Indo-Asian women that in Caucasian women but similar in Indo-Asian men and Caucasian men. This difference is likely to be social or environmental in

origin. In developing strategies to prevent CHD in South Asians consideration should be given to possible diversity of CHD risk factors between culturally different Indo-Asian groups.

### 2.2.2 Study 2

This considered the ethnic variation in C-reactive protein: UK resident Indo-Asians compared with Caucasians. The increased prevalence of coronary heart disease (CHD) in UK resident Indo-Asians is unexplained by the traditional cardiovascular risk factors of dyslipidaemia, hypertension, smoking and diabetes mellitus (74-76). C-reactive protein (CRP) has been implicated in the pathogenesis of CHD but the data on ethnic variation in CRP is conflicting (77). I therefore investigated whether CRP could help explain the increased prevalence of CHD in Indo-Asians. For this study I measured CRP, using a highly sensitive assay, in 102 men (63 Caucasians and 39 Indo-Asians) and 89 women (58 Caucasians and 31 Indo-Asians). All subjects, aged between 40 and 70 years, were nondiabetic and non-smokers.

#### 2.2.2.1 Results

Serum CRP correlated ($P < 0.05$) positively with coronary risk. Serum HDL cholesterol concentrations were lower ($P<0.05$) in Indo-Asian women when compared with Caucasian women, but otherwise the ethnic groups were matched for calculated coronary risk and cardiovascular risk factors. Serum CRP concentrations were similar in Indo-Asians (women 2.29 (1.52) mg/l [mean (SD)]; men 1.77 (1.46) mg/l) and Caucasians (women 2.23 (1.54) mg/l; men 1.94 (1.45) mg/l).

#### 2.2.2.2 Summary and significance

This work showed that altered CRP concentrations do not appear to be implicated in the increased prevalence of CHD in UK resident Indo-Asians.

### 2.2.3 Study 3

In this study, I investigated the relationship between low maternal vitamin B12 status and lower cord blood HDL cholesterol in white Caucasians living in the UK. Previous studies in South Asian population show that low maternal vitamin B12 associates with insulin resistance

and small for gestational age in the offspring (78). Low vitamin B12 status is attributed to vegetarianism in these populations (79). It is not known whether low B12 status is associated with metabolic risk of the offspring in whites, where the childhood metabolic disorders are increasing rapidly (80). Here, I studied whether maternal B12 levels associate with metabolic risk of the offspring at birth.

#### 2.2.3.1 Methods

This was a cross-sectional study of 91 mother-infant pairs (n = 182), of white Caucasian origin living in the UK. Blood samples were collected from white pregnant women at delivery and their newborns (cord blood). Serum vitamin B12, folate, homocysteine as well as the relevant metabolic risk factors were measured.

#### 2.2.3.2 Results

The prevalence of low serum vitamin B12 (<191 ng/L) and folate (<4.6 µg/L) were 40% and 11%, respectively. Maternal B12 was inversely associated with offspring's Homeostasis Model Assessment 2-Insulin Resistance (HOMA-IR), triglycerides, homocysteine and positively with HDL-cholesterol after adjusting for age and BMI. In regression analysis, after adjusting for likely confounders, maternal B12 is independently associated with neonatal HDL-cholesterol and homocysteine but not triglycerides or HOMA-IR.

#### 2.2.3.3 Summary and conclusions

Our study shows that low B12 status is common in white women and is independently associated with adverse cord blood cholesterol.

### 2.2.4 A summary of Project 2

This project studied the intra individual variation in laboratory analytes due to ethnicity. Often reference ranges are not specific for ethnic variation and therefore when clinically assessing the test results one might overlook the natural variation that may occur due to ethic differences in the patient population.

Data presented in the studies demonstrates significant differences in result results between different ethnicities and therefore highlights the need for greater awareness of those

differences when interpreting clinical biochemistry results. These papers have been cited 53 times and through those citations have increased awareness of the significance of ethnic origin as a source of variability in laboratory results. More specifically the citations include reviews on insulin resistance, glycated haemoglobin and renal function and papers describing the wider impact on, for example, other ethnic minorities such as aboriginal Australians.

## 2.3 Project 3. Variation in laboratory results due to pre-existing conditions causing an elevation in interferants that affect common laboratory tests.

Many conditions will have an effect on the metabolism of many common laboratory tests. Some of those changes may be have appropriate sensitivity and specificity to be markers for the disease, others analytes will not and are seen as consequential observations. However significant rises in some analytes (as a non-specific consequence of the original condition) can cause an interference in the testing of others. This will cause a variation in results. My work in this area has investigated whether there is a significant effect of lipaemia on other commonly requested tests as this is often quoted as a reason for the not being able to issue a result. In addition I studied whether the observation of a raised prolactin seen in rheumatoid arthritis, is genuine or laboratory artefact. Finally, a series of case investigations into the often reported pseudo-pseudo hypercalcaemia in Waldenstroms macroglobulinaemia, and whether this was due to an interference in the calcium method by the high protein concentrations observed in that condition.

### 2.3.1 Study 1

This investigation was to quantify the effect that interference from lipaemia has on common laboratory tests. Lipaemia is reported to interfere in many routine assays. Many reagent suppliers provide information on the effect of lipaemia in their assays, but this is often vague, not quantified and may not be instrument-specific (81-82). Lipaemia, like haemolysis and icterus, causes chromophoric interference in photometric analyses due to high background readings, interference at the measured wavelength and light scattering caused by the interfering substance (82-83). The interference may be dose-dependent for some analytes but not for all (82). The interference from lipaemia can be minimised in a number of ways, including the use of a sample blank reading, kinetic analysis, changing the wavelength at which the reaction is read to one at which there is minimal absorbance from the interferant (84-85), and the use of commercial preparations that clear the lipid content from serum (86).

In the laboratory setting, staff use different methods – such as visual inspection, lipaemia index, serum indices and triglyceride concentration – to determine the degree of turbidity from lipaemia. These assessments, however, may be inaccurate as the degree of interference from lipaemia is method- and instrument-dependent (81-82). In this study, I aimed to evaluate the effects of lipaemia and LipoClear, a non-toxic polymer for serum lipid clearance, on 14

tests commonly analysed on the Bayer Opera analyser, prior to the introduction of Lipoclear into our routine laboratory repertoire.

#### 2.3.1.1 Materials and methods

A total of 14 analytes were measured in up to 44 serum samples with either no lipaemia or varying degrees of lipaemia (mean serum triglyceride 6.89 (range 0.58–284) mmol/L) using methods recommended for use by the instrument manufacturer. Twelve samples had serum triglyceride <2 mmol/L; 20 samples had a serum triglyceride >2 and <10 mmol/L, and 12 samples had a serum triglyceride >10 mmol/L. Each analyte was determined before and after treatment with Lipoclear (Phisec International, UK) on a Bayer Opera analyser (Bayer AG, Germany). A 0.5 mL serum sample was added to 0,1 mL LipoClear, mixed and left to stand for 5 min. The mixture was centrifuged and the supernatant analysed. Results were multiplied by 1.2 to correct for the initial dilution. Significance differences before and after the use of LipoClear were calculated using the paired t-test and Wilcoxon matched pairs for parametric and non-parametric data.

#### 2.3.1.2 Results

Table 2.3 shows that with the exception of alanine transaminase (ALT), amylase and bicarbonate, significant differences in the other analyte values before and after treatment with Lipoclear were seen using standard statistical techniques. When analytical CV was taken into account, only phosphate, total protein, cholesterol and triglyceride showed significant analytical change.

| Analyte | Analytical CV | 2.8x CV | % Difference | Significance |
|---|---|---|---|---|
| Urea | 2.5 | 7.0 | +4.4 | Not significant |
| Creatinine | 2.6 | 7.3 | +2.1 | Not significant |
| Albumin | 1.3 | 3.6 | -2.4 | Not significant |
| Alkaline Phosphatase | 4.9 | 13.7 | -1.0 | Not significant |
| Alanine Transaminase | 3.0 | 8.4 | 0 | Not significant |
| Amylase | 3.5 | 9.8 | 0 | Not significant |
| Phosphate | 1.6 | 4.5 | -9.5 | Significant |
| Glucose | 2.6 | 7.3 | +3.3 | Not significant |
| Bicarbonate | 4.6 | 12.9 | +4.8 | Not significant |
| Total protein | 2.3 | 5.6 | -14.5 | Significant |
| Total bilirubin | 3.7 | 10.4 | +4.5 | Not significant |

| | | | | |
|---|---|---|---|---|
| Calcium | 2.1 | 5.9 | -3.9 | Not significant |
| Triglyceride | 1.2 | 3.4 | -74 | Significant |
| Cholesterol | 2.5 | 7.0 | -54 | Significant |

Table 2.3 Comparison of analyte coefficients of variation with % difference in analyte result before and after lipid extraction

#### 2.3.1.3 Summary and significance

This study showed that most methodologies used on the Bayer Opera appeared to be subject to statistically significant interference from lipaemia when evaluated by standard statistical methods, but these do not consider the analytical imprecision of assays. When the analytical CV was taken into account, most of the differences failed to achieve critical significance. Only phosphate, protein, cholesterol and triglyceride values remained critically different after the addition of LipoClear.

These findings support a previous study of LipoClear (86) and were expected as Lipoclear, a non-ionic polymer, precipitates lipoproteins and phospholipids. Lipaemia did not critically affect measurement of other analytes, probably because the Bayer Opera performs an initial blank reading at the start of the reaction, supporting previous reports recommending the use of serum blanks in minimising lipaemic interference (81-82). However, these results are at variance with the manufacturer's method sheets, which indicate lipaemia interference with ALT, amylase, glucose, bicarbonate and calcium methods.

I concluded, LipoClear does reduce lipaemia but most methodologies are often sufficiently robust to avoid interference from lipaemia. Therefore, I recommend that individual laboratories quantify interference from lipaemia w for their specific methods and instruments, as the interference could be analyser- and/or reagent-specific. Only if there is significant interference should the use of lipid clearing agents be considered.

### 2.3.2 Study 2

It has been suggested that prolactin may have a pathogenic role in rheumatoid arthritis (RA), since in vitro studies have shown that prolactin enhances inflammatory responses (87-88). Indeed, increased serum prolactin concentrations have been reported in patients with RA (89-90). Furthermore, dopamine agonists, which suppress pituitary secretion of prolactin, may be a

useful adjunct to treatment in patients with RA (91). Prolactin circulates in several different molecular forms, predominantly monomeric prolactin but also as small but variable amounts of big prolactin and "big, big prolactin' (macroprolactin). The binding of prolactin to an immunoglobulin forms macroprolactin. Since macroprolactin is less physiologically active and is less effectively cleared than free unbound prolactin, the total concentration of serum prolactin increases. Depending on the immunoassay used, macroprolactin may account for 4-5% of cases of hyperprolactinaemia (92) and has also been reported in normoprolactinaemic samples (93).

Systemic lupus erythematosus (SLE), an autoimmune disease with an increased prevalence of serum autoantibodies, is associated with macroprolactinaemia (94). RA is also associated with an increased frequency of circulating antibodies and macroprolactin has been reported in patients with RA (95-96). It is therefore possible that macroprolactin could account for the hyperprolactinaemia observed in RA.

#### 2.3.2.1 Material and methods

Sixty women with RA and 31 women with osteoarthritis, who served as controls, were studied. A blood sample for serum prolactin was collected at least 2h after awakening, between 09.30 and 12.00h. Polyethylene glycol (PEG) was used to precipitate and remove big prolactin and macroprolactin from serum samples. Serum prolactin was therefore measured before (total prolactin) and after (free or monomeric prolactin) precipitation with PEG 6000 (97-98) using the Architect prolactin assay (Abbott Laboratories, Diagnostics Division, IL, USA).

#### 2.3.2.2 Statistical analysis

Data were normally distributed. Unpaired and paired Student's t tests were therefore used to assess differences in variables between groups and within groups respectively. Results are expressed as mean (S.D). Pearson’s linear correlation was used to measure the significance of association between variables.

| | RA | Controls | P |
|---|---|---|---|
| Number of subjects | 60 | 31 | |
| Age (yr) | 56.7 (12.9) | 53.1 (16.1) | 0.287 |
| ESR (mm/hr) | 27.7 | - | - |

| | | | |
|---|---|---|---|
| Total prolactin (mU/l) | 225.6 (104.6) | 175.0 (68.5) | 0.0387 |
| Free prolactin (mU/l) | 201.6 (95.4) | 154.0 (60.9) | 0.0127 |

Table 2.4 Clinical and biomedical results of women with RA arthritis and their controls. Data are mean (S.D).

#### 2.3.2.3 Results

Demographic, clinical and biochemical data are shown in Table 2.4. No subjects had hyperprolactinaemia or macroprolactinaemia. Serum concentrations of total and free (monomeric) prolactin were higher ($P<0.05$) in women with RA (225.6 (104.6) and 201.6 (95.4) mu/l respectively than in controls 175.0 (68.5) and 154.0 (60.9) mU/ respectively). Although serum prolactin fell following precipitation in women with RA and controls, this was not significant ($P=0.1936$ and $P = 0.2877$ respectively). In women with RA, there were no correlations between ESR and total prolactin ($r= 0.1163$; $P = 0.3762$) or free prolactin ($r=0.07725$; $P = 0.5574$), but ESR inversely correlated with the percentage of free prolactin of the total prolactin ($r=-0.3278$; $P=0.01.06$).

#### 2.3.2.4 Summary and significance

Although no subjects in this study had hyperprolactinaemia, I report higher serum prolactin concentrations in women with RA compared with controls. This result is consistent with other studies similarly reporting higher total prolactin concentrations in patients with RA than in controls, but differs from studies reporting similar or lower prolactin concentrations in patients with RA. In addition, I report for the first time higher free prolactin concentrations in subjects with RA. It is therefore unlikely that the different total prolactin concentrations reported in various studies are solely due to varying macroprolactin cross- reactivity in different prolactin assays.

Prolactin has a role in immunomodulation and it has been proposed that prolactin is a risk factor for the development of autoimmunity (95). However, it remains unclear whether the higher prolactin concentrations are the cause or consequence of RA.

I confirmed higher free prolactin concentrations in subjects with RA. I found no evidence to support the notion that low biological activity macroprolactin contributes to the elevated prolactin concentrations observed in RA.

### 2.3.3 Study 3

This was a case report on Pseudo-pseudohypercalcaemia and apparent primary hyperparathyroidism in Waldenström's macroglobulinaemia, which is a malignant disorder of B lymphocytes characterised by high serum concentrations of monoclonal IgM, a lymphoplasmacytic infiltrate of the bone marrow and an elevated serum viscosity. (99) Hypercalcaemia is unusual in Waldenström's macroglobulinaemia (100) but a characteristic feature of primary hyperparathyroidism. Although Waldenström's macroglobulinaemia is uncommon, primary hyperparathyroidism is relatively common. It is, therefore, possible that the two conditions may coexist. I describe a case of apparent primary hyperparathyroidism due to pseudo-pseudohypercalcaemia in association with Waldenström's macroglobulinaemia.

#### 2.3.3.1 Case report

A 74-year-old man was diagnosed with Waldenström's macroglobulinaemia when he presented in May 2002 with lethargy, weight loss and nasal bleeds, and an IgM K paraprotein of 31.5 g/l (IgM reference range: 0.5–2 g/l). He received chemotherapy courses and in February 2003 was referred to the urologists with prostatism. His rectal examination was normal; however, his serum prostate-specific antigen (PSA) was raised (reference range 0.1–6.5 g/1; age 270 years). A four-quadrant prostatic biopsy followed, but did not show malignant infiltration of the prostate. At an oncology review in April 2003, it was noticed that he had asymptomatic hypercalcaemia (serum adjusted calcium level 2.86 mmol/l, reference range 2.17-2.66 mmol/l, Arsenazo III dye binding method). A persistently elevated serum PSA of 52.2 g/l measured in May 2003 had instigated a second four-quadrant prostatic biopsy, which was inconclusive of a diagnosis of prostatic carcinoma. Serum calcium levels remained elevated at 2.89 mmol/l. The possibility of hypercalcaemia due to metastatic bone disease, although uncommon in patients with prostate cancer despite the high frequency of skeletal metastases, prompted an isotope bone scan; however, it did not show any metastatic lesions. The diagnosis of primary hyperparathyroidism was then considered on the basis of the presence of a consistently elevated serum adjusted calcium level of 3.03 mmol/l with inappropriately non-suppressed two serum parathyroid hormone results (4 and 44 pmol/l; reference range 1.6-6.9 pmol/l, DPC immulite) and a normal serum creatinine level of 94 umol/l. Subsequently, an ultrasound of the neck was performed, which suggested the presence of a parathyroid adenoma in the posterior inferior part of the left thyroid lobe. However, in July 2003, on metabolic review, the presence of a normal serum ionised calcium level of 1.10 mmol/l (reference range 1.11-1.3 mmol/l measured on a Radiometer ABL700) and a normal fasting

urine calcium excretion of 0.01.2 mmol/l GF (reference range Cae <0.045 mmol/l GF) has excluded genuine hypercalcaemia despite the repeatedly elevated serum adjusted Calcium level of 3.1 mmol/l.

The diagnosis of pseudo hypercalcaemia was made and was postulated to be due to an abnormal binding of calcium molecules to the IgM paraprotein (100). This was, however, excluded when gel filtration chromatography showed a similar pattern between the patient's sample and that of a normal control. Both samples showed two peaks of calcium: the first peak associated with albumin and the second peak represented the complexed calcium; however, none was associated with the IgM paraprotein 3, thus excluding calcium-paraprotein binding as a cause of the falsely elevated serum calcium levels. Additional investigation with measurement of serum calcium level on the same patient's sample (serum adjusted calcium level of 3.1 mmol/l) with the o-cresolphthalein complexone (CPC) method gave a calcium level of 2.19 mmol/l (reference range 2.1-2.55 mmol/l), and with atomic absorption spectrophotometry gave a calcium level of 2,4 mmol/l (reference range 2,2- 2.6 mmol/l). All these findings led to the conclusion of interference of the IgM paraprotein with the Arsenazo III dye binding calcium method.4

In November 2003, histological examination of repeat four quadrant prostatic biopsies in the presence of a PSA of 79 g/1 (reference range 0.1–6.5 g/l, age 270 years) had confirmed the diagnosis of an adenocarcinoma for which he is currently receiving treatment.

**2.3.3.2 Summary and significance**

The presence of pseudo-pseudo-hypercalcaemia in association with Waldenström's macroglobulinaemia had resulted in an erroneous provisional diagnosis of primary hyperparathyroidism. In patients with Waldenström's macroglobulinaemia, genuine hypercalcaemia is rare (99-100) and pseudo-hypercalcaemia is even rarer (101-102). Indeed, there were only two case reports of pseudo-hypercalcaemia in Waldenström's macroglobulinaemia due to either calcium binding to the IgM paraprotein or paraprotein interference in the calcium Arsenazo III method. The findings for this patient are consistent with the notion of IgM paraprotein interference with the calcium Arsenazo III method and the consequent pseudo-pseudo-hypercalcaemia. The mechanism of the interference is likely to be increased turbidity produced by paraprotein interaction with the acidic medium of the calcium Arsenazo III reagent and not with the alkaline CPC method (101).

It is important to identify patients with falsely elevated serum calcium levels in patients with paraproteinaemias to prevent unnecessary investigations, erroneous diagnosis and potentially inappropriate treatment.

### 2.3.4 A summary of Project 3

These studies clearly showed significant variation in laboratory results due to pre-existing conditions, with the pre-existing condition causing an elevation in interferants that affect common laboratory tests. I demonstrate that a possible source of variation may be due to the excess presence of an analyte which is secondary to the primary investigation, such as lipaemia or haemolysis affecting liver function tests. My work also deals with the solutions for dealing with this potential interferants. These papers have been cited 52 times in biochemical and disease specific journals, of interest is the citing of the papers in texts on effect on analytes not covered in my work (such as the effect of lipaemia on caeruloplasim), the relevance in and implications of investigation of the clinical conditions (rheumatoid arthritis and antipsychotic medication).

## 2.4 Additional studies

These two studies demonstrate how the high variability in CRP would render it ineffective in its incorporation into coronary heart disease risk assessment and the effect of inappropriate equipment selection for the diagnosis of hypoglycaemia and how it can lead to inaccurate results and therefore unnecessary investigations

### 2.4.1 High variability in CRP and the effectiveness of its incorporation into CHD risk assessment

CRP at the time of study was being proposed as a suitable marker for CVD screening and assessment. Proposed role were put forward that necessitated low non-pathophysiological variability, especially at the low concentrations observed in CVD.

Therefore for this study, I wanted to make an observation about the limited clinical utility of single hs-C-reactive protein (CRP) measurement in coronary heart disease (CHD) risk assessment (103). It has been suggested that CRP values can be incorporated into CHD risk assessment to improve prediction of CHD morbidity (104). Assessment of CHD risk usually involves opportunistic screening in primary care (105).

#### 2.4.1.1 Materials and Methods

I measured hs-CRP by immunoassay (DPC, Los Angeles CA, USA) in 434 subjects from primary care in whom a laboratory-based CHD risk score calculation had been requested.

#### 2.4.1.2 Results

113 (26.0%) subjects had hs-CRP concentrations above 6.0 mg/L detection limit of ordinary CRP assays) and 32 subjects (7.3% had CRP values >15 mg/L, suggesting an active inflammatory process probably due to a minor illness.

#### 2.4.1.3 Summary and significance

These results suggest that using CRP during opportunistic screening may falsely increase CHD risk score, leading to inappropriate patient management. Furthermore, inflammatory processes, indicated by elevated CRP, may be associated with lowering of total cholesterol and high density cholesterol concentrations (106), which may in turn influence calculated CHD risk scores. The results have shown that a significant number of patients who have undertaken

opportunistic screening may have minor or subclinical illness that could affect their CHD risk scores and adversely influence their management. Health care practitioners should be aware of the potential limitations of the use of CRP and opportunistic screening in CHD risk assessment.

### 2.4.2 Inappropriate assay selection causes increased variation in results and possible misinterpretation of results.

This paper was a series of three case histories demonstrating glucose meter hypoglycaemia. These highlighted the risk of using equipment with an inappropriate assay sensitivity to demonstrate low blood glucose.

This work was particularly relevant at the time because glucose meter technology was novel and was optimised for the intended use at high, or diabetic, glucose concentrations. The meters were no optimised for low concentrations, even though hypoglycaemia in diabetic patients is not uncommon.

The diagnosis of hypoglycaemia depends upon the demonstration of a low blood glucose concentration during a spontaneous symptomatic episode. Glucose monitoring devices may misdiagnose many healthy individuals with nonspecific symptoms as having hypoglycaemia.

#### 2.4.2.1 Case 1

A 31-year-old housewife gave a 20-year history of intermittent fatigue and `shakes' unrelated to food or exercise. She attributed these symptoms to hypoglycaemia when a nurse recognized the `symptoms of hypoglycaemia' and checked her capillary blood glucose on a meter, which gave a low reading (1.9mmol/L) (unconfirmed however by laboratory measurement) during inpatient investigations for abdominal pain 1 year previously.

Her GP then provided her with blood glucose reagent strips. She visually recorded her glucose being as low as 1±2mmol/L when symptomatic, and was therefore referred for assessment.
During an exercise test following an 18-h fast her plasma venous glucose rose from 4.3 to 4.7mmol/L and her insulin concentrations were low, at 525 pmol/L. During a prolonged (5-h) 75 g glucose load test her nadir plasma venous glucose was 5.5mmol/L at 240min, but she

experienced `shakes and tiredness' when her plasma venous glucose was 6.9mmol/L at 180min. I explain that hypoglycaemia was unlikely to explain her symptoms. I suggested that she should have a blood sample collected when symptomatic and taught her how to collect capillary blood into a fluoride capillary tube. She was unhappy with our explanation and never re-attended.

#### 2.4.2.2 Case 2

A 29-year-old female nurse gave a 3-year history of intermittent disorientation, sweats and lethargy unrelated to food or exercise. While at work, during two of these attacks finger-prick blood samples gave low glucose meter readings (2.0mmol/L). She was referred for further assessment. An exercise test following an 18-h fast and a 5-h 75 g glucose load test, during which she was asymptomatic, provided no evidence for hypoglycaemia. During one of her symptomatic episodes a glucose meter reading was 1.1mmol/L, but simultaneously collected laboratory capillary blood glucose was 5.5mmol/L. She accepted that her symptoms were not due to hypoglycaemia and that her previous readings were due either to faulty technique or to a faulty meter.

#### 2.4.2.3 Case 3

While at work during a period of personal stress, a 24-year-old female nurse felt dizzy with sweats, nausea and `shakes'. A nursing colleague using a glucose meter found her to have a reading of 1.6mmol/L. She was therefore given oral glucose, which led to a resolution of the symptoms after 30min. During a further similar attack 3 months later she was seen in an Accident and Emergency department with a label of hypoglycaemia and her glucose meter reading was confirmed as `low'. She was given oral glucose and discharged with recommendations that she be investigated further. An exercise test following an 18-h fast and a 5-h 75 g glucose load test, during which she was asymptomatic, provided no evidence for hypoglycaemia. I explained that her symptoms were unlikely to be due to hypoglycaemia. As her personal stress resolved she had no further symptomatic episodes.

#### 2.4.2.4 Summary and significance

Glucose meters are justifiably popular and have advanced the management of diabetic patients. However, glucose meters, and especially visually read glucose test strips, are unsuitable for the diagnosis of spontaneous hypoglycaemia in the domestic environment, as

many of the methods used may be unreliable in the hypoglycaemic range (107-108). Faulty meters, faulty blood strips, faulty patient preparation and faulty analytical technique may also add to the considerable risk of erroneous results (109). Glucose meters may however be useful in the clinical environment (e.g. in Accident and Emergency departments) as a rapid guide to the need for further blood collection (for confirmation and further investigation), followed immediately by the administration of glucose to relieve symptoms.

In the cases described here the subjects were initially `investigated' in three different hospitals, suggesting that `hypoglycaemia' was diagnosed using different types of meter. This suggests that any errors were probably due to faulty technique, but does not exclude instrument or reagent error. It also implies that the use of glucose meters to investigate vague symptoms for hypoglycaemia may be widespread. It is likely that the simplicity and ready availability of glucose meters has led to their inappropriate use in the investigation of nonspecific symptoms for the possibility of hypoglycaemia. An important adverse consequence of mislabelling healthy people with a disease is a reduction in their quality of life, and in some individuals the adoption of a sick role (`the worried well'). The mislabel of `hypoglycaemia' in these patients can however often be difficult to rectify, perhaps because patients who present with symptoms of hypoglycaemia may have abnormal psychological profiles (110).

The misdiagnosis of hypoglycaemia may also result in unnecessary and wasteful investigations, and may in part explain why 57% of samples received in a supra-regional laboratory for the investigation of hypoglycaemia were inappropriate, having glucose values of greater than 3.0mmol/L (111). We suggest that the use of glucose meters to diagnose spontaneous hypoglycaemia (without laboratory confirmation) is inappropriate, wasteful of resources and may adversely affect the health of the individual. Patients with symptoms suggestive of neuroglycopenia should be appropriately assessed and investigated. I recommend that training of personnel in the use of glucose meters should include the inappropriateness of their routine use in investigating spontaneous hypoglycaemia in non-diabetic adults.

It must be recognised that since publication glucose meter technology has developed considerably. It is now uncommon for meters not to be able to detected glucose concentrations in the hypoglycaemic range. However with the expansion of point of care testing in to the fields of virology and molecular testing, the same principles hold. What is the meter detecting and what is the analytical range it covers? Until that is elucidated for each point of care test, there is a risk of variability in test reporting that healthcare professionals need to be aware of.

### 2.4.3 A summary of Project 4

These two projects demonstrate how inappropriate test selection can cause increased variability in test results and therefore potentially inappropriate result interpretation. The two projects explore the inappropriate incorporation of CRP into cardiac risk stratification and the inappropriate use of glucose meters in the assessment of hypoglycaemia and the potential misinterpretation of results that could arise from their use. These projects have been cited 14 times in best practice guidelines on glucose meters and clinical guidelines for the assessment of hypoglycaemia, additionally in a paper on CRPs role in cardiovascular risk assessment.

## 2.5 References


1 Hallworth MJ, (2011). The '70% claim': what is the evidence base? *Annals of Clinical Biochemistry;*48(6): 487–488

2 Jones RG, Johnson OA, Batstone G (2014). Informatics and the Clinical Laboratory. *Clinical Biochemist Rev*iews; 35(3): 177–192.

3 Methven S, Perisanidou L, Nicholas J, Dawnat A, (2017). UK Renal Registry, 19th Annual report: Chapter 8, Biochemical Variable amongst UK Adult Dialysis patients in 2015. *Nephron*;137( 1):189-234

4 Castelli WP, 1988. Cholesterol and lipids in the risk of coronary heart disease-the Framingham Heart Study. *The Canadian journal of cardiology*; 4; 5A-10A

5 Cirillo M, Lombardi C, Luciano MG, Biliancio G, Anastasio P, DeSanto NG (2010). Estimation of GFR a comparison of new and established equations. *American Journal of Kidney Diseases*;56(4):802-4

6 Kavsa k P, Don- Wauchope AC, Hill SA, Worster A, (2016). Acceptable analytical variation may exceed high-sensitivity cardiac Tropinin I cut offs in early rule out and rule in AMI algorithms. *Clinical Chemistry*;62(6):887-9

7 Franzini C (1995). Relevance of analytical and biological variations to quality and interpretation of test results: examples of application to haematology. *Annali-Istituto Superiole di Sanita:* 31 (1):9-13

8 Lippi G, Guidi GC, Mattiuzzi C, Plebani M, 2006. Preanalytical variability: the dark side of the moon in laboratory testing. *Clinical Chemistry and Laboratory Medicine*; 44(4):358-365

9 Fraser CG, Petersen PH, Libeer JC, Ricos C, (1997). Proposals for setting generally acceptable quality goals solely based on biology. *Annals of Clinical Biochemistry*;34:8-12

10 Westgard JO, (1999). The need for a system of quality standards for modern quality management. *Scandinavian Journal of Clinical and Laboratory Investigation*; 59 (7) 483-486

11 Fraser CG (2001). Biological Variation: from principle to practice. *American Association for Clinical Chemistry*

12 Fraser CG (2004). Test result variation and the quality of evidence based clinical guidelines. *Clinica Chimica Acta*; 346 (1): 19-24

13 Dallas Jones GR (2016). Measurement uncertainty for clinical laboratories - a revision of the concept. *Clin Chem Lab Med*. 1;54(8):1303-7

14 Lundberg GD (1981). Acting on significant laboratory results. JAMA; 245(17):1762-1763

15 Plebani M, Laposata M, Lundberg GD (2011). Brain to brain cycle, 40 years after its introduction. *American Journal of Clinical Pathology*;136 (6): 829-833

16 Gama R, Anderson NR, Marks V (2000). 'Glucose Meter Hypoglycaemia' : often a non-disease. *Annals of Clinical Biochemistry*;37(5):731-732.

17 Carraro P, Zago T, Plebani M (2012). Exploring the initial steps of the testing process: frequency and nature of preanalytical errors. *Clinical Chemistry*; 58 (3): 638-642

18 Kemp, GM, Bird, CE, Barth, JH (2012). Short-term interventions on wards fail to reduce preanalytical errors: results of two prospective controlled trials. *Ann Clin Biochem* 2012; 49: 166–169

19 Frank H. Wians (2009). Clinical Laboratory Tests: Which, Why, and What Do The Results Mean? *Laboratory Medicine*. 40(2);105–113

20 Laposata M, Dighe A (2007). Pre-Pre and Post Post analytical error: High incidence patient safety hazards involving the clinical laboratory. *Clinical Chemistry and Laboratory Medicine*; 45(6): 712-719

21 Wians F. Clinical Laboratory Tests, (2015): Which, Why and What do the results mean? *Laboratory Medicine*; 40(2): 105-113

22 Wytze P. Oosterhuis, Hassan Bayat, David Armbruster, Abdurrahman Coskun, Kathleen P. FreemanI, Anders Kallner, David Koch, Finlay Mackenzie, Gabriel Migliarino, Matthias Orth, Sverre Sandber, Marit S. Sylte, Sten Westgard, Elvar Theodorsson (2017). The use of error and uncertainty methods in the medical laboratory. *Clinical Chemistry and Laboratory Medicine (CCLM).* 56 (2);209–219.

23 Lippi G (2009). Governance of preanalytical variability: Travelling the right path to the bright side of the moon. *Clinica Chimica Acta*;404(1):32-36

24 Ricos C, Alvarez V, Cava F, Garcia-Lario JV, Hernandez A, Jimenez CV, Minchinela J, Perich C, Simon M (1999). Current databases on biologic variation: pros, cons and progress. *Scandinavian Journal of Clinical Laboratory investigation*;59(7):491-500

25 ISO 15189. Medical laboratories - Requirements for quality and competence. Geneva, Switzerland: International Organization for Standardization; 2012

26 Gama R, Elfatih A, Anderson NR (2002). Ethnic differences in total and HDL Cholesterol concentrations: Caucasians compared with predominantly Punjabi Sikh Indo-Asians. *Annals of Clinical Biochemistry*;39:609-611.

27 Chatha K, Anderson NR, Gama R (2002). Ethnic variation in C-reactive protein: UK resident Indo-Asians compared with Caucasians. *Journal of Cardiovascular Risk*;99(3):139-141.

28 Adaikalakoteswari A, Vatish M, Lawson A, Wood C, Sivakumar K, McTernan PG, Webster C, Anderson N, Yajnik CS, Tripathi G, Saravanan P (2015). Low maternal vitamin B12 status is associated with lower cord blood HDL cholesterol in white Caucasians living in the UK. *Nutrients*;7(4):2401-14.

29 Ram S, Blumberg D, Netwon P, Anderson NR, Gama R (2004). Raised serum prolactin in rheumatoid arthritis: genuine or laboratory artefact? *Rheumatology;*43(10):1272-4.

30 Elfaith A, Anderson NR, Fahie-Wilson MN, Gama R (2007). Pseudo-pseudohypercalcaemia, apparent primary hyperparathyroidism and Waldenstrom's macroglobulinaemia. *Journal Clinical Pathology;60(4):436-7*

31 Elfatih A, Chatha K, Anderson NR, Gama R (2014). Limited clinical utility of high sensitivity plasma C-reactive protein assays. *Annals of Clinical Biochemistry*;39(5):534-537.

32 Anderson NR, Nicholas J, Holland MR, Gama R (2003). Effect of a protease inhibitor on in vitro stability of intact parathyroid hormone. *Annals of Clinical Biochemistry*;40(2):188-190.

33 Anderson NR, Gama R (2001). Stability of intact parathyroid hormone in blood samples. *Annals of Clinical Biochemistry*;38 (3):288-289.

34 Anderson NR, Chatha K, Holland MR, Gama R (2003). Effect of sample tube type and time to separation on in vitro stability of C-Reactive protein. *British Journal of Biomedical Science*;60(3):164-5.

35 Anderson NR, Chatha K, Holland MR, Gama R (2004). Interference caused by the contents of serum separator tubes in the Vitros CRP assay protein concentration between quantitation in serum with an without using serum separator tube. *Annals of Clinical Biochemistry*;41:171-2.

36 Anderson NR, Slim S, Gama R, Holland MR (2003). Lipaemia: An over-rated interference*? British Journal Biomedical Science*;60(3):141-3.

37 Locatelli, F., Cannata-Andía, J. B., Drüeke, T. B., Hörl, W. H., Fouque, D., Heimburger, O., & Ritz, E. (2002). Management of disturbances of calcium and phosphate metabolism in chronic renal insufficiency, with emphasis on the control of hyperphosphataemia. *Nephrology Dialysis Transplantation*, 17(5), 723-731.

38 Capuzzi, D. M., & Freeman, J. S. (2007). C-reactive protein and cardiovascular risk in the metabolic syndrome and type 2 diabetes: controversy and challenge. *Clinical Diabetes*, 25(1), 16-22.

39 Haq, I. U., Jackson, P. R., Yeo, W. W., & Ramsay, L. E. (1995). Sheffield risk and treatment table for cholesterol lowering for primary prevention of coronary heart disease. *The Lancet*, 346(8988), 1467-1471.

40 Compton C. Garbage in Garbage out (2018). The hidden reason laboratory test results may not be a reliable as they seem *The pathologist*.; 318, 1-16.

41 Ana-Maria Simundic, William A Bartlett, Callum G Fraser (2015). Biological variation: a still evolving facet of laboratory medicine. *Annals of Clinical Biochemistry* 52(2) 189-190

42 Evans T. Pathology Networks. Identification of 29 potential pathology networks, allowing for the transformation of pathology services into a series of networks across the country. NHS Improvement

43 Wood P.J (1992). The measurement of parathyroid hormone. *Annals of Clinical Biochemistry*; 29(1):11–21

44 Levin GE, Nisbet JA (1994). Stability of parathyroid hormone at room temperature. *Annals of Clinical Biochemistry*; 31(5):497–500

45 Walker KS, Seth J (2000). Stability of parathyroid hormone in blood from renal patients on haemodialysis. *Annals of Clinical Biochemistry*; 37(6):800–1

46 Newman DJ, Ashby JP (1988) Clinical and laboratory evaluation of a two site immuno-radiometric assay for intact parathyroid hormone. *Annals of Clinical Biochemistry*; 25(6):654—60

47 Pouliss M, Landis RC, Taylor KM (2001). Aprotinin: is it prothrombotic? *Perfusion; 16: 401–9*

48 Omar H, Chamberlin A, Walker W, Wood PJ(2001). Immulite 2000 parathyroid hormone assay: stability of parathyroid hormone in EDTA blood kept at room temperature for 48 h. *Annals of Clinical Biochemistry*; 38(5). 561–63

49 Karppi J, Akerman KK, Parviainen M (2000). Suitability of collection tubes with separator gels for collecting and storing blood samples for therapeutic drug monitoring. *Clinical Chemistry*; 38(4): 313-20

50 Walker KS, Seth J (2000), Stability of parathyroid hormone in blood from renal patients on haemodialysis. *Annals of Clinical Biochemistry*; 37(6): 800-1

51 Russell D, Henley R (1995). The stability of parathyroid hormone in blood and serum samples at 4$^{o}$C and at room temperature. *Annals of Clinical Biochemistry*; 32(2): 216-7

52 Liuzzo G, Biasucci LM, Gallimore JR (1994). The prognostic value of C-reactive protein and serum C-amyloid. A protein in severe unstable angina. *New England Journal of Medicine*; 331(7): 417-24

53 Morrow DA, Rifai N, Antman EM (1998). C-reactive protein is a potent predictor of mortality independently of, and in conjunction with troponin T in acute coronary syndromes. *American College of Cardiology*; 31: 1460-5

54 de Winter RJ, Bholasing R, Lijmer JG (1999). Independent prognostic value of C-reactive protein and troponin I in patients with unstable angina or non Q-wave myocardinal infarction. *Cardiovascular Reviews*; 42: 240-5

55 Haverkate F, Thompson SG, Pyke SD, Callimore JR, Pepys MB (1997). Production of C-reactive protein and risk of coronary events in stable and unstable angina. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. *Lancet*; 349(9050): 462-6

56 Rifai N, Ridker PM (2001). High sensitivity C-reactive protein: A novel and promising marker of coronary heart disease. *Clinical Chemistry*; 47(3): 403-11

57 Meier-Ewert HK, Ridker, PM, Rifai N, Price N, Dinges DF, Mullington JM (2001). Absence of diurnal variation of C-reactive protein concentration in healthy human subjects. *Clinical Chemistry*; 47(3); 426-30

58 Ockene IS, Matthews CE, Rifai N, Ridker PM, Reed G, Stanek E (2001). Variability and classification accuracy of serial high-sensitivity C-reactive protein measurement in healthy adults. *Clinical Chemistry*; 47(3): 444-50

59 Roberts WL, Moulton L, Law TC (2001). Evaluation of nine automated high-sensitivity C-reactive protein methods: implications for clinical and epidemiological applications. *Clinical Chemistry*; 47(2): 418-25

60 Karppi J, Akerman KK, Parviainen M (2000). Suitability of collection tubes with separator gels for collecting and storing blood samples for therapeutic drug monitoring. *Clinical Chemistry Laboratory Medicine*; 38(4): 313-20

61 Anderson NR, Gama R (2001). Stability of intact parathyroid hormone in blood samples. *Annals of Clinical Biochemistry*; 38(3): 288

62 Anderson NR, Chatha K, Holland MR, Gama R (2003). Effect of sample tube type and time to separation on in vitro stability of C-reactive protein *British Journal of Biomedical Science*; 60 (3): 164-5

63 Chang C, Lu J, Chien T, Kao J, Lin J, Shih P (2003). Interface caused by the contents of serum separator tubes in the Vitros CRP assay. *Annals of Clinical Biochemistry*; 40: 249-51

64 Karppi J, Akerman KK, Parviainen M (2000). Suitability of collection tubes with separator gels for collecting and storing blood samples for therapeutic drug monitoring. *Clinical Chemistry Laboratory Medicine*; 38(4): 313-20

65 Anderson NR, Gama R (2001). Stability of intact parathyroid hormone in blood samples. *Annals of Clinical Biochemistry*; 38(3): 288

66 Rifai N, Ridker PM (2001). High-sensitivity C reactive protein: a novel and promising marker of coronary heart disease. *Clinical Chemistry*; 47(3): 403-11

67 Bhopal R (2002). Epidemic of cardiovascular disease in South Asians. British Medical Journal; 324: 625-6

68 McKeigue PM (1992), Coronary heart disease in Indians. Pakistanis and Bangladeshis: aetiology and possibilities for prevention, *British* Heart Journal; 67: 341-2

69 Bhopal R, Unwin N, White M. Yallop J, Walker L, Alberti KGMM (1999). Heterogeneity of coronary heart disease in Indian, Pakistani, Bangladeshi and European ongin populations: cross sectional study. *British Heart Journal*; 319(7204): 215-20

70 Anderson KM, Odell PM. Wilson PWF, Kannel WB (1991). Cardiovascular disease risk profiles. *American Heart Journal*; 121(1): 293-8

71 Legato MJ (2000). Dyslipidaemia, gender, and the role of high density lipoprotein cholesterol: implications for therapy. *American Journal of Cardiology*; 86 (12): 15-18

72 Enas EA. Garg A. Davidson MA, Nair VM, Huet BA. Yusuf S, (1996), Coronary heart disease and its risk factors in first generation immigrant Asian Indians to the United States of America. *Indian Heart Journal*; 48(4): 343-53

73 Shanmugasundaram KR, Suresh S, Misra KP, Jayakrishnan TK (1983). Plasma lipoprotein cholesterol in India in healthy persons and those with coronary heart disease. *Atherosclerosis*; 46(1): 129-35

74 McKeigue PM, Shah B, Marmot MG (1991). Relation of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in South Asians. *Lancet*;337: 382-5

75 McKeigue PM, Shah B, Marmot MG (1991). Relation of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in Indo-Asians. *Lancet*; 337:382-385.

76 Mather HM, Keen H (1985). The Southall diabetes survey; prevalence of known diabetes in Asians and Europeans. *British Medical Journal*; 291:1081-1084

77 Hughes LO, Cruickshank JK, Wright J, Raftery EB (1989). Disturbances of insulin in British Asian sand white men surviving myocardial infarction. *British Medical Journal*; 299:537-541.

78 Saravanan P., Yajnik C.S (2010). Role of maternal vitamin B12 on the metabolic health of the offspring: A contributor to the diabetes epidemic? *British Journal of Vascular Disease*;10:109–114.

79 Guerra-Shinohara E.M., Morita O.E., Peres S., Pagliusi R.A., Sampaio Neto L.F., D'Almeida V., Irazusta S.P., Allen R.H., Stabler S.P (2004). Low ratio of S-adenosylmethionine to S-adenosylhomocysteine is associated with vitamin deficiency in Brazilian pregnant women and newborns. *American Journal of Clinical Nuitrition;*;80:1312–1321.

80 Saravanan P, Wood C, Anderson N (2010). B12 deficiency is more common than folate deficiency in early pregnancy: Do we need to consider B12 fortification? *Diabetologia.*;53:S151–S152.

81 Glick MR, Ryder KW, (1987) Analytical systems ranked by freedom - from interferences. *Clinical Chemistry*; 33: 1453–8.

82 Glick MR, Ryder KW, Jackson SA (1986). Graphical comparisons of CV interferences in clinical chemistry instrumentation. *Clinical Chemistry*; 32: 470–5.

83 Brady J, OLeay N (1994). Interference due to lipaemia in routine photometric analysis – survey of an underrated problem. *Annals of Clinical Biochemistry*; 31: 281—8.

84 O'Leary N, Pembrooke A, Duggan PE, (1992) Improving accuracy of phosphatase glucose oxidase procedure for glucose determinations on discrete analysers. *Clinical Chemistry*; 38: 298–302.

85 Randall AC, Garcia-Webb P. Beilby JP, (1990) Interference by haemolysis, icterus and lipaemia in the assays on the Beckman Synchron CX5 and methods for correction. *Annals of Clinical Biochemistry*; 27: 345-52

86 Rogers LC. Smith FA. Evaluation of LipoClear, (1989), a non-ionic polymer system, for the removal of interfering lipaemia. *Clinical Chemistry*; 35: 1103.

87 Neidhart M. Prolactin in autoimmune diseases (1998). *Proceedings of the Society of Experimental Biological Medicine;*217:408-19.

88 Matera L, Mori M, Geuna M, Buttiglieri S, Palaestro G (2000). Prolactin in autoimmunity and antitumor disease defence. *Journal of Neuroimmunology*;109:47-55.

89 Walker SE, Jacobson JD (2000). Roles of prolactin and gonadotropin releasing hormone in rheumatic diseases. *Rheumatology Disease Clinical North America;*26:713–36.

90 Seriolo B, Ferretti V, Sulli A, Fasciolo D, Cutolo M (2002). Serum prolactin concentrations in male patients with rheumatoid arthritis (2002). *Annals of New York Academy of Science;*966:258-62.

91 McMurray RW (2001). Bromocriptine in rheumatic and autoimmune disease. *Arthritis Rheum*atology;31:21-32.

92 Miyai K, Ichihara K., Kondo K., Mori S (1986). Asymptomatic hyperprolactinaemia and prolactinoma in general population-mass screening by paired assays of serum prolactins. *Clinical Endocrinology*;25:549–54.

93 Ram S, Anderson NR, Elfatih A. Gama R, (2003). Do normo-prolactinaemic samples contain macroprolactin? In: Martin SM, ed*. Proceedings of the ACB National Meeting. London: Association of Clinical Biochemists*; 80.

94 Liederman S, Garfinkiel M. Daimilano S (2002). Prolactin and IgG-prolactin complex levels in patients with rheumatic arthritis. *Annals New York Academy of Science;*966:252-7.

95 Leanos A, Pascoe D, Fraga A, Blanco-Favela F (1998). Anti-prolactin autoantibodies in systemic lupus erythematosus patients with associated hyperprolactinemia*. Lupus*;7:398-403.

96 Berczi I, Cosby H. Hunter T, Baragar F. McNeilly AS, Friesen HG (1987). Decreased bioactivity of circulating prolactin in patients with rheumatoid arthritis. *British Journal of Rhuematology;*26:433–6.

97 Nagy E, Chalmers IM, Baragar FD, Friesen HG, Berczi I (1991). Prolactin deficiency in rheumatoid arthritis. *Journal of Rheumatology*; 18:1662-8.

98 Smith TP, Suliman AM, Fahie-Wilson MN, McKenna T.J (2002). Gross variability in the detection of prolactin in sera containing big big prolactin (macroprolactin) by commercial immunoassays. *Journal of Clinical Endocrinology and Metabolism;* 87:5410–15.

99 Dimopoulos MA, Panayiotidis, P, Kouropoulos LA, (2000). Waldenshrom's macroglobulincemica: features, complications and management. *Journal of Clinical Oncology;*18:21.4-26.

100 2. Dimopoulos MA, Galani E, Matsouka C (1999). Waldenshrom's macroglobulincemia. *Hematol ogy Oncology Clinical North America;* 13:1351-66.

101 Side L, Fahie-Wilson MN, Mills MJ (1995). Proalcaemia due to calcium binding IgM paraprotein in Wealdenstrom's macroglobulinoemia. *Journal of Clinical Pathology*;48:961-2

102 Rhys J, Oleesky D, Issa B (1997). Pseudohypercalcaemia in two patients with IgM paraproteinaemia. *Annals of Clinical Biochemistry*;34:694-6.

103 Campbell B, Badrick T, Flatman R, Kanowski D (2002). Limited utility of high sensitivity plasma C-reactive protein assays. *Annals of Clinical Biochemistry ;*39: 85-8.

104 Ridker PM (2001). High sensitivity C-reactive protein: potential adjunct for global risk assessment in the primary prevention of cardiovascular disease. *Circulation* ;103: 1813-27.

105 The National Service framework of coronary heart disease (2000). *Modern standards and service models.* London: Department of Health.

106 Sammalkorpi KT, Valtonen VV, Maury CP (1990). Lipoproteins and acute phase response during acute infection. Interrelationships between C-reactive protein and serum amyloid A and lipoproteins. *Annals of Medicine*; 22: 397-401

107 Trajanoski Z, Brunner GA, Gfrerer RJ, Wach P, Pieber TR (1996). Accuracy of home glucose meters during hypoglycaemia. *Diabetes Care*; 19: 1412-5

108 Bergman M, Migake C, Felig P (1989). Misdiagnosis of gestational diabetes and hypoglycaemia using portable blood glucose meters. *Archives of Internal Medicine*; 149: 2602-3

109 Southgate HJ, Marks V (1986). Measurement of hypoglycaemia by reflocheck. *Practical Diabetes ;*3: 206-7

110 Lefebvre PJ, Andreani D, Marks V, Creutzfeldt W (1988). Statement on postprandial hypoglycaemia. *Diabetes Care* ; 11: 439-40

111 Marks V, Teale JD (1996). Investigation of hypoglycaemia. *Clinical Endocrinology;* 44: 133-6

# Appendix A. Publications relevant to this thesis

Anderson NR, Nicholas J, Holland MR, Gama R (2003). Effect of a protease inhibitor on in vitro stability of intact parathyroid hormone. Annals of Clinical Biochemistry;40(2):188-190.

Anderson NR, Gama R (2001). Stability of intact parathyroid hormone in blood samples. Annals of Clinical Biochemistry;38 (3):288-289.

Anderson NR, Chatha K, Holland MR, Gama R (2003). Effect of sample tube type and time to separation on in vitro stability of C-Reactive protein. British Journal of Biomedical Science;60(3):164-5.

Anderson NR, Chatha K, Holland MR, Gama R (2004). Interference caused by the contents of serum separator tubes in the Vitros CRP assayeactive protein concentration between quantitation in serum with and without using serum separator tube. Annals of Clinical Biochemistry; 41:171-2.

Gama R, Elfatih A, Anderson NR (2002). Ethnic differences in total and HDL Cholesterol concentrations: Caucasians compared with predominantly Punjabi Sikh Indo-Asians. *Annals of Clinical Biochemistry*;39:609-611.

Chatha K, Anderson NR, Gama R (2002). Ethnic variation in C-reactive protein: UK resident Indo-Asians compared with Caucasians. *Journal of Cardiovascular Risk;*99(3):139-141.

Adaikalakoteswari A, Vatish M, Lawson A, Wood C, Sivakumar K, McTernan PG, Webster C, Anderson N, Yajnik CS, Tripathi G, Saravanan P (2015). Low maternal vitamin B12 status is associated with lower cord blood HDL cholesterol in white Caucasians living in the UK. *Nutrients;*7(4):2401-14.

Anderson NR, Slim S, Gama R, Holland MR (2003). Lipaemia: An over-rated interference? British Journal of Biomedical Science;60(3):141-3.

Ram S, Blumberg D, Netwon P, Anderson NR, Gama R (2004). Raised serum prolactin in rheumatoid arthritis: genuine or laboratory artefact? *Rheumatology*:43(10):1272-4.

Elfaith A, Anderson NR, Fahie-Wilson MN, Gama R (2007). Pseudo-pseudohypercalcaemia, apparent primary hyperparathyroidism and Waldenstrom's macroglobulinaemia. *Journal Clinical Pathology;*60(4):436-7

Elfatih A, Chatha K, Anderson NR, Gama R (2014). Limited clinical utility of high sensitivity plasma C-reactive protein assays. *Annals of Clinical Biochemistry;*39(5):534-537.

Gama R, Anderson NR, Marks V (2000). 'Glucose Meter Hypoglycaemia': often a non-disease. *Annals of Clinical Biochemistry*;37(5):731-732.

*Short Report*

# Effect of a protease inhibitor on *in vitro* stability of intact parathyroid hormone

NR Anderson[1], J Nicholas[2], MR Holland[1] and R Gama[1]

**Addresses**
[1]Department of Clinical Chemistry
[2]Department of Renal Medicine
New Cross Hospital
Wolverhampton, WV10 0PQ, UK

**Correspondence**
Mr N Anderson
E-mail: neil.anderson@rwh-tr.nhs.uk

## Abstract

**Background** We investigated whether increased protease activity explains the increased *in vitro* degradation of intact parathyroid hormone (iPTH) observed in serum when compared to EDTA plasma.

**Methods** Pre-dialysis blood samples for iPTH were taken from 11 patients with chronic renal failure and collected into plain glass tubes, tubes containing 200 KIU/mL aprotinin (a protease inhibitor) and EDTA tubes. All sample aliquots were separated at 20 min, 1 h, 2 h, 4 h, 8 h and 24 h post collection.

**Results** Over 24 h, iPTH concentrations remained unchanged in EDTA tubes. iPTH concentrations were significantly lower in both plain tubes ($P < 0.01$) and aprotinin tubes ($P < 0.001$) at 24 h when compared to the baseline sample (20 min). At 24 h, iPTH concentrations in EDTA tubes were higher than in plain tubes ($P < 0.001$) and aprotinin tubes ($P < 0.01$). The addition of aprotinin to plain tubes significantly reduced the degradation of iPTH ($P < 0.05$) at 24 h.

**Conclusion** Aprotinin significantly reduces the *in vitro* degradation of iPTH in plain tubes at 24 h from 24·7% to 9·6%. We suggest that increased protease activity contributes to the decline in serum iPTH over time. As this is observed in serum and not plasma it suggests that the increased protease activity may be due to the clotting process.

*Ann Clin Biochem* 2003; **40**: 188–190

## Introduction

Stability studies on intact parathyroid hormone (iPTH) have reported that any *in vitro* degradation of iPTH in unseparated blood samples may be sample tube- and time-dependent.[1,2] The greater degradation of iPTH observed in serum compared to EDTA plasma[3] remains unexplained. Increased *in vitro* degradation of iPTH has been reported in patients who have high concentrations of circulating proteases.[4] Additionally, it has been demonstrated that the addition of protease inhibitors may arrest the *in vitro* decline in iPTH over 24 h,[2] but this may be assay- and sample population-dependent.

Thrombin plays a significant *in vivo* role in platelet aggregation as part of the clotting process. Initiation of platelet aggregation requires protease-dependent binding of thrombin to platelet thrombin receptors.[5] This increase in protease activity may be responsible for the *in vitro* degradation of PTH. We therefore investigated whether the addition of aprotinin (a potent protease inhibitor) influenced the *in vitro* stability of serum iPTH in unseparated samples from patients with chronic renal disease.

## Materials and methods

We venesected 11 patients with chronic renal failure prior to them receiving dialysis. Blood samples were collected into 10-mL plain glass tubes (Z10/GN, LIP, Equipment and Services Ltd, Shipley, UK), with and without 2000 KIU of aprotinin (Bayer AG, Germany), and 2·7-mL EDTA tubes (Sarstead Monovet 2·7 mL KE, Aktiengesellschaft & Co, Germany).

Samples were then transported to the laboratory on ice and remained unseparated at room temperature (17–23°C) until centrifugation. At 20 min (baseline), 1 h, 2 h, 4 h, 8 h and 24 h, a 1-mL aliquot was taken from each tube and centrifuged. The resultant supernatant was frozen at −20°C until analysis, which was

carried out in one batch using the DPC Immulite iPTH assay (intra-assay coefficient of variation 4·8) (LKPH, Diagnostic Products Corporation, Los Angeles, CA, USA) immediately following thawing of the aliquot. Following logarithmic transformation, the data were normally distributed. Statistical analysis of the transformed data was by parametric repeat measures ANOVA with Tukey–Kramer post-test comparison. Results are given as pre-transformed data expressed as median (95% confidence intervals).

## Results

Concentrations of iPTH in each tube type over time are shown in Table 1. The iPTH concentration in the plain sample separated at 24 h was significantly lower than in those separated at baseline ($P<0·001$), 1 h ($P<0·001$), 2 h ($P<0·001$), 4 h ($P<0·001$) and 8 h ($P<0·001$). There were no significant differences between any other time points. Between baseline and 24 h the decline in iPTH in plain tubes was 24·7%. The iPTH value in the aprotinin sample separated at 24 h was significantly lower than those separated at baseline ($P<0·01$), 1 h ($P<0·001$), 2 h ($P<0·001$), 4 h ($P<0·001$) and 8 h ($P<0·001$). There were no significant differences between any other time points. Between baseline and 24 h the decline in iPTH in aprotinin tubes was 9·6%. There were no differences in iPTH concentrations between the EDTA samples separated at any time point.

iPTH concentration was significantly lower in plain tubes when compared with aprotinin tubes ($P<0·05$) at 24 h. iPTH concentration was also significantly lower in plain tubes ($P<0·001$) and aprotinin tubes ($P<0·01$) when compared with EDTA tubes at 24 h. There were no other significant between-tube differences at any other time points.

## Discussion

The unchanged plasma iPTH concentrations in EDTA tubes, left unseparated for up to 24 h in pre-dialysis samples collected from patients with chronic renal failure, confirms that iPTH is stable for up to 24 h in unseparated EDTA samples.[3] Although we found no significant difference in baseline results between any of the tubes, Omar *et al.* reported significantly higher baseline iPTH values in EDTA tubes compared with plain tubes in samples collected from patients attending a renal stone clinic.[6] These differences could be explained by the different mixture of PTH fragments in each of the different sample populations, which may have different *in vitro* stability.

In this study, serum iPTH concentrations were stable when left unseparated for up to 8 h in plain and aprotinin-containing tubes. The addition of aprotinin to plain tubes significantly reduced the decline in iPTH at 24 h. However, there was still significant difference in iPTH in aprotinin tubes when compared with EDTA tubes at 24 h. Levin and Nesbit[2] using the Nichols Institute, Allegro iPTH assay, found that addition of two protease inhibitors (aprotinin and leupeptin) eliminated the decline in iPTH that we observed at 24 h when compared to EDTA tubes. It is possible that the combination of protease inhibitors confers greater protection against *in vitro* iPTH instability. However, as both studies used different iPTH assays and different study populations, this is not clear.

Our findings suggest that increased protease activity could explain, in part, the decline in serum iPTH in blood samples left unseparated for greater than 8 h. Aprotinin is a serine protease inhibitor and probably acts *in vivo* by inhibiting the thrombin-induced proteolytic activation of platelets.[5] This mechanism may explain the *in vitro* observation that iPTH decline is observed in serum and not in EDTA plasma, suggesting that the increased protease activity may be due to the clotting process.

We conclude that plain and EDTA tubes are appropriate sample tubes for collection of iPTH in patients with renal disease, if left unseparated for up to 8 h. Increased protease activity may solely or partially

Table 1. *Intact parathyroid hormone concentrations (pmol/L) in different sample tubes against time*

| Time (h) | Number of samples | Tube type | | |
|---|---|---|---|---|
| | | Plain | Aprotinin | EDTA |
| 0·3 | 11 | 46·5 (20·2–62·4) | 43·7 (19·1–61·8) | 51·3 (20·7–65·6) |
| 1 | 11 | 51·7 (20·6–61·4) | 45·8 (20·4–60·0) | 45·8 (20·6–62·5) |
| 2 | 11 | 47·3 (19·9–61·7) | 48·9 (23·3–62·5) | 52·4 (21·6–63·4) |
| 4 | 11 | 46·6 (20·9–65·3) | 47·4 (20·7–64·2) | 50·8 (20·9–67·1) |
| 8 | 11 | 46·6 (20·2–62·9) | 45·0 (20·6–64·4) | 53·3 (22·2–67·8) |
| 24 | 11 | 30·7†† (13·9–48·5) | 34·3†, c* (16·9–56·4) | 49·2, a***, b** (19·9–63·9) |

Results are median (95% confidence limits). Within-tubes, the only significant differences observed compared with baseline (0·3 h) were at 24 h: †$P<0·01$, ††$P<0·001$. Between-tubes, the only significant differences were observed at 24 h: a, EDTA compared to plain tube, ***$P<0·001$; b, EDTA compared to aprotinin tube, **$P<0·01$; c, aprotinin tube compared to plain tube, *$P<0·05$.

contribute to the decline in serum iPTH in blood samples left unseparated for longer than 8 h.

**Acknowledgements**

We acknowledge the help of the staff in the Renal Dialysis Unit, New Cross Hospital, Wolverhampton, and the support of DPC UK who provided the reagents for this study.

## References

1 Wood PJ. The measurement of parathyroid hormone. *Ann Clin Biochem* 1992; **29**: 11–21

2 Levin GE, Nisbet JA. Stability of parathyroid hormone at room temperature. *Ann Clin Biochem* 1994; **31**: 497–500

3 Walker KS, Seth J. Stability of parathyroid hormone in blood from renal patients on haemodialysis. *Ann Clin Biochem* 2000; **37**: 800–1

4 Newman DJ, Ashby JP. Clinical and laboratory evaluation of a two-site immuno-radiometric assay for intact parathyroid hormone. *Ann Clin Biochem* 1988; **25**: 654–60

5 Pouliss M, Landis RC, Taylor KM. Aprotinin: is it prothrombotic? *Perfusion* 2001; **16**: 401–9

6 Omar H, Chamberlin A, Walker V, Wood PJ. Immulite 2000 parathyroid hormone assay: stability of parathyroid hormone in EDTA blood kept at room temperature for 48 h. *Ann Clin Biochem* 2001; **38**: 561–63

*Accepted for publication 26 September 2002*

College of Cardiology Committee for the redefinition of myocardial infarction. *J Am Coll Cardiol* 2000; **36**: 959–69

## Stability of intact parathyroid hormone in blood samples

We wish to comment on the recent study of Walker and Seth, which reports that intact parathyroid hormone (iPTH) in blood collected into ethylenediaminetetraacetic acid (EDTA) tubes is more stable at room temperature than in blood collected into 'plain' tubes.[1]

We feel, however, that a clear distinction needs to be made between plain serum tubes and gel serum tubes (as used by Walker and Seth). We have compared the stability of iPTH in blood samples taken into gel tubes (Sarstead Monovet 4.7 mL, Z GEL) and plain glass tubes (Lip Z10/GN).

Blood was drawn from nine patients with chronic renal failure before commencement of haemodialysis. Immediately after venesection, samples were split between the plain and the gel tubes. Samples were then separated after 20 min at room temperature and serum frozen at $-20^{\circ}C$ until assayed for iPTH using the DPC Intact PTH assay on the Immulite automated immunoassay analyser.

Intact PTH values were 13% higher ($P<0{\cdot}01$, two-tailed paired *t*-test) in plain serum tubes [mean (SD): 7·6 (2.57) pmol/L] than in gel serum tubes [6·7 (2·19) pmol/L] (*see* Fig. 1). We suggest

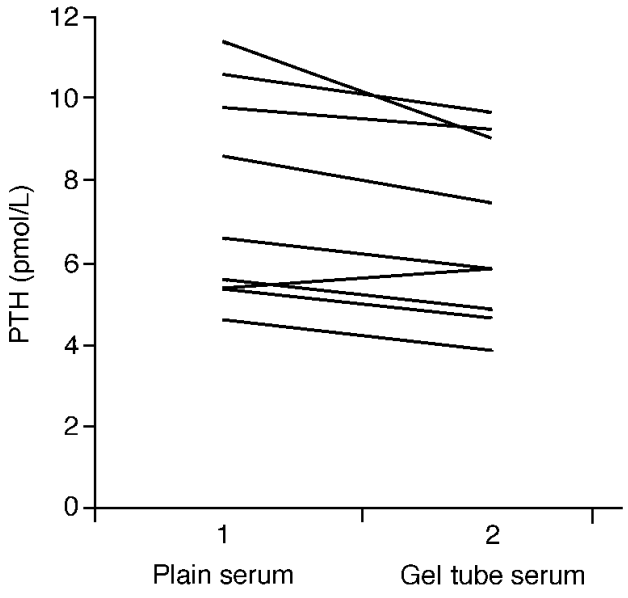



FIGURE 1. *Stability of intact PTH at room temperature. Blood was collected in plain serum tubes (1) and gel tubes (2). All samples separated by 20 min and serum stored at −20°C until analysis.*

that these differences in iPTH values are attributable to the gel barrier or clot activator or both, especially since these results are consistent with other studies reporting a similar 'gel effect' on other immunoassays.[2]

DPC also recommended that blood samples when collected into EDTA should fill the tube. Otherwise, the EDTA concentration in the sample will proportionately increase, affecting the Immulite substrate alkaline phosphatase reaction and thus lowering the iPTH result.

These factors may help explain both the variation between EDTA plasma and (gel) serum as reported[1] and the differences between this study, which reports that 'serum' iPTH collected in gel tubes in unstable at 3 h,[1] and other studies which have reported that iPTH collected into plain serum tubes is stable for up to 8 h.[3,4]

In conclusion, we suggest that differences by Walker and Seth may be due to the gel separator rather than to the differences between serum and EDTA plasma. Further evaluation regarding iPTH stability in whole blood is, therefore, required.

N R ANDERSON
R GAMA
Department of Clinical Chemistry,
New Cross Hospital,
Wolverhampton WV10 0QP, UK

### REFERENCES

1 Walker KS, Seth J. Stability of parathyroid hormone in blood from renal patients on haemodialysis. *Ann Clin Biochem* 2000; **37**: 800–1
2 Karppi J, Akerman KK, Parviainen M. Suitability of collection tubes with separator gels for collecting and storing blood samples for therapeutic drug monitoring. *Clin Chem Lab Med* 2000;**38**:313–20
3 Russell D, Henley R. The stability of parathyroid hormone in blood and serum samples at 4°C and at room temperature. *Ann Clin Biochem* 1995; **32**: 216–17
4 Michelangeli VP, Heyma PO, Colman PG, Ebeling PR. Evaluation of a new, rapid and automated immunochemiluminometric assay for the measurement of serum intact parathyroid hormone. *Ann Clin Biochem* 1997; **34**: 97–103

### Authors' reply

Anderson and Gama correctly point out the need to consider the potential effects of separator gel in blood collection tubes on immunoassay results and raise the question as to whether this could account for the difference we observed between EDTA plasma and serum

samples. We would first acknowledge that our short report did not make the type of serum tube absolutely clear and we apologize for this. We used white-capped Sarstedt S Monovettes (cat no. 03.1397), which contain clotting activator coated on plastic barrier beads but no gel, as opposed to the brown-top tubes in the same range which contain in addition a gel separator. The differences we observed are not, therefore, due to the presence of gel, or to the plastic beads, as the samples were aliquoted into clean plastic tubes within 15 min of collection, but could indeed be due to the clotting activator. We can confirm, in the light of DPC's recent recommendation, that EDTA blood tubes should be filled completely to avoid assay interference from high concentrations of EDTA, and that we used fully filled EDTA blood tubes.

This recommendation from DPC, and Anderson and Gama's observations, further emphasize the need for laboratory and clinical staff to be aware of the subtle (or in some cases not so subtle) effects that blood collection systems can have on PTH results. There is clearly no substitute for checking the validity of the particular combination of blood collection system and PTH assay method used locally.

Kay Walker
John Seth
Department of Clinical Biochemistry,
Royal Infirmary of Edinburgh,
Lauriston Place,
Edinburgh EH3 9YW, UK

## Effect of inflammatory response on trace element and vitamin status

The statements by Sattar *et al.*[1] on the nadirs of serum zinc and selenium and their significance require clarification.

In support of our earlier cited references on the 6 $\mu$mol/L lower limit for zinc, we report that Halstead and Smith[2] measured plasma zinc in 324 patients in 12 groups, with a variety of disorders/conditions, and found minimum concentrations ranging from 5·8 $\mu$mol/L (liver disease) to 11·9 $\mu$mol/L (tuberculosis). Although Sattar *et al.*[1] correctly report that the lowest mean plasma zinc observed by Fraser *et al.*[3] in eight cardiac patients was $4{\cdot}9 \pm 1{\cdot}7$ $\mu$mol/L, at least part of this low value can be explained by the haemodilution that occurs as part of the coronary bypass procedure and which complicates interpretation of changes in concentrations of metalloproteins following cardiac surgery. Their observed nadir in plasma zinc, which occurred 9 h post-operatively, was accompanied by a decrease in plasma copper from 18·3 to 13·3 $\mu$mol/L. This decrease must have involved haemodilution since caeruloplasmin (copper) is a positive acute phase reactant. The plasma copper level returned to the pre-operative value 48 h after surgery, at which time the mean plasma zinc was $6{\cdot}8 + 1{\cdot}6$ $\mu$mol/L and the C-reactive protein concentration had peaked. 72 h post-operatively, the mean zinc and copper concentrations were respectively 8·2 $\mu$mol/L and 20·3 $\mu$mol/L. These observations vindicate our 6·0 $\mu$mol/L lower limit for plasma zinc. Moreover, such transient changes would be avoided by using our 3–5 day interval between monitoring trace elements.[4]

Sattar *et al.*[1] are correct; nadirs of 0·2 $\mu$mol/L for plasma selenium have been observed in intensive care unit (ICU) patients. Hawker *et al.*[5] reported plasma selenium concentrations for 175 ICU patients and found that only five (2·9%) had concentrations $\leqslant 0{\cdot}3$ $\mu$mol/L, and of these three (1·7%) had levels below 0·2 $\mu$mol/L. The lowest concentrations were seen in patients with surgical sepsis and with decompensated alcoholic liver disease. Forceville *et al.*[6] also observed plasma selenium levels as low as 0·2 $\mu$mol/L (and up to 0·72 $\mu$mol/L) in ICU patients with severe sepsis and septic shock. Thus, our limit of about 0·3 $\mu$mol/L appears at first sight to be too stringent. However, 0·2 $\mu$mol/L is typical of selenium deficiency in Keshan disease-endemic areas ($< 0{\cdot}25$ $\mu$mol/L),[7] and its finding in ICU patients is, to us and to others,[5,6] a cause for concern.

Forceville *et al.*[6] gave selenium supplementation to their ICU patients with systemic immune response syndrome and observed decreases in plasma selenium in non-surviving patients and slight increases in plasma selenium in surviving patients. They concluded that a prolonged decrease in plasma selenium could explain the three-fold increase in morbidity and mortality in patients with low plasma selenium ($\leqslant 0{\cdot}7$ $\mu$mol/L) on admission, compared with other ICU patients. Although Hawker *et al.*[5] concluded that true selenium deficiency was unlikely in their ICU patients, they referred specifically to their patients' first week in ICU. They went on to say that '. . . true selenium deficiency may develop within the first few weeks of an acute illness' and '. . . in the absence of supplementation, up to half of body selenium stores could be

endocarditis in a child. *Pediatr Infect Dis J* 2002; **21**: 984–6.
13 Barker KF, O'Driscoll JC, Bhargava A. *Staphylococcus lugdunensis*. *J Clin Pathol* 1991; **44**: 873–4.
14 Wachtler M, Strobel E, Koch U *et al*. Native mitral valve endocarditis caused by *Staphylococcus lugdunensis* in a 22-year-old woman. *Infection* 2002; **30**: 251–3.
15 Kaabia N, Seauarda D, Lena G, Drancourt M. Molecular identification of *Staphylococcus lugdunensis* in a patient with meningitis. *J Clin Microbiol* 2002; **40**: 1824–5.
16 Weinstein MP, Mirrett S, Van Pett L *et al*. Clinical importance of identifying coagulase-negative staphylococci isolated from blood cultures: evaluation of microscan rapid and dried overnight Gram-positive panels versus a conventional reference method. *J Clin Microbiol* 1998; **36**: 2089–92.
17 Patel R, Piper KE, Rouse MS *et al*. Frequency of isolation of *Staphylococcus lugdunensis* among staphylococcal isolates causing endocarditis. *J Clin Microbiol* 2000; **38**: 4262–3.
18 Leung MJ, Nuttall N, Mezur M *et al*. Case of *Staphylococcus schleiferi* endocarditis and a simple scheme to identify clumping factor positive staphylococci. *J Clin Microbiol* 1999; **37**: 3353–6.
19 Schritzler N, Meilicke R, Conrads G, Frank D, Haase G. *Staphylococcus lugdunensis*: report of a case of peritonitis and an easy-to-perform screening strategy. *J Clin Microbiol* 1998; **36**: 812–3.
20 Tee WSN, Yen Soh S, Lin R, Loo LH. *Staphylococcus lugdunensis* carrying the *mecA* gene causes catheter-associated bloodstream infection in premature neonate. *J Clin Microbiol* 2003; **41**: 519–20.

# Effect of sample tube type and time to separation on *in vitro* levels of C-reactive protein

N. R. ANDERSON, K. CHATHA, M. R. HOLLAND and R. GAMA
*Department of Clinical Chemistry, New Cross Hospital, Wolverhampton WV10 0QP, West Midlands, UK*

Highly sensitive C-reactive protein (hs-CRP) is assuming increased importance in the evaluation of patients with coronary artery disease (CAD). It is a prognostic indicator in acute coronary syndromes[1-3] and a predictor of future coronary events in those with and without overt CAD.[4,5] As small changes in hs-CRP concentration potentially have considerable clinical impact, all aspects of the analytical process need to be evaluated.

Highly sensitive CRP has undergone numerous evaluations on pre-analytical and analytical variability. In healthy individuals, hs-CRP exhibits no diurnal variation,[6] and hs-CRP shows little variation over a 12-month period, with a similar stability of measurement to total cholesterol.[7] Analytical variability of hs-CRP assays has been assessed recently by Roberts and colleagues, who reported coefficients of variation (CV) <10% at 0.15 mg/L.[8]

It is widely recognised that sample collection tube type may affect analyte concentrations. In particular, 'gel' tube effects have been described for anticonvulsant drugs[9] and intact parathyroid hormone.[10] However, the effect of sample collection tube type on hs-CRP concentration has not been studied.

This study aims to assess the stability of serum and plasma hs-CRP in different collection tubes over a 24-hour period, using the DPC Immulite highly sensitive CRP assay.

Blood samples were collected from seven patients with chronic renal failure (on dialysis) into a 10 mL plain glass tube (tube A; Lip Z10/GN), a 2.7 mL EDTA tube (tube B [2.7 ml KE]; Sarstedt Monovet, Germany) and a 4.2 mL gel tube (tube C [4.2 ml,Z GEL]; Sarstedt Monovet).

Samples were transported to the laboratory on ice and remained unseparated at room temperature (17–23°C) until centrifuged. At 20 min (baseline), 1, 2, 4, 8 and 24 h, a 1 mL sample was taken from each tube and centrifuged. The resultant supernatant was frozen at –20°C until analysis, which was carried out in one batch using a DPC Immulite high sensitivity CRP assay (LKCR, Diagnostic Products, Los Angeles; intra-assay CV = 4.1%), immediately after thawing. Statistical analysis was by repeat measures ANOVA and, where significant, was followed by Tukey-Kramer post-test comparison.

Results of tube-type effect and time on hs-CRP values are shown in Table 1. Significant differences were observed in samples separated at 0.3 h (ANOVA $P=0.0192$), and hs-CRP values were significantly higher ($P<0.0.5$) in gel tubes than in plain and EDTA tubes. Otherwise, there were no significant between-tube differences in hs-CRP concentration.

*Correspondence to: Mr N Anderson*
*Email: neil.anderson@rwh-tr.nhs.uk*

**Table 1.** Mean CRP concentration (mg/L) for each sample tube against time

| | Time | | | | | |
|---|---|---|---|---|---|---|
| | 0.3 h | 1 h | 2 h | 4 h | 8 h | 24 h |
| Tube A (Plain) | 17.0(17.3) | 16.6(16.8) | 17.2(17.9) | 17.4(17.7) | 17.7(18.1) | 17.8(17.6) |
| | A/C⁺ | | | | | |
| Tube B (EDTA) | 16.8(17.1) | 17.2(17.8) | 16.7(16.4) | 16.0(16.1) | 15.9(16.6) | 17.2(18.2) |
| | B/C⁺ | | | | | |
| Tube C (Gel) | 18.5(18.3) | 17.6(17.7) | 17.4(17.3) | 17.5(17.9) | 16.6(16.3)* | 16.7(16.9)* |

Results are mean (Standard Deviation)
Within sample tube C (Gel), compared to 0.3 h * = $P<0.05$
Between sample tubes. A/C ⁺ = $P<0.05$, B/C ⁺ = $P<0.05$

Within the tube type, there were no significant differences in hs-CRP concentration in samples taken into plain tubes and EDTA tubes over the 24-h period. In gel tubes, however, there were significant differences over time (*ANOVA* $P=0.03$), with hs-CRP concentration significantly lower ($P<0.05$) in the samples separated at 8 and 24 h, compared with that in the basal sample separated at 0.3 h. In gel tubes, hs-CRP concentration decreased by 9.7% over 24 h.

These results demonstrate a significant decline in hs-CRP concentration over time, when blood is collected into a gel tube, and support previous studies reporting the effect of gel tubes on other analytes such as therapeutic drugs[9] and intact parathyroid hormone.[10]

Several studies have proposed that hs-CRP be used as a prognostic indicator in acute coronary syndromes[1-3] or a predictor of future coronary events.[4,5] However, the significant decline in hs-CRP in gel collection tubes observed over 24 h in this study could lead to the misclassification of patients in samples left unseparated for eight or more hours. This may be particularly important for samples collected in the community for evaluation of CAD risk.

In conclusion, results from this study suggest that caution should be exercised in interpreting hs-CRP results when the sample is collected into a gel tube, as we report a significant decline in hs-CRP concentration over time. □

*The authors acknowledge the assistance of DPC for providing the reagents used in this study.*

## References


1 Liuzzo G, Biasucci LM, Gallimore JR *et al.* The prognostic value of C-reactive protein and serum amyloid A protein in severe unstable angina. *N Engl J Med* 1994; **331**: 417–24.

2 Morrow DA, Rifai N, Antman EM *et al.* C-reactive protein is a potent predictor of mortality independently of and in combination with troponin T in acute coronary syndromes: aTIMI 11A substudy. *J Am Coll Cardiol* 1998; **31**: 1460–5.

3 de Winter RJ, Bholasing R, Lijmer JG *et al.* Independent prognostic value of C-reactive protein and troponin I in patients with unstable angina or non Q-wave myocardial infarction. *Cardiovasc Res* 1999; **42**: 240–5.

4 Haverkate F, Thompson SG, Pyke SD, Gallimore JR, Pepys MB. Production of C-reactive protein and risk of coronary events in stable and unstable angina. European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. *Lancet* 1997; **349**: 462–6.

5 Rifai N, Ridker PM. High sensitivity C-reactive protein: A novel and promising marker of coronary heart disease. *Clin Chem* 2001; **47**: 403–11.

6 Meier-Ewert HK, Ridker PM, Rifai N, Price N, Dinges DF, Mullington JM. Absence of diurnal variation of C-reactive protein concentrations in healthy human subjects. *Clin Chem* 2001; **47**: 426–30.

7 Ockene IS, Matthews CE, Rifai N, Ridker PM, Reed G, Stanek E. Variability and classification accuracy of serial high-sensitivity C-reactive protein measurement in healthy adults. *Clin Chem* 2001; **47**: 444–50.

8 Roberts WL, Moulton L, Law TC *et al.* Evaluation of nine automated high-sensitivity C-reactive protein methods: implications for clinical and epidemiological applications. Part 2. *Clin Chem* 2001; **47**: 418–25.

9 Karppi J, Akerman KK, Parviainen M. Suitability of collection tubes with separator gels for collecting and storing blood samples for therapeutic drug monitoring. *Clin Chem Lab Med* 2000; **38**: 313–20.

10 Anderson NR, Gama R. Stability of intact parathyroid hormone in blood samples. *Ann Clin Biochem* 2001; **38**: 288.


# Enzyme-linked immunosorbent assay for $\beta_2$-glycoprotein I quantitation: the importance of variability in the plastic support

F. LIN[a], C. FEIGHERY[b], J. GUERIN [a], H. O'BYRNE [a] and J. JACKSON[c]

[a]*Department of Biological Sciences, Dublin Institute of Technology, Dublin;* [b]*Department of Immunology, St James's Hospital, Dublin; and* [c]*Trinity College, Dublin, Republic of Ireland*

$\beta_2$-glycoprotein I (β2GPI) is a poorly understood plasma protein that is thought to be an important autoantigen in the antiphospholipid syndrome. In addition, β2GPI may be a key player in the phospholipid-dependent coagulation pathway, and in the clearance of liposomes, phosphatidylserine-expressing cells and foreign particles.[1-3] It shows a high propensity to bind negatively charged surfaces, including phospholipids and irradiated plastic plates.[10] Binding to phospholipids is accompanied *in vitro* by the inhibition of intrinsic coagulation pathway activation,[4] ADP-induced platelet aggregation,[5] prothrombinase activity of activated platelets,[6] as well as the anticoagulant activity of activated protein C.[11]

As relatively little is known about the pathophysiological role of β2GPI, a reliable assay to determine plasma levels would further our understanding of its functions in health and disease. However, non-specific binding of β2GPI to plastic surfaces could significantly decrease the accuracy and reliability of an enzyme-linked immunosorbent assay (ELISA) designed to measure it.

In order to study the effect of non-specific binding of β2GPI to plastic and to determine a normal range for β2GPI concentration in sera from female and male subjects, this study establishes a capture ELISA based on the protocol designed by McNally *et al.*[12] Nunc A/S (Kamstrup, Roskilde, Denmark) provides two commonly used sets of γ-irradiated plates; one certified for consistency in adsorption of protein, the other uncertified. Here, we test six batches of γ-irradiated Nunc Maxisorp 96-well flat-bottomed polystyrene ELISA plates for reproducibility; one of which was certified by the manufacturer for homogeneity in adsorption of IgG.

Briefly, the ELISA plates were incubated overnight at 4°C with 100 μL mouse monoclonal anti-human β2GPI (Chemicon International Inc., Temecula, CA, USA) at a

*Correspondence to: Dr John Jackson*
*Email: jjackso2@tcd.ie*

References

1 Ferns GAA, O'Dowd D, Wark G, Collins N. Molecular diagnostics in routine practice: quality issues and application to complex disease. *Ann Clin Biochem* 2003; **40**: 309–12
2 Muller CR. Quality control in mutation analysis: the European Molecular Genetics Quality Network. *Eur J Pediatr* 2001; **160**: 464–7
3 Dequeker E, Ramsden S, Grody WW, Stenzel TT, Barton D. Quality control in molecular genetic testing. *Nat Rev Genet* 2001; **2**: 717–24

## Author's reply

I am pleased that our article has raised awareness of the external quality assurance (EQA) schemes for genetic testing. The article was principally targeted at biochemistry laboratories rather than specialist genetics laboratories, and, although it was not our intention to provide a comprehensive list of EQA schemes available for tests of monogenic disorders, we had hoped to provide sufficient information for those engaged in genetic testing to source an appropriate scheme. We note that several of the schemes listed under the European Molecular Genetics Quality Network (EMQN) in our article are now also available from UK NEQAS. However, for many of the tests that may be particularly useful for our colleagues in specialist biochemistry laboratories, EQA schemes are not available nationally, if indeed they are available at all.

With respect to error rates, the figure quoted by us was taken directly from an article from the EMQN and was clearly a matter of concern to its members. Presumably they have used these data as a stimulus to improve the quality of genetic testing by encouraging participation in EQA schemes. Whilst the figures quoted by Dr Ramsden for a more recent estimation of error rates are an improvement, one wonders what the error rates are for tests that currently do not have established national schemes.

**Gordon Ferns**
Clinical and Molecular Diagnostics Laboratory
Royal Surrey County Hospital
Guildford GU2 7XX, UK

## Interference caused by the contents of serum separator tubes in the Vitros CRP assay

We wish to comment on the study of Chang *et al.*[1] who report higher serum C-reactive protein (CRP) concentrations, using the Vitros CRP assay, in samples collected into gel separator tubes when compared with plain tubes. We also have studied the stability of CRP, using the DPC Immulite high-sensitivity CRP assay, in samples collected into plain glass tubes, EDTA tubes gel tubes and separated at 20 min (baseline) and 1 h, 2 h, 8 h and 24 h.[2]

In samples separated at baseline, measured CRP concentrations were significantly higher in gel tubes than in plain and EDTA tubes. Otherwise, there were no significant differences between tube CRP concentrations at any time point. In gel tubes, CRP concentrations were significantly lower at 8 h and 24 h when compared with the sample separated at baseline. There were no significant differences in CRP concentrations in samples taken into plain or EDTA tubes over 24 h.

These findings of higher baseline serum CRP concentrations in gel tubes compared with plain tubes are consistent with those of Chang *et al.*[1] We additionally report a significant decline in CRP concentrations in gel tubes by 8 h post sampling.[2] These findings of discrepant results from different collection tubes are similar to previous studies reporting the effect of gel tubes on other analytes, including therapeutic drugs[3] and intact parathyroid hormone.[4]

Serum CRP concentrations, often in the 'conventional' reference range, have an important role in the evaluation of cardiovascular risk and are of prognostic value in acute coronary syndromes.[5] We therefore disagree with the assertion of Chang *et al.* that between-tube differences in CRP concentrations are only of importance at high CRP concentrations and are unlikely to cause a problem in interpretation at lower CRP concentrations.

We suggest that these between-tube differences are more likely to influence clinical interpretation of CRP at lower concentrations especially where CRP is used as an aid to assess cardiovascular risk evaluation and prognosis of acute coronary syndromes.

We report an intra-assay coefficient of variation of 3.36% at a CRP concentration of 10.7 mg/L, using the Immulite high-sensitivity CRP assay. However, there is an additional 8.8–10.1% variation (according to the sample tube type) and a 10.7% decline in CRP concentrations if gel tubes are used for sample collection. Such variability due to sample tube type could lead to potential misclassification of patients under evaluation for cardiovascular risk and acute coronary syndrome.

**NR Anderson**
**K Chatha**
**MR Holland**
**R Gama**
Department of Clinical Chemistry
New Cross Hospital

References

1 Chang C, Lu J, Chien T, Kao J, Lin J, Shih P, *et al.* Interference caused by the contents of serum separator tubes in the Vitros CRP assay. *Ann Clin Biochem* 2003; **40**: 249–51

2 Anderson NR, Chatha K, Holland MR, Gama R. Effect of sample tube type and time to separation on *in vitro* stability of C-reactive protein *Br J Biomed Sci* 2003; **60**: 164–5
3 Karppi J, Akerman KK, Parviainen M. Suitability of collection tubes with separator gels for collecting and storing blood samples for therapeutic drug monitoring. *Clin Chem Lab Med* 2000; **38**: 313–20
4 Anderson NR, Gama R. Stability of intact parathyroid hormone in blood samples. *Ann Clin Biochem* 2001; **38**: 288
5 Rifai N, Ridker PM. High-sensitivity C reactive protein: a novel and promising marker of coronary heart disease. *Clin Chem* 2001; **47**: 403–11

## Routine transferrin saturation measurements in liver clinic patients increases detection of hereditary haemochromatosis

The use of serum transferrin saturation (TFS), as described by Poullis *et al.*[1] as an initial screening test for genetic haemochromatosis (HH) in patients with a high risk of the disease confirms our own findings.[2] Over a number of years we have developed a targeted screening approach to detect HH in patients with early liver disease. The rationale for our programme is the initial screening with TFS for the iron overload phenotype in patients with a biochemical marker of liver disease [raised serum alanine aminotransferase (ALT) found in any routine biochemistry request] followed by genetic testing to identify the C282Y genotype in those patients so identified.

Over a 29-month period, all routine samples sent to the laboratory for liver function tests that had a raised ALT (greater than 50 iu/L) were selected for TFS. If the TFS was greater than 55%, a further fasting TFS was requested. If the fasting TFS was greater than 50%, patients were invited to attend the haematology clinic for genetic testing and assessment of iron overload. From 8770 samples with raised ALT, 376 samples (5%) had a raised TFS. A fasting TFS was requested on all of these, 194 samples being obtained. Seventy patients had a raised fasting TFS and genetic testing was performed on 66 of these. There were 32 HFE C282Y homozygotes detected. Over the same period of time the laboratory had referred on clinical grounds alone, 68 gene tests from all other clinicians in the hospital. Only seven C282Y homozygotes were detected by these traditional clinical methods. Thus, the diagnostic yield from our study was 32/66 (48%) and 7/68 (10%) from traditional methods.

Targeted screening using a raised ALT and subsequent TFS is an effective method for detecting C282Y homozygotes who are likely to be affected by the disease, and is five times more successful at detecting C282Y homozygotes than traditional clinical methods. Our study also suggests that patients so identified are at an early stage of the disease before irreversible liver damaged has occurred. This is in contrast to the findings of Poullis *et al.* who screened patients attending a liver clinic (presumably at a later stage of the diagnostic process) and found that seven out of the 18 patients with mutations had established liver cirrhosis.

**David Lloyd**
**Manju Bhavnani**
**John Marples**
Departments of Clinical Biochemistry and Haematology
Royal Albert Infirmary
Wigan WN1 2NN, UK

### References

1 Poullis A, Moodie L, Ang L, Finlayson CJ, Levin GE, Maxwell JD. Routine transferring saturation measurement in liver patients increases detection of hereditary haemochromatosis. *Ann Clin Biochem* 2003; **40**: 521–7
2 Bhavnani M, Lloyd D, Bhattacharyya A, Marples J, Elton P, Wormwood L. Screening for gentic haemochromatosis in blood samples with raised alanine aminotransferase. *Gut* 2000; **46**: 707–10

## Authors' reply

The comments by Lloyd *et al.* support our recommendation that transferrin saturation (TFS) be used as a first-line test in subjects with abnormal liver function presenting for investigation.[1] The differences in diagnostic yield and stage of liver disease they report can be explained by the differences in study designs.

Our 60-month prospective study was pragmatic, based on typical UK clinical practice. Non-fasting TFS was used because most clinic patients are in the non-fasting state when seen and when phlebotomy is requested. In our study, elevated concentrations in non-fasting TFS were not repeated but when selecting those for HFE genotyping we used a lower cut-off limit than the Bhavnani study (45% *v* 60%).[2] In the Bhavnani group, the diagnostic yield in those with an elevated non-fasting TFS was 32/376 (8.5%). By repeating a fasting TFS this improved the yield to 32/70 (46%), which suggests that this could be a useful step in the phenotypic screening of at risk individuals.

The strengths of our study are the completeness of data (41% of subjects with elevated TFS were not genotyped in the study by Bhavnani *et al.*). The histology data is also unique to our study. Lloyd *et al.* speculate that they detected subjects at an earlier stage of liver disease, however this speculation was based on their finding that C282Y homozygotes only had midly

# Ethnic differences in total and HDL cholesterol concentrations: Caucasians compared with predominantly Punjabi Sikh Indo-Asians

R Gama, AB Elfatih and NR Anderson

**Address**
Department of Clinical Chemistry
New Cross Hospital
Wolverhampton
West Midlands WV10 0QP, UK

**Correspondence**
Dr R Gama
E-mail: dr.gama@rwh-tr.nhs.uk

## Abstract

**Background** In comparison with Caucasians, Indo-Asians resident in the UK have similar total cholesterol but lower HDL cholesterol (HDLC) concentrations. It is however possible that cardiovascular risk factors may vary between culturally different Indo-Asians.

**Methods** We present data on 223 Indo-Asians (129 men, 94 women) and 787 Caucasians (421 men, 366 women) in whom a laboratory-based coronary heart disease (CHD) risk score calculation had been requested.

**Results** Total cholesterol concentrations were similar in Indo-Asians and Caucasians. HDLC concentrations were higher ($P<0.001$) in Caucasians [1·4 (1·3–1·4) mmol/L; median (95% confidence intervals)] than in Indo-Asians [1·2 (1·2–1·3) mmol/L]. Indo-Asian women [1·2 (1·2–1·3) mmol/L], Indo-Asian men [1·2 (1·2–1·3) mmol/L] and Caucasian men [1·2 (1·2–1·3) mmol/L] had similar HDLC concentrations but these were all lower ($P<0.001$) than those in Caucasian women [1·4 (1·3–1·4) mmol/L].

**Conclusion** We confirm low HDLC concentrations in Indo-Asians, but propose that this is solely due to low HDLC concentrations in Indo-Asian women. Since Indo-Asians in Wolverhampton are predominantly Punjabi Sikhs, we suggest that the difference between this study and previous reports may be due to heterogeneity of CHD risk factors within culturally diverse Indo-Asians.

*Ann Clin Biochem* 2002; **39**: 609–611

An increased prevalence of coronary heart disease (CHD) in Indo-Asians resident in the UK is unexplained by differences in cardiovascular risk factors of hypercholesterolaemia, hypertension, smoking or a family history of CHD.[1,2] Although diabetes mellitus is more common in Indo-Asians, it does not fully explain the increased prevalence of CHD.[1,2]

Low serum HDL cholesterol (HDLC) concentrations, widely reported in Indo-Asians, may contribute to the increased prevalence of CHD amongst this group.[1,2] However, it has been suggested that there may be a difference in cardiovascular risk factors between culturally different Indo-Asian groups.[3] We therefore compared serum total cholesterol (TC) and HDLC in Indo-Asian and Caucasian subjects residing in Wolverhampton, UK, for whom a laboratory CHD risk assessment had been requested.

## Subjects and methods

We provide general practitioners (GPs) with a laboratory-based 10-year CHD risk score calculation based on the Framingham equation.[4] This calculation uses the individual's age, sex, systolic blood pressure (BP), TC concentration, HDLC concentration, and the presence or absence of smoking, diabetes mellitus and left ventricular hypertrophy. Clinical information was collected from a sticker fixed on the pathology request form, which also requested data on ethnicity (Caucasian, Afro/Caribbean, Asian, Chinese and 'other'). Patients were selected at the discretion of their GPs. Since most laboratory CHD risk assessment requests in Indo-Asians were on subjects aged 40–60 years, we restricted the study to this age group. Other exclusion

criteria included overt macrovascular disease and lipid-lowering medication.

Serum TC and HDLC concentrations were measured automatically (DiaSys Diagnostic Systems GmbH & Co, Holzheim, Germany and Bio-Stat Diagnostic Systems, Stockport, UK, respectively). Inter-assay and intra-assay coefficients of variation for TC were respectively 1·22% and 0·61% and for HDLC were respectively 1·93% and 0·75%.

Data were non-parametric. The Mann–Whitney *U*-test was therefore used to assess the differences between Indo-Asians and Caucasians. The Kruskal–Wallis test with Dunn post-test comparison was then used to assess the differences between ethnic male and female subgroups. Results are expressed as medians with 95% confidence intervals in parentheses.

Table 1. *Cardiovascular risk factors in Indo-Asians and Caucasians*

| | Caucasian | Indo-Asian | *P* value |
|---|---|---|---|
| *n* | 787 | 223 | |
| Age (years) | 52 (51·2–52·0) | 49 (48·9–50·5) | <0·0001 |
| Diabetics (%) | 16·9 | 24·8 | 0·3055 |
| Current smokers (%) | 40·7 | 12·2 | <0·0001 |
| Total cholesterol (mmol/L) | 5·8 (5·7–5·9) | 5·7 (5·6–5·9) | 0·0769 |
| HDL cholesterol (mmol/L) | 1·4 (1·3–1·4) | 1·3 (1·2–1·3) | <0·0001 |
| Total:HDL cholesterol ratio | 4·6 (4·5–4·7) | 4·8 (4·6–5·0) | 0·004 |
| Systolic BP (mmHg) | 140 (139–142) | 136 (133–138) | 0·002 |
| 10-year CHD risk (%) | 10·6 (10·1–11·2) | 9·4 (8·6–10·2) | 0·0714 |

Results are medians (95% confidence intervals).

## Results

Complete demographic, clinical and biochemical data sets were collected in 94 Indo-Asian women, 129 Indo-Asian men, 366 Caucasian women and 421 Caucasian men. Results are shown in Tables 1 and 2. Serum TC concentration was similar in Caucasians and Indo-Asians but Indo-Asians had a lower average HDLC concentration ($P<0·001$) and therefore a higher TC:HDLC ratio ($P<0·005$). Men and women in the two ethnic groups had similar TC concentrations. Indo-Asian women, Indo-Asian men and Caucasian men had similar HDLC concentrations but these were on average lower ($P<0·001$) than those in Caucasian women. The TC:HDLC ratio was similar within each gender group. Indo-Asian and Caucasian men had a higher TC:HDLC ratio than did Caucasian women ($P<0·001$) but a similar TC:HDLC ratio compared to Indo-Asian women.

## Discussion

We found that TC concentration in Indo-Asians and Caucasians was similar and this is consistent with previous studies.[1,2] The lower HDLC concentration found in Indo-Asians when compared with Caucasians was consistent with previous studies.[1,2] However, we suggest this is due to higher HDLC concentrations in Caucasian women, since HDLC concentrations were similar in Indo-Asian women, Indo-Asian men and Caucasian men.

Table 2. *Cardiovascular risk factors in women and men within the ethnic groups*

| | Women | | Men | |
|---|---|---|---|---|
| | Caucasian | Indo-Asian | Caucasian | Indo-Asian |
| *n* | 366 | 94 | 421 | 129 |
| Age (years) | 52 (50·7–51·8) | 49 (48·4–50·9) | 52 (50·4–51·5) | 49 (48·9–50·8) |
| Diabetics (%) | 16·7 | 21·3 | 20·7 | 27·1 |
| Current smokers (%) | 41·8$^{b}$ | 0$^{acd}$ | 48·4$^{b}$ | 20·9$^{b}$ |
| Total cholesterol (mmol/L) | 5·8 (5·7–6·0) | 5·4 (5·3–5·8) | 5·7 (5·6–5·9) | 5·7 (5·6–6·0) |
| HDL cholesterol (mmol/L) | 1·4$^{bcd}$ (1·4–1·5) | 1·2$^{a}$ (1·2–1·3) | 1·2$^{a}$ (1·2–1·3) | 1·2$^{a}$ (1·2–1·3) |
| Total:HDL cholesterol ratio | 4·0$^{cd}$ (4·0–4·4) | 4·5 (4·3–4·8) | 4·7$^{a}$ (4·7–5·1) | 4·9$^{a}$ (4·8–5·3) |
| Systolic BP (mmHg) | 140$^{b}$ (138–142) | 130$^{ac}$ (129–138) | 143$^{b}$ (142–145) | 140 (135–141) |
| 10-year CHD risk (%) | 7·7$^{cd}$ (7·2–8·3) | 5·6$^{cd}$ (5·6–7·9) | 13·5$^{ab}$ (12·6–14·4) | 10·4$^{ab}$ (10·4–12·5) |

Results are medians (95% confidence intervals). $^{a}P<0·05$ compared with Caucasian women; $^{b}P<0·05$ compared with Asian women; $^{c}P<0·05$ compared with Caucasian men; $^{d}P<0·05$ compared with Asian men. CHD=coronary heart disease.

Previous studies have found higher HDLC concentrations in Caucasian and Indo-Asian women compared with men.[5,6] The absence of a sex difference in HDLC concentration in Indo-Asians in this study, however, has been previously reported in some indigenous Indian communities[7] but this finding remains unexplained. In women, the lower average HDLC concentration in Indo-Asians, compared with those in Caucasians, is unlikely in this study to be ethnic in origin, since Indo-Asian men and Caucasian men had similar HDLC concentrations. The lower HDLC concentrations in Indo-Asian women may be related to lifestyle (for example, exercise, menopausal status and alcohol intake) or medication (e.g. oral contraceptive pill or hormone replacement therapy), both of which affect HDLC concentrations. It is also possible that the higher than 'expected' HDLC in Indo-Asian men may be likewise influenced by lifestyle factors.

It has been reported that there may be a variation of CHD risk factors within culturally diverse Indo-Asians.[3] In this study the Indo-Asian subjects were predominantly Punjabi Sikhs, whereas other studies have often included culturally diverse Indo-Asian groups. The results in this study are consistent with a previous study which observed similar HDLC concentrations in Sikh Indo-Asian men and Caucasian men but lower HDL concentrations in Muslim and Hindu Indo-Asian men,[8] supporting the notion of diversity of CHD risk factors amongst Indo-Asians.[3]

In conclusion, HDLC concentrations are lower in Indo-Asian women that in Caucasian women but similar in Indo-Asian men and Caucasian men. We suggest that this difference is likely to be social or environmental in origin. In developing strategies to prevent CHD in South Asians,[1,2] consideration should be given to possible diversity of CHD risk factors between culturally different Indo-Asian groups.

## References


1 Bhopal R. Epidemic of cardiovascular disease in South Asians. *BMJ* 2002; **324**: 625–6

2 McKeigue PM. Coronary heart disease in Indians, Pakistanis and Bangladeshis: aetiology and possibilities for prevention. *Br Heart J* 1992; **67**: 341–2

3 Bhopal R, Unwin N, White M, Yallop J, Walker L, Alberti KGMM, *et al.* Heterogeneity of coronary heart disease in Indian, Pakistani, Bangladeshi and European origin populations: cross sectional study. *BMJ* 1999; **319**: 215–20

4 Anderson KM, Odell PM, Wilson PWF, Kannel WB. Cardiovascular disease risk profiles. *Am Heart J* 1991; **121**: 293–8

5 Legato MJ. Dyslipidaemia, gender, and the role of high density lipoprotein cholesterol: implications for therapy. *Am J Cardiol* 2000; **86** (12A): 15L–18L

6 Enas EA, Garg A, Davidson MA, Nair VM, Huet BA, Yusuf S. Coronary heart disease and its risk factors in first generation immigrant Asian Indians to the United States of America. *Indian Heart J* 1996; **48**: 343–53

7 Shanmugasundaram KR, Suresh S, Misra KP, Jayakrishnan TK. Plasma lipoprotein cholesterol in India in healthy persons and those with coronary heart disease. *Atherosclerosis* 1983; **46**: 129–35

8 McKeigue PM, Shah B, Marmot MG. Relation of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in South Asians. *Lancet* 1991; **337**: 382–5


*Accepted for publication 6 June 2002*

# Ethnic variation in C-reactive protein: UK resident Indo-Asians compared with Caucasians

K. Chatha, N.R. Anderson and R. Gama

**Background** The increased prevalence of coronary heart disease (CHD) in UK resident Indo-Asians is unexplained by the traditional cardiovascular risk factors of dyslipidaemia, hypertension, smoking and diabetes mellitus. C-reactive protein (CRP) has been implicated in the pathogenesis of CHD but the data on ethnic variation in CRP is conflicting. We therefore investigated whether CRP could help explain the increased prevalence of CHD in Indo-Asians.

**Design and methods** We measured CRP, using a highly sensitive assay, in 102 men (63 Caucasians and 39 Indo-Asians) and 89 women (58 Caucasians and 31 Indo-Asians). All subjects, aged between 40 and 70 years, were nondiabetic and nonsmokers.

**Results** Serum CRP correlated ($P < 0.05$) positively with coronary risk. Serum HDL cholesterol concentrations were lower ($P < 0.05$) in Indo-Asian women when compared with Caucasian women, but otherwise the ethnic groups were matched for calculated coronary risk and cardiovascular risk factors. Serum CRP concentrations were similar in Indo-Asians (women 2.29 (1.52) mg/l {mean (SD)}; men 1.77 (1.46) mg/l) and Caucasians (women 2.23 (1.54) mg/l; men 1.94 (1.45) mg/l).

**Conclusions** Altered CRP concentrations does not appear to be implicated in the increased prevalence of CHD in UK resident Indo-Asians. *J Cardiovasc Risk* 9 : 139–141


**Journal of Cardiovascular Risk** 2002, **9** : 139–141

**Keywords: CRP, coronary heart disease, ethnic groups, Indo-Asians**

Department of Clinical Chemistry, New Cross Hospital, Wolverhampton, UK.

Correspondence and requests for reprints to: Dr R. Gama, Department of Clinical Chemistry, New Cross Hospital, Wolverhampton, West Midlands, WV10 0QP, UK.
Tel: 01902 643056; fax: 0044 902 642813;
e-mail: dr.gama@rwh-tr.nhs.uk

Conflicts of interest: None

**Received** 01 October 2001 **Revised** 18 March 2002
**Accepted** 26 March 2002

## Introduction

An increased prevalence of coronary heart disease (CHD) in UK Indo-Asians resident in the UK, in comparison with Caucasians, is well-recognized [1–3]. This increased CHD risk is not explained by the conventional cardiovascular risk factors of hypertension, hypercholesterolaemia, smoking or a family history of CHD [2–4]. Although diabetes mellitus is more common in Indo-Asians [4–6], it does not fully explain the increased prevalence of CHD since most Indo-Asians with CHD do not have diabetes mellitus [7]. It has suggested that other more novel cardiovascular risk factors including the metabolic syndrome [2,4,6,8], socio-economic deprivation [9] and hyperhomocysteinaemia [10] may contribute to the increased prevalence of coronary heart disease in Indo-Asians when compared with Caucasians.

Chronic inflammation is a key feature of atherosclerosis [11]. C-reactive protein (CRP), an acute phase reactant, has recently been implicated as an independent CHD risk factor [12–15]. The possibility of ethnic differences in CRP as a CHD risk factor has been recently studied [16,17]. The data are, however, conflicting, reporting either higher [16] or similar [17] CRP concentrations in Indo-Asians when compared with Caucasians. Since Indo-Asians constitute 13.7% of the local population, we compared CRP concentrations in 70 Indo-Asians and 153 Caucasians resident in Wolverhampton, UK.

## Subjects and methods

As part of primary prevention of CHD in Wolverhampton, UK we have been providing general practitioners with a laboratory calculated 10-year CHD risk score using a computer program written for the laboratory computer system (LMX, Bayer Diagnostics Ltd, Newbury, UK). The CHD risk calculation, based on the Framingham equation, uses the individual's age, sex, systolic blood pressure (BP), serum cholesterol, serum HDL cholesterol, and the presence or absence of smoking, diabetes mellitus and left ventricular hypertrophy [18]. Patients were selected at the discretion of their GPs for laboratory-based CHD risk score calculation. Exclusion criteria for this study included $< 40$ years and $> 70$ years, arterial vascular disease, diabetes mellitus, smoking, hypolipidaemic therapy and CRP concentrations $> 6$ mg/l. The clinical information was collected from a self-adhesive sticker fixed on the pathology request form, which also

provided data on ethnicity (supplied as Caucasian, Afro/Carribean, Asian, Chinese and 'other').

We studied 102 men (39 Indo-Asians) and 89 women (31 Indo-Asians) in whom data collection was complete. Since gender affects CHD risk, we investigated possible ethnic differences in CRP separately in males and female.

Serum CRP was measured using a high sensitivity automated immunoassay method (DPC, Los Angeles, CA, USA) on the Immulite Analyser. Serum total and HDL cholesterol were measured by routine automated kit methods (DiaSys Diagnostic Systems GmbH & Co., Holzheim, Germany and Bio-Stat Diagnostic Systems, Stockport, UK respectively). Analytical sensitivity of the CRP assay was 0.1 mg/l. Respective interassay and intra-assay coefficient of variation were for CRP 3.6% and 3.4%; total cholesterol 1.22% and 0.61%; and HDL cholesterol 1.93% and 0.75%.

Data were normally distributed, unpaired *t*-test was therefore used to compare the significance of differences between groups. Pearson's correlation (*r*) was used to measure the significance of association between variables. Results are expressed as means with standard deviations in parentheses. Data were analysed using the statistical package GraphPad Instat version 3.00 for Windows 95 (GraphPad Software, San Diego, USA).

## Results

The results are shown in Table 1. In summary, CRP concentrations were similar in Indo-Asian and Caucasian subjects. Indo-Asian women had lower ($P < 0.05$) serum HDL cholesterol concentrations than Caucasian women but HDL cholesterol concentrations were similar in Indo-Asian men and Caucasian men. There was no difference in calculated cardiac risk, systolic BP, serum cholesterol and serum total: HDL cholesterol ratio between Indo-Asians and Caucasians.

In men, serum CRP correlated positively with calculated CHD risk ($r = 0.2764$; $P = 0.0049$) and with serum total:HDL cholesterol ratio ($r = 0.2707$; $P = 0.0059$) and negatively with serum HDL ($r = -0.2400$; $P = 0.0151$). In women, serum CRP correlated positively with age ($r = 0.2277$; $P = 0.0343$). There were no other significant correlations.

## Discussion

We report similar serum CRP concentrations in Indo-Asians and Caucasians. These results are consistent with the study by Forouhi and colleagues, who similarly reported no significant differences in CRP between Indo-Asians and Caucasians [16], but different from the report of Chambers *et al.*, who observed higher CRP concentrations in Indo-Asian men [17]. This difference in CRP results in the different studies could be due to selection of patient groups, different study groups, different analytical techniques and type I or II statistical errors.

We excluded subjects with diabetes mellitus and smokers because these factors are associated with increased serum CRP concentrations [19,20].

Table 1 **CRP and cardiovascular risk factors in: (a) Indo-Asian and Caucasian men**

| | Indo-Asian | Caucasian | P value |
|---|---|---|---|
| Number | 39 | 63 | |
| Age (years) | 52.1 (7.0) | 55.2 (7.7) | 0.0613 |
| CRP (mg/l) | 1.77 (1.46) | 1.94 (1.45) | 0.5779 |
| Cholesterol (mmol/l) | 5.85 (0.89) | 5.79 (0.99) | 0.7666 |
| HDL cholesterol (mmol/l) | 1.32 (0.32) | 1.36 (0.37) | 0.5475 |
| Total:HDL Cholesterol ratio | 4.70 (1.25) | 4.50 (1.20) | 0.4214 |
| Systolic BP(mmHg) | 136.2 (17.1) | 139.8 (21.7) | 0.3811 |
| % 10 year CHD risk | 11.1 (6.4) | 11.9 (6.5) | 0.5842 |

**(b) Indo-Asian and Caucasian women**

| | Indo-Asians | Caucasian | P value |
|---|---|---|---|
| Number | 31 | 58 | |
| Age (years) | 53.3 (9.4) | 56.1 (7.9) | 0.1475 |
| CRP (mg/l) | 2.29 (1.52) | 2.23 (1.54) | 0.8607 |
| Cholesterol (mmol/l) | 5.58 (1.20) | 5.93 (0.84) | 0.1064 |
| HDL cholesterol (mmol/l) | 1.39 (0.45) | 1.67 (0.42) | 0.0040 |
| Total:HDL Cholesterol ratio | 4.26 (1.23) | 3.76 (1.12) | 0.0542 |
| Systolic BP(mmHg) | 136.5 (16.4) | 136.2 (22.8) | 0.9529 |
| % 10 year CHD risk | 6.6 (5.4) | 6.1 (4.3) | 0.6164 |

Results are means (SD).

The difference in CRP levels reported in the various studies could, therefore, have been due to the inclusion of diabetics and smokers in the study of Chambers *et al.* [16] and smokers in the study by Forouhi and co-workers [17]. As CRP correlates with CHD risk [19], the results in this study are especially noteworthy since Indo-Asian and Caucasian study groups were matched for calculated CHD risk and conventional cardiovascular risk factors.

It is possible that variations in body mass index (BMI) could explain the ethnic differences in CRP since obesity is associated with increased CRP levels [19]. Indo-Asians, however, appear to have the same BMI as Caucasians [21] which was confirmed in other two studies on ethnic variation of CRP which reported similar BMI but different CRP values in Indo-Asians and Caucasians [16,17]. We do not have data on BMI but this is therefore unlikely to affect our results.

An alternative explanation for the differing CRP results could be due to ethnic variation within study subgroups since it has been recently reported that there are differences in cardiovascular risk factors within culturally diverse Indo-Asians [21]. In their study Chambers *et al.* reported lower HDL cholesterol concentrations in Indo-Asian men [16], which is a widely accepted view [2,3,21]. In our study and that of Forouhi *et al.* [17], HDL cholesterol concentrations were similar in Indo-Asian and Caucasian men. Since Indo-Asians resident in Wolverhampton are predominantly Punjabi Sikhs, our results are consistent with a previous report of similar HDL cholesterol concentrations in Sikh and Caucasian men [4]. We are unaware of the exact cultural composition of Indo-Asians in this and other studies [16,17], but it is possible that heterogeneity within the ethnic groups could account for the conflicting data on CRP in Indo-Asians.

In conclusion, we report that CRP concentrations are similar in Indo-Asian and Caucasian with comparable calculated CHD risk. We have no evidence to suggest that altered CRP concentrations are implicated in the increased cardiovascular morbidity observed in UK resident Indo-Asians.

## References


1 Balarajan R. Ethnicity and variations in mortality form coronary heart disease. *Health Trends* 1996; **28**:45–51.

2 McKeigue PM, Miller GJ, Marmot MG. Coronary heart disease in Indo-Asians overseas: a review. *J Clin Epidemiol* 1989; **42**:597–609.

3 McKeigue PM. Coronary heart disease in Indians, Pakistanis and Bangladeshis: aetiology and possibilities for prevention. *Br Heart J* 1992; **67**:341–342.

4 McKeigue PM, Shah B, Marmot MG. Relation of central obesity and insulin resistance with high diabetes prevalence and cardiovascular risk in Indo-Asians. *Lancet* 1991; **337**:382–385.

5 Mather HM, Keen H. The Southall diabetes survey; prevalence of known diabetes in Asians and Europeans. *BMJ* 1985; **291**:1081–1084.

6 McKeigue PM, Marmot MG, Syndercombe, Court YD, Cottier DE, Rahman S, *et al.* Diabetes, hyperinsulinaemia, and coronary risk factors in Bangladeshis in east London. *Br Heart J* 1988; **60**:390–396.

7 Hughes LO, Cruickshank JK, Wright J, Raftery EB. Disturbances of insulin in British Asian and white men surviving myocardial infarction. *BMJ* 1989; **299**:537–541.

8 McKeigue PM, Ferrie JE, Pierpoint T, Marmot MG. Association of early-onset coronary heart disease in Indo-Asian men with glucose intolerance and hyperinsulinemia. *Circulation* 1993; **87**:152–161.

9 Williams R, Bhopal R, Hunt K. Coronary risk in a British Punjabi Population: Comparative profile of non-biochemical factors. *Int J Epidemiol* 1994; **23**:28–37.

10 Chambers JC, Obeid OA, Refsum H, Ueland P, Hackett D, Hooper J, *et al.* Plasma homocysteine concentrations and risk of coronary heart disease in UK Indian Asian and European men. *Lancet* 2000; **355**:523–527.

11 Ross R. Atherosclerosis – An inflammatory disease. *N Eng J Med* 1999; **340**:115–126.

12 Koenig W, Sund M, Frohlich M, Fischer HG, Lowel H, Doring A, *et al.* C-Reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: results from the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) Augsburg Cohort Study, 1984 to 1992. *Circulation* 1999; **99**:237–242.

13 Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med* 2000; **342**:836–843.

14 Pepys MB, Berger A. The renaissance of C reactive protein. *BMJ* 2001; **322**:4–5.

15 Ridker PM. High-sensitivity C-reactive protein. Potential adjunct for the global risk assessment in the primary prevention of cardiovascular disease. *Circulation* 2001; **103**:1813–1818.

16 Chambers JC, Eda S, Bassett P, Karim Y, Thompson SG, Gallimore JR, *et al.* C-reactive protein, insulin resistance, central obesity, and coronary heart disease risk in Indian Asians from the United Kingdom compared with European whites. *Circulation* 2001; **104**:145–150.

17 Forouhi NG, Sattar N, McKeigue PM. Relation of C-reactive protein to body fat distribution and features of the metabolic syndrome in Europeans and South Asians. *Int J Obes Relat Metab Disord* 2001; **25**:1327–1331.

18 Anderson KM, Odell PM, Wilson PWF, Kannel WB. Cardiovascular disease risk profiles. *Am Heart J* 1991; **121**:293–298.

19 Rhode LEP, Hennekens CH, Ridker PM. Survey of C-reactive protein and cardiovascular risk factors in apparently healthy men. *Am J Cardiol* 1999; **84**:1018–1022.

20 Hulthe J, Wikstrand J, Fagerberg B. Relationship between C-reactive protein and intima-media thickness in carotid and femoral arteries and to antibodies against oxidized low-density lipoprotein in healthy men: the Atherosclerosis and Insulin Resistance (AIR) study. *Clin Sci* 2001; **100**:371–378.

21 Bhopal R, Unwin N, White M, Yallop J, Walker L, Alberti KGMM, *et al.* Heterogeneity of coronary heart disease in Indian, Pakistani, Bangladeshi and European origin populations: cross sectional study. *BMJ* 1999; **319**:215–220.

*Nutrients* **2015**, *7*, 2401-2414; doi:10.3390/nu7042401

OPEN ACCESS

*nutrients*

**ISSN 2072-6643**

www.mdpi.com/journal/nutrients

*Article*

# Low Maternal Vitamin B12 Status Is Associated with Lower Cord Blood HDL Cholesterol in White Caucasians Living in the UK

**Antonysunil Adaikalakoteswari [1], Manu Vatish [2], Alexander Lawson [3], Catherine Wood [4], Kavitha Sivakumar [1], Philip G. McTernan [1], Craig Webster [3], Neil Anderson [4], Chittaranjan S. Yajnik [5], Gyanendra Tripathi [1,*] and Ponnusamy Saravanan [1,4,6,*]**

1 Warwick Medical School, University of Warwick, Coventry CV2 2DX, UK; E-Mails: A.K.Antonysunil@warwick.ac.uk (A.A.); K.Sivakumar@warwick.ac.uk (K.S.); P.G.McTernan@warwick.ac.uk (P.G.M.)

2 Nuffield Department of Obstetrics & Gynaecology, University of Oxford, Oxford OX3 9DU, UK; E-Mail: manu.vatish@obs-gyn.ox.ac.uk

3 Department of Pathology, Heartlands Hospital, Birmingham B9 5SS, UK; E-Mails: alexander.lawson@heartofengland.nhs.uk (A.L.); craig.webster@heartofengland.nhs.uk (C.W.)

4 Academic department of Diabetes and Metabolism, George Eliot Hospital, Nuneaton CV10 7DJ, UK; E-Mails: Catherine.Wood@geh.nhs.uk (C.W.); neil.anderson@geh.nhs.uk (N.A.)

5 Diabetes Research Centre, KEM Hospital, Pune 411011, India; E-Mail: csyajnik@hotmail.com

6 WISDEM Centre, University Hospital Coventry and Warwickshire, Coventry CV2 2DX, UK

* Authors to whom correspondence should be addressed; E-Mails: P.Saravanan@warwick.ac.uk (P.S.); G.Tripathi@warwick.ac.uk (G.T.); Tel.: +44-2476968668; Fax: +44-2476968653.

*Received: 29 January 2015 / Accepted: 24 March 2015 / Published: 2 April 2015*

---

**Abstract:** Background and Aims: Studies in South Asian population show that low maternal vitamin B12 associates with insulin resistance and small for gestational age in the offspring. Low vitamin B12 status is attributed to vegetarianism in these populations. It is not known whether low B12 status is associated with metabolic risk of the offspring in whites, where the childhood metabolic disorders are increasing rapidly. Here, we studied whether maternal B12 levels associate with metabolic risk of the offspring at birth. Methods: This is a cross-sectional study of 91 mother-infant pairs ($n$ = 182), of white Caucasian origin living in the UK. Blood samples were collected from white pregnant women at delivery and their newborns (cord blood). Serum vitamin B12, folate, homocysteine as well as the relevant metabolic risk factors were measured. Results: The prevalence of low serum vitamin B12

(<191 ng/L) and folate (<4.6 μg/L) were 40% and 11%, respectively. Maternal B12 was inversely associated with offspring's Homeostasis Model Assessment 2-Insulin Resistance (HOMA-IR), triglycerides, homocysteine and positively with HDL-cholesterol after adjusting for age and BMI. In regression analysis, after adjusting for likely confounders, maternal B12 is independently associated with neonatal HDL-cholesterol and homocysteine but not triglycerides or HOMA-IR. Conclusions: Our study shows that low B12 status is common in white women and is independently associated with adverse cord blood cholesterol.

**Keywords:** vitamin B12; maternal; offspring; metabolic risk; lipids

## 1. Introduction

The prevalence of childhood obesity is increasing rapidly [1,2]. Recently, the Early Childhood Longitudinal Study demonstrated that 27.3% of children were either overweight or obese by the time they enter kindergarten in the United States [1]. Higher rate of childhood obesity is a likely contributor for the increasing incidence of type 2 diabetes (T2D) earlier in life as well as pre-gestational and gestational diabetes (GDM) in women [3]. It is known that childhood obesity independently predicts obesity and metabolic disorders in the adulthood [4]. Children born with lower HDL and higher triglyceride levels were small for gestational age (SGA) and had higher abdominal circumference [5]. It is known that both higher abdominal circumference and SGA are associated with future development of T2D and GDM [6,7] in many populations.

Although current adverse lifestyle (nutrition and physical inactivity) contributes to obesity, a growing body of evidence links nutrient imbalance in early life to the development of metabolic disorders in childhood and in adults [8]. Many studies support this link including the Dutch-famine study. Individuals exposed to nutritional imbalance during pregnancy are likely to be obese, have early onset of coronary artery disease, T2D and worse cognitive performances as adults [9]. Emerging evidence from clinical studies show that key maternal micronutrients involved in the one-carbon metabolism (1-C) can cause adverse metabolic programming. Independent studies from South Asia have demonstrated that children born to mothers with low vitamin B12 [10,11] and higher folate [12] have greater insulin resistance. In addition, low maternal B12 levels independently contributed to the risk of small for gestational age (SGA), which has been shown to increase the metabolic risk of the offspring [13]. Vegetarianism is the likely cause of high prevalence of low B12 levels in these population [14]. In a Brazilian pregnancy cohort, low maternal B12 was associated with lower levels of the methyl donor (*S*-adenosyl methionine—SAM) in the cord blood [15]. A study in a Chinese population demonstrated that low maternal B12 is common during pregnancy and is associated with an altered methylation pattern of the insulin growth factor 2 (IGF2) promoter region in the cord blood [16], highlighting a potential role of B12 on fetal growth. Further, animal studies showed that maternal vitamin B12 deficiency resulted in higher adiposity, insulin resistance, blood pressure [17] and adverse lipid profile in the offspring [18,19]. These investigations provide evidence that low maternal B12 could be an independent determinant of adverse metabolic phenotypes in the offspring.

Recently, we demonstrated in Europeans and Indians with T2D that vitamin B12 deficiency is associated with adverse lipid profile [20]. Re-analysis of the UK National Diet and Nutrition Survey data showed that low vitamin B12 levels (<191 ng/L) is common in the adult population (10%) and in women of reproductive age (14%) [21]. Our preliminary study of white pregnant women showed that the rate of low B12 status was as high as 20% at 16–18 weeks of gestation [22].

Despite the evidence that vitamin B12 deficiency is a potential contributor for adverse offspring metabolic phenotypes and the prevalence of low B12 status is increasing in White Caucasian population, the link between maternal B12 status and metabolic risk at birth is unexplored in the White Caucasian population. Therefore, the objective of our study was to investigate whether maternal B12 levels in white women independently associate with the metabolic risk at birth.

## 2. Methods

### *2.1. Study Population*

The study was conducted in University Hospital Coventry Warwickshire (UHCW), Coventry, UK. All study participants were pregnant women delivering at 39–40 weeks of gestation. The Coventry local research ethics committee approved the study, and all patients gave written informed consent (Research Ethics Committees 07/H1210/141). Women with known chronic diseases were excluded. Maternal data including parity, smoking, BMI and birth weight were collected from pregnancy records. Folic acid supplement use collected but detailed dietary history was not recorded. Maternal BMI measured routinely at the first pregnancy visit (before 10 weeks of gestation). We collected 182 maternal venous and cord blood samples (91 mother-newborn pairs) at the time of delivery. Extrapolating from our preliminary studies [21,22], we anticipated around 20%–25% of the mothers to have low levels of vitamin B12 (<191 ng/L). To detect a similar proportion of low B12 status a sample size of 100–120 was required. The samples were collected in the fasting state, in tubes without anticoagulant and centrifuged at 2000 rpm/10 min. Serum was separated, aliquoted and stored at −80 °C until analysis.

### *2.2. Analytical Determinations*

Serum glucose, cholesterol, triglycerides, HDL cholesterol were determined using an auto analyser Synchron CX7 (Beckman Coulter, Fullerton, CA, USA) based on enzymatic colorimetric assays. Insulin was measured using Invitrogen ELISA kit (Camarillo, CA, USA) according to manufacturer's instructions. LDL cholesterol was calculated using Friedewald formula. Insulin resistance (HOMA-IR) was calculated by the Homeostasis Model Assessment 2 computer model (HOMA2) using fasting insulin and glucose levels [10]. Serum B12 and folate were determined by electrochemiluminescent immunoassay using a Roche Cobas immunoassay analyzer (Roche Diagnostics UK, Burgess Hill, UK). Similar to other studies [20,23,24], we have used 191–663 ng/L for serum Vitamin B12 and 4.6–18.7 μg/L for serum folate as normal range, respectively. The inter-assay coefficient of variations for B12 and folate were 3.9% and 3.7%, respectively. To avoid potential bias, all the biochemical analyses were conducted in a single batch to minimise assay variation. All the laboratory personnel were blinded and did not have any access to the clinical data. Serum homocysteine was determined by stable isotopic dilution analysis liquid chromatography (LC-MS/MS) [25] using a Waters Equity UPLC system

(Waters, Milford, CT, USA) coupled to an API 4000 tandem mass spectrometer (Applied Biosystems, Warrington, UK). Due to the uncertainty of defining deficiencies of serum vitamin B12 and folate levels during pregnancy and cord blood, the terms "low B12 status" and "low folate status" were used throughout the manuscript if the levels were below 191 ng/L and 4.6 μg/L, respectively.

### *2.3. Statistical Analysis*

Continuous data are reported either as mean ± standard deviation (SD) or geometric mean with 95% confidence intervals (CI). Categorical data are reported in numbers (percentages). The distributions of the maternal and neonatal parameters such as vitamin B12, folate, cholesterol, triglycerides, HDL, LDL, glucose, insulin, HOMA-IR and homocysteine concentrations were skewed; these data were log-transformed before analyses. Student's *t*-test was used for comparison of groups. Bivariate correlations were done using Pearson test. Variables that showed significant associations with dependent variable (neonatal metabolic risk factors) were included as independent variables in the multiple linear regression analyses. To facilitate comparison, dependent and independent variables were converted into standard deviation scores (SDS). The data are presented as SD change in offspring outcome per SD change in maternal vitamin B12, folate and homocysteine. Associations between maternal vitamin B12, folate and homocysteine concentrations and offspring outcomes were examined in multivariate linear regression using 3 models. Model 1: unadjusted; Model 2: adjusted for maternal age, BMI, glucose, insulin, parity, folic acid supplement use, smoking, vitamin B12, folate and homocysteine; Model 3: Model 2 + respective maternal variable. All tests were two-sided, and $p$ values of <0.05 were considered to be statistically significant. All analyses were performed using SPSS Statistics version 21 (IBM Corp, Armonk, NY, USA).

## 3. Results

### *3.1. B12, Folate and Homocysteine Status*

The clinical characteristics of mothers and neonates are shown in Table 1. The prevalence of serum low vitamin B12 and folate status in women during pregnancy were 40% and 11% in mothers and 29% and 0% in neonates, respectively (Table 1). In cord blood, all the biochemical parameters were significantly lower than in maternal serum, except for the B12 and folate levels (Table 1). Children born to mothers with low B12 status had significantly lower B12 levels compared to those born to mothers with normal levels (Table 2). Mothers with higher parity and smoking had lower B12 levels. Those with self-reported folic acid supplement use had higher B12 and lower homocysteine levels (Table 3). Maternal B12, folate and homocysteine showed strong positive correlation with the respective offspring indices (B12: $r = 0.648$, folate: $r = 0.706$, homocysteine: $r = 0.756$, all $p < 0.0001$) (Supplementary Figure S1a–c). Neonatal homocysteine showed negative correlation with maternal B12 and folate (B12: $r = -0.409$, $p < 0.0001$; folate: $r = -0.346$, $p < 0.001$; Supplementary Figure S2a,b).

**Table 1.** Clinical characteristics of mothers and neonate.

| | Mother | Neonate |
|---|---|---|
| | ***n* = 91** | ***n* = 91** |
| Age (years) | 32.7 ± 5.9 [a] | - |
| Weight (Kg) | 77.7 ± 18.1 | 3.57 ± 0.26 |
| Height (m) | 1.62 ± 0.09 | - |
| BMI (early pregnancy) (kg/m$^2$) | 29.4 ± 6.2 | - |
| Glucose (mmol/L) | 4.37 ± 0.42 | 3.88 ± 0.52 |
| Insulin (mIU/L) | 11.6 (12.9, 17.4) [b] | 8.01 (8.62, 11.9) |
| Triglycerides (mmol/L) | 2.69 (2.62, 3.06) | 0.23 (0.22, 0.26) |
| Cholesterol (mmol/L) | 6.48 (6.31, 6.89) | 1.68 (1.63, 1.84) |
| LDL cholesterol (mmol/L) | 3.53 (3.46, 3.95) | 0.82 (0.79, 0.95) |
| HDL cholesterol (mmol/L) | 1.56 (1.53, 1.72) | 0.74 (0.72, 0.83) |
| HOMA-IR | 1.37 (1.55, 2.09) | 0.99 (1.04, 1.41) |
| Vitamin B12 (ng/L) | 218 (213, 289) | 290 (292, 418) |
| Low B12 status (%) | 36 (40) [c] | 26 (29) |
| Folate (μg/L) | 10.5 (10.9, 13.2) | 16.8 (16.4, 17.7) |
| Low folate status (%) | 10 (11) | 0 |
| Homocysteine (μmol/L) | 6.23 (6.02, 7.54) | 5.76 (5.64, 6.85) |

[a] Mean ± SD (all such values); [b] Geometric mean (95% CI) (all such values); [c] Numbers (percentages) (all such values).

**Table 2.** Clinical characteristics of mothers and neonate according to maternal B12 levels.

| | Mothers | | Neonate | |
|---|---|---|---|---|
| | **Maternal B12 ≥191 (ng/L)** | **Maternal B12 <191 (ng/L)** | **Maternal B12 ≥191 (ng/L)** | **Maternal B12 <191 (ng/L)** |
| | ***n* = 55** | ***n* = 36** | ***n* = 55** | ***n* = 36** |
| Age (years) | 33.0 ± 6.2 [a] | 32.3 ± 5.6 | - | - |
| Weight (Kg) | 74.3 ± 15.8 | 82.9 ± 20.8 * | 3.58 ± 0.31 | 3.57 ± 0.18 |
| Height (m) | 1.62 ± 0.07 | 1.61 ± 0.11 | - | - |
| BMI (early pregnancy) (kg/m$^2$) | 28.4 ± 6.1 | 30.8 ± 6.4 * | - | - |
| Glucose (mmol/L) | 4.40 ± 0.46 | 4.36 ± 0.34 | 3.85 ± 0.52 | 3.94 ± 0.52 |
| Insulin (mIU/L) | 10.4 (11.7, 18.0) [b] | 13.7 (12.7, 18.6) | 8.27 (8.33, 12.75) | 7.64 (7.30, 12.40) |
| Triglycerides (mmol/L) | 2.49 (2.37, 2.93) | 3.04 (2.82, 3.48) * | 0.21 (0.20, 0.24) | 0.26 (0.23, 0.32) ** |
| Cholesterol (mmol/L) | 6.23 (5.99, 6.72) | 6.86 (6.51, 7.43) * | 1.72 (1.64, 1.92) | 1.62 (1.51, 1.83) |
| LDL cholesterol (mmol/L) | 3.29 (3.16, 3.82) | 3.91 (3.67, 4.38) * | 0.79 (0.76, 0.90) | 0.87 (0.77, 1.09) |
| HDL cholesterol (mmol/L) | 1.61 (1.54, 1.80) | 1.49 (1.42, 1.70) | 0.79 (0.76, 0.91) | 0.67 (0.62, 0.77) * |
| HOMA-IR | 1.18 (1.37, 2.14) | 1.68 (1.57, 2.27) | 0.94 (0.95, 1.41) | 1.08 (0.98, 1.63) |
| Vitamin B12 (ng/L) | 288 (265, 378) | 146 (139, 155) *** | 367 (354, 544) | 202 (187, 234) *** |
| Low B12 status (%) | 0 | 36 (40) [c] | 8 (14.5) | 18 (50) |
| Folate (μg/L) | 11.7 (11.5, 14.6) | 9.0 (8.5, 12.4) * | 17.6 (17.0, 18.5) | 15.7 (14.9, 17.2) ** |
| Low folate status (%) | 3 (5.5) | 7 (19.4) | 0 | 0 |
| Homocysteine (μmol/L) | 5.50 (5.26, 6.18) | 7.53 (6.69, 10.1) *** | 4.97 (4.74, 5.59) | 7.09 (6.58, 8.96) *** |

[a] Mean ± SD (all such values); [b] Geometric mean (95% CI) (all such values); [c] Numbers (percentages) (all such values); * *p*-value compared to maternal B12 (≥191 ng/L) group; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

**Table 3.** Vitamin B12, folate and homocysteine in mothers and neonate according to maternal smoking status, parity and folate supplement use.

| | Smoking | *n* = 91 | Vitamin B12 (ng/L) | Folate (µg/L) | Homocysteine (µmol/L) |
|---|---|---|---|---|---|
| **Maternal** | No (%) | 55 | 245 (227, 357) | 10.7 (10.6, 13.9) | 6.15 (5.61, 8.08) |
| | Yes (%) | 45 | 189 (176, 224) ** | 10.1 (9.7, 13.3) | 6.33 (5.90, 7.45) |
| **Neonate** | No (%) | 55 | 327 (305, 502) | 16.9 (16.4, 18.2) | 5.42 (5.06, 6.58) |
| | Yes (%) | 45 | 252 (232, 364) * | 16.5 (15.8, 17.7) | 6.21 (5.80, 7.68) |
| | **Parity** | | | | |
| **Maternal** | Para 0 (%) | 18 | 248 (203, 332) | 13.7 (11.8, 17.9) | 6.25 (5.21, 8.03) |
| | Para 1 (%) | 48 | 224 (201, 347) | 11.1 (10.8, 14.1) | 6.12 (5.73, 7.43) |
| | Para ≥2 (%) | 34 | 195 (179, 239) * | 8.2 (7.6, 11.6) ** | 6.4 (5.31, 8.97) |
| **Neonate** | Para 0 (%) | 18 | 327 (264, 479) | 17.9 (16.6, 19.6) | 5.61 (4.50, 7.59) |
| | Para 1 (%) | 48 | 284 (258, 487) | 16.9 (16.3, 18.1) | 5.91 (5.53, 7.30) |
| | Para ≥2 (%) | 34 | 283 (250, 396) | 16.0 (15.2, 17.4) * | 5.67 (5.08, 7.15) |
| | **Folate supplement users** | | | | |
| **Maternal** | Yes (%) | 85 | 224 (216, 305) | 11.1 (11.3, 14.0) | 6.06 (5.77, 7.44) |
| | No (%) | 15 | 187 (154, 245) | 6.8 (5.5, 9.3) *** | 7.42 (6.02, 9.61) * |
| **Neonate** | Yes (%) | 85 | 311 (306, 445) | 17.4 (17.0, 18.2) | 5.57 (5.40, 6.53) |
| | No (%) | 15 | 213 (134, 391) | 13.5 (12.0, 15.6) *** | 7.01 (5.34, 10.33) * |

Data are geometric mean (95% CI); * *p*-value compared to geometric mean in the respective group(s); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

### *3.2. Maternal B12 and Metabolic Risk of Offspring*

Maternal B12 adjusted for age and BMI was inversely associated with metabolic risk factors such as triglycerides ($r = -0.219$; $p = 0.047$), HOMA-IR ($r = -0.232$; $p = 0.041$), homocysteine ($r = -0.423$; $p = 0.0001$) and positively with HDL-cholesterol ($r = 0.315$; $p = 0.004$) (Figure 1a–d) in the offspring. Despite similar birth weight, offspring of low B12 mothers had significantly lower HDL-cholesterol, higher triglycerides and homocysteine than those of normal B12 mothers (Table 2). Multiple regression analysis was carried out to assess whether maternal B12 independently associated with these metabolic risk factors in the offspring by adjusting for likely confounders. The model included maternal age, parity, smoking, folic acid supplement use, BMI, glucose, insulin, folate and homocysteine as independent variables. In addition, for offspring's lipid parameters, respective maternal variable was also included in the model (maternal triglycerides for offspring's triglycerides, *etc.*). After all these adjustments, maternal B12 was independently associated with the offspring's HDL and homocysteine. Though similar trends were seen for the triglycerides and HOMA-IR, these were not statistically significant. No sex-specific changes were seen in any of these analyses (data not shown). Maternal B12 explained 5.1% of the variation in offspring's HDL and 10.6% in homocysteine (Table 4).

**Table 4.** Association of maternal B12, folate and homocysteine with neonate metabolic risk factors.

| Maternal Variable (SDS) | Neonate's Metabolic Risk Factors (SDS) | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Triglycerides *** | | | **Cholesterol *** | | | **HDL *** | | | **LDL *** | | | **Insulin *** | | | **Glucose *** | | | **Homocysteine *** | |
| Maternal B12 * | β | 95% CI | *p* | β | 95% CI | *p* | β | 95% CI | *p* | β | 95% CI | *p* | β | 95% CI | *p* | β | 95% CI | *p* | β | 95% CI | *p* |
| Model 1 | −0.148 | (−0.38, 0.09) | 0.210 | 0.109 | (−0.11, 0.33) | 0.317 | 0.296 | (0.07, 0.52) | 0.010 | −0.044 | (−0.26, 0.17) | 0.691 | 0.070 | (−0.21, 0.21) | 0.516 | −0.005 | (−0.22, −0.21) | 0.960 | −0.381 | (−0.58, −0.18) | <0.001 |
| Model 2 | −0.086 | (−0.38, 0.21) | 0.562 | 0.178 | (−0.08, 0.44) | 0.173 | 0.294 | (0.05, 0.54) | 0.018 | 0.070 | (−0.26, 0.39) | 0.672 | −0.063 | (−0.17, 0.29) | 0.593 | −0.088 | (−0.14, −0.31) | 0.438 | −0.200 | (−0.35, −0.05) | 0.009 |
| Model 3 | −0.079 | (−0.39, 0.23) | 0.609 | 0.170 | (−0.09, 0.43) | 0.198 | 0.295 | (0.08, 0.51) | 0.009 | 0.056 | (−0.28, 0.39) | 0.736 | −0.062 | (−0.17, 0.29) | 0.602 | −0.093 | (−0.12, 0.30) | 0.378 | | | |
| Maternal Folate * | | | | | | | | | | | | | | | | | | | | | |
| Model 1 | −0.109 | (−0.33, 0.11) | 0.326 | −0.210 | (−0.42, 0.001) | 0.051 | −0.025 | (−0.25, 0.19) | 0.825 | −0.173 | (−0.38, 0.04) | 0.111 | 0.040 | (−0.17, 0.25) | 0.705 | −0.124 | (−0.33, 0.09) | 0.243 | −0.327 | (−0.53, −0.13) | 0.002 |
| Model 2 | −0.084 | (−0.43, 0.26) | 0.625 | −0.204 | (−0.49, 0.08) | 0.160 | −0.236 | (−0.51, 0.04) | 0.091 | −0.079 | (−0.40, 0.24) | 0.625 | 0.133 | (−0.15, 0.42) | 0.357 | −0.035 | (−0.31, 0.24) | 0.799 | −0.004 | (−0.19, 0.18) | 0.966 |
| Model 3 | −0.093 | (−0.45, 0.26) | 0.600 | −0.200 | (−0.49, 0.09) | 0.169 | −0.209 | (−0.46, 0.04) | 0.099 | −0.082 | (−0.41, 0.24) | 0.616 | 0.136 | (−0.15, 0.43) | 0.353 | −0.039 | (−0.29, 0.22) | 0.763 | | | |
| Maternal Homocysteine * | | | | | | | | | | | | | | | | | | | | | |
| Model 1 | 0.218 | (−0.00, 0.44) | 0.050 | 0.290 | (−0.07, 0.51) | 0.009 | 0.119 | (−0.13, 0.36) | 0.336 | 0.265 | (0.06, 0.47) | 0.013 | 0.001 | (−0.21, 0.21) | 0.993 | 0.121 | (−0.09, 0.34) | 0.269 | 0.752 | (0.62, 0.89) | <0.001 |
| Model 2 | 0.137 | (−0.19, 0.47) | 0.473 | 0.206 | (−0.08, 0.49) | 0.156 | 0.093 | (−0.23, 0.41) | 0.556 | 0.224 | (−0.09, 0.54) | 0.160 | 0.037 | (−0.23, 0.31) | 0.785 | 0.057 | (−0.21, 0.32) | 0.669 | 0.696 | (0.52, 0.87) | <0.001 |
| Model 3 | 0.126 | (−0.23, 0.48) | 0.474 | 0.230 | (−0.07, 0.53) | 0.129 | 0.158 | (−0.13, 0.45) | 0.278 | 0.248 | (−0.08, 0.57) | 0.130 | 0.043 | (−0.24, 0.32) | 0.759 | 0.089 | (−0.16, 0.34) | 0.469 | | | |

* Log transformed for statistical comparisons. β represents SDS change in the dependent variable per SDS change in the independent variable. Model 1: unadjusted; Model 2: Maternal age, BMI, glucose, insulin, parity, folic acid supplement use, smoking, vitamin B12, folate and homocysteine; Model 3: Model 2 + respective maternal variable such as a—Model 2 + maternal triglycerides, b—Model 2 + maternal cholesterol, c—Model 2 + maternal HDL, d—Model 2 + maternal LDL, e—Model 2 + maternal insulin, f—Model 2 + maternal glucose, g—Model 2 + maternal homocysteine.

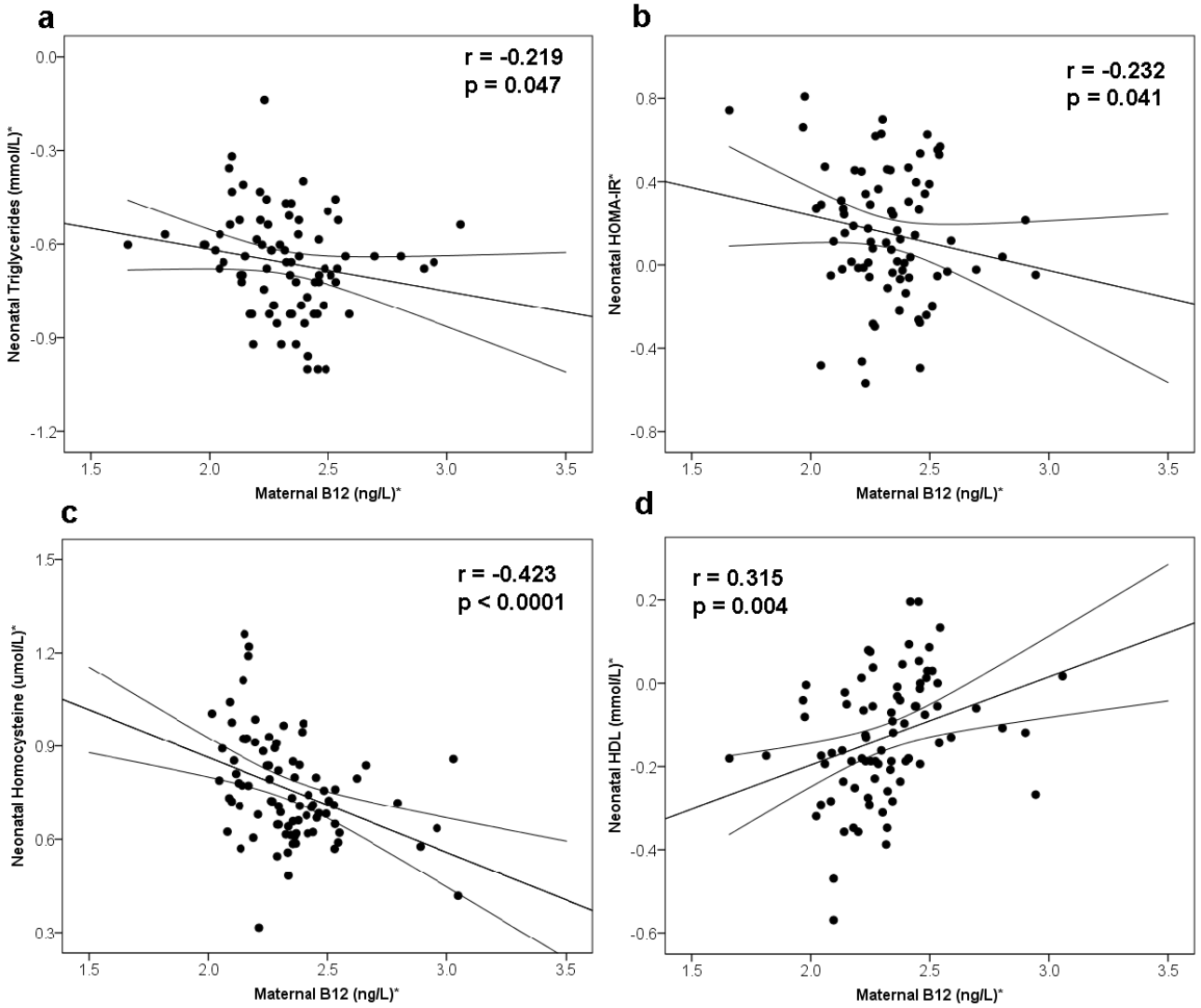



**Figure 1.** Correlation between maternal B12 (adjusted for age and BMI) and metabolic risk factors of neonates. (**a**) Maternal B12 and neonatal triglycerides, (**b**) Maternal B12 and neonatal Homeostasis Model Assessment 2-Insulin Resistance (HOMA-IR), (**c**) Maternal B12 and neonatal homocysteine, (**d**) Maternal B12 and neonatal HDL. ***** Log-transformed for statistical comparisons.

### *3.3. Maternal Folate and Homocysteine and Metabolic Risk of Offspring*

Maternal folate negatively associated with offspring's cholesterol ($r = -0.214$; $p = 0.045$), LDL ($r = -0.233$; $p = 0.030$) and homocysteine ($r = -0.346$; $p = 0.001$). Maternal homocysteine positively associated with offspring's triglycerides ($r = 0.239$; $p = 0.030$), cholesterol ($r = 0.247$; $p = 0.022$) and LDL ($r = 0.244$; $p = 0.026$), however, these associations diminished after adjusting for all likely confounders (Table 4).

## 4. Discussion

Our study is the first to show that maternal vitamin B12 levels adversely associated with markers of metabolic risk at birth, in particular lipid profiles. Our observed rates of low B12 status in mothers (40%)

is common at the time of delivery though it is not as high as in the South Asian population [10,11,26]. Haemodilution and increased nutrient demand by the growing fetus [27] are known contributors to low B12 levels during pregnancy. In addition, consumption of processed foods, improving hygiene and reheating of cooked food, all known to reduce the bioavailable B12 in food products, could have contributed to lower B12 levels in this population [20,28]. The presence of higher homocysteine in the low B12 group suggest that these low levels are clinically significant and represent true insufficiency at the tissue level.

Our findings show that low maternal B12 status was associated with offspring's insulin resistance, lower HDL and higher triglycerides (Figure 1a,b,d). However, when multivariate analysis was used to assess the effect of B12 across the spectrum, only HDL was statistically significant after adjusting for the possible confounders (Table 4). In support of this, adverse lipid profile (higher total cholesterol and triglycerides) was noticed in rats born to vitamin B12 restricted dams [18,19]. In addition, we have recently demonstrated that adipocytes cultured in low B12 condition showed increased cholesterol levels and was due to hypomethylation of cholesterol transcription factor (SREBF1 and LDLR) [29]. The clinical findings observed in this study thus add evidence that low maternal B12 status adversely affects lipid profile in the offspring. We did not see any significant association between maternal and neonatal lipids (data not shown). While this was surprising, it was similar to other observations, where only lipids from GDM mothers associated with foetal lipids and not from non-GDM mothers [30,31].

Our study also showed that maternal B12 showed a stronger inverse association with neonatal homocysteine than folate (Supplementary Figure S2a,b). In multiple regression analysis, after adjusting for the possible confounders, only maternal B12 and not folate, was independently associated with neonatal homocysteine (Table 4). The association between maternal folate and neonatal homocysteine became insignificant, when maternal homocysteine was added in the stepwise regression model (Table 4). This suggests that the effect of folate on neonatal homocysteine is likely to be mediated through maternal homocysteine while the effect of B12 could be partly independent of maternal homocysteine. Similar to our findings, Molloy *et al.* showed in an Irish population that low maternal B12 levels predicted hyperhomocysteinemia in both the newborns and the mothers [32]. Thus, our findings confirm that in folate replete populations, B12 is the strongest driver of homocysteine [10], an established metabolic risk factor [33]. Our study also showed that the BMI was higher in the low B12 group (Table 2). Similar observations were seen other studies [29–31]. The cause and effect of this relationship is not known. Theoretically this could have contributed to higher maternal lipids and in turn higher lipids in cord blood. However, we did not see any correlation between maternal and cord lipids and our regression analysis adjusted for maternal lipids (Table 4).

The plausible biochemical reasons that low maternal B12 status increase the metabolic risk in the offspring might be, firstly, in the cytoplasm, vitamin B12 acts as a cofactor for conversion of homocysteine to methionine, the direct precursor of *S*-adenosylmethionine (SAM) which is the common donor required for methylation of DNA, protein and lipids [10,26,34]. Secondly, in mitochondria, vitamin B12 also acts a cofactor for the conversion of methylmalonyl Co-A (MM-CoA) to succinyl Co-A. Thus, low vitamin B12 causes higher MM-CoA levels. This in turn can inhibit carnitine palmitoyl transferase-1 (CPT-1), the rate-limiting enzyme for fatty acid β-oxidation, thereby increasing lipogenesis [10,35]. As these mechanisms involve methylation of DNA, this might lead to higher metabolic risk in the offspring by adverse epigenetic programming in addition to directly affecting β-

oxidation of fatty acids. *In vivo* and interventional studies are required to identify the exact mechanisms and prove the causality.

Similar to B12, low maternal folate levels also showed adverse correlations with the metabolic risk markers of the offspring but these differences disappeared in regression models. Women with highest B12 and folate levels gave birth to children with lowest homocysteine levels compared to those with lowest B12 and folate levels (7.80 *vs.* 4.85 μmol/L, $p < 0.001$; Supplementary Table S1). Taken together, these findings suggest that optimising the circulating levels of these two B vitamins during pregnancy, is likely to be beneficial to the offspring.

Strengths and limitations: Our study is cross sectional and from a single-centre. However, this is the first study to report the associations between maternal B12 and lipid profiles in the offspring. A prospective cohort of women from before or early pregnancy would have been a better model. As the pathophysiological link, if any, between maternal nutrient status and offspring metabolic risk seem to happen earlier in pregnancy, such longitudinal study would have strengthened our findings [9,10]. Our findings call for such studies to be conducted urgently. Studies have reported that B12 levels progressively decline during pregnancy [36]. Therefore, the effect size we observed during late pregnancy might have been an overestimate if early pregnancy samples were tested. We did not use the microbiological assay for B12 measurements, which is known to be more sensitive at the lower levels of B12. This may have underestimated the rate of low B12 status [37] and in turn, the association with the metabolic risk factors in the offspring. We did not have a detailed socioeconomic status of the participants. It is known that lower socioeconomic status is an important confounder of adverse lipid profiles and BMI but the link between socioeconomic status and B12 is not known. Therefore, this is also a limitation of our observation and future studies should collect detailed socioeconomic status. Finally, although our sample size was adequate to demonstrate the low B12 status, it was probably too small to demonstrate the independent associations between maternal folate and homocysteine status and cord blood lipids.

In summary, our study shows that maternal vitamin B12 plays an important role in lipid metabolism in the offspring and that their restriction *in utero* may predispose them to the increased metabolic risk. However, these findings need to be replicated, ideally in a larger cohort of pregnant women from early pregnancy. In addition, *in vivo* and interventional studies are required to prove the exact mechanisms and the potential causal link. If proven, optimizing B12 levels of young women around the peri-conceptional period, could offer novel opportunities to reduce the burden of obesity and related metabolic disorders of the next generation.

## Acknowledgments

The authors acknowledge all the clinic staffs including the labour ward at University Hospital Coventry Warwickshire (UHCW) and George Eliot Hospital (GEH), the assistance of clinical research nurses (Selvin Selvamoni and Jackie Farmer) and research coordinators (Amitha Gopinath and Karen Rouault) in supporting the recruitment of the women. The funding body did not have any input on the design, objectives or the analysis of the results.

The study was supported by Diabetes Research Fund, George Eliot NHS trust, Nuneaton, UK and RCUK block grant (funding open access publication charges).

## Author Contributions

Adaikala Antonysunil performed the statistical analysis, interpreted the data and drafted the initial manuscript. Manu Vatish contributed to patient recruitment, study design and reviewed the manuscript for intellectual content. Alexander Lawson & Craig Webster involved in the measurement of homocysteine by LC-MS and data collection of all the samples in Heartlands Hospital, Birmingham. Catherine Wood & Neil Anderson involved in the measurement of vitamin B12, folate and data collection of all the samples at George Eliot Hospital, Nuneaton. Kavitha Sivakumar contributed to patient recruitment, sample collection, storage and data collection. Philip G. McTernan co-ordinated and supported the data collection, contributed to the other biochemical analysis and made important contributions to the design of the study and reviewed the manuscript for important intellectual content. Chittaranjan S. Yajnik made important contributions to the design of the study, revised and reviewed the manuscript for important intellectual content. Ponnusamy Saravanan & Gyanendra Tripathi conceived the research question, designed the study, contributed to data interpretation, critically reviewed the manuscript, and approved the final manuscript as submitted. Ponnusamy Saravanan is the guarantor of this work and had full access to all the data presented in the study and takes full responsibility for the integrity and the accuracy of the data analysis. All authors approved the final manuscript as submitted.

## Conflicts of Interest

The authors declare no conflict of interest.

## References

1. Cunningham, S.A.; Kramer, M.R.; Narayan, K.M. Incidence of childhood obesity in the united states. *N. Engl. J. Med.* **2014**, *370*, 403–411.
2. Weiss, R.; Kaufman, F.R. Metabolic complications of childhood obesity: Identifying and mitigating the risk. *Diabetes Care* **2008**, *31*, S310–S316.
3. Ignell, C.; Claesson, R.; Anderberg, E.; Berntorp, K. Trends in the prevalence of gestational diabetes mellitus in southern sweden, 2003–2012. *Acta Obstet. Gynecol. Scand.* **2014**, *93*, 420–424.
4. Lawlor, D.A.; Benfield, L.; Logue, J.; Tilling, K.; Howe, L.D.; Fraser, A.; Cherry, L.; Watt, P.; Ness, A.R.; Davey Smith, G.; *et al.* Association between general and central adiposity in childhood, and change in these, with cardiovascular risk factors in adolescence: Prospective cohort study. *BMJ* **2010**, *341*, doi:10.1136/bmj.c6224.
5. Nayak, C.D.; Agarwal, V.; Nayak, D.M. Correlation of cord blood lipid heterogeneity in neonates with their anthropometry at birth. *Indian J. Clin. Biochem. IJCB* **2013**, *28*, 152–157.
6. Whincup, P.H.; Kaye, S.J.; Owen, C.G.; Huxley, R.; Cook, D.G.; Anazawa, S.; Barrett-Connor, E.; Bhargava, S.K.; Birgisdottir, B.E.; Carlsson, S.; *et al.* Birth weight and risk of type 2 diabetes: A systematic review. *JAMA* **2008**, *300*, 2886–2897.
7. Pettitt, D.J.; Jovanovic, L. Low birth weight as a risk factor for gestational diabetes, diabetes, and impaired glucose tolerance during pregnancy. *Diabetes Care* **2007**, *30* (Suppl. 2), S147–S149.
8. Barker, D.J.; Osmond, C.; Kajantie, E.; Eriksson, J.G. Growth and chronic disease: Findings in the Helsinki birth cohort. *Ann. Hum. Biol.* **2009**, *36*, 445–458.

9. Roseboom, T.J.; Painter, R.C.; van Abeelen, A.F.; Veenendaal, M.V.; de Rooij, S.R. Hungry in the womb: What are the consequences? Lessons from the dutch famine. *Maturitas* **2011**, *70*, 141–145.
10. Yajnik, C.S.; Deshpande, S.S.; Jackson, A.A.; Refsum, H.; Rao, S.; Fisher, D.J.; Bhat, D.S.; Naik, S.S.; Coyaji, K.J.; Joglekar, C.V.; *et al.* Vitamin B12 and folate concentrations during pregnancy and insulin resistance in the offspring: The pune maternal nutrition study. *Diabetologia* **2008**, *51*, 29–38.
11. Stewart, C.P.; Christian, P.; Schulze, K.J.; Arguello, M.; LeClerq, S.C.; Khatry, S.K.; West, K.P. Low maternal vitamin B-12 status is associated with offspring insulin resistance regardless of antenatal micronutrient supplementation in rural Nepal. *J. Nutr.* **2011**, *141*, 1912–1917.
12. Krishnaveni, G.V.; Veena, S.R.; Karat, S.C.; Yajnik, C.S.; Fall, C.H. Association between maternal folate concentrations during pregnancy and insulin resistance in Indian children. *Diabetologia* **2014**, *57*, 110–121.
13. Dwarkanath, P.; Barzilay, J.R.; Thomas, T.; Thomas, A.; Bhat, S.; Kurpad, A.V. High folate and low vitamin B-12 intakes during pregnancy are associated with small-for-gestational age infants in South Indian women: A prospective observational cohort study. *Am. J. Clin. Nutr.* **2013**, *98*, 1450–1458.
14. Saravanan, P.; Yajnik, C.S. Role of maternal vitamin B12 on the metabolic health of the offspring: A contributor to the diabetes epidemic? *Br. J. Diabetes Vasc. Disease* **2010**, *10*, 109–114.
15. Guerra-Shinohara, E.M.; Morita, O.E.; Peres, S.; Pagliusi, R.A.; Sampaio Neto, L.F.; D'Almeida, V.; Irazusta, S.P.; Allen, R.H.; Stabler, S.P. Low ratio of *S*-adenosylmethionine to *S*-adenosylhomocysteine is associated with vitamin deficiency in Brazilian pregnant women and newborns. *Am. J. Clin. Nutr.* **2004**, *80*, 1312–1321.
16. Ba, Y.; Yu, H.; Liu, F.; Geng, X.; Zhu, C.; Zhu, Q.; Zheng, T.; Ma, S.; Wang, G.; Li, Z.; *et al.* Relationship of folate, vitamin B12 and methylation of insulin-like growth factor-II in maternal and cord blood. *Eur. J. Clin. Nutr.* **2011**, *65*, 480–485.
17. Sinclair, K.D.; Allegrucci, C.; Singh, R.; Gardner, D.S.; Sebastian, S.; Bispham, J.; Thurston, A.; Huntley, J.F.; Rees, W.D.; Maloney, C.A.; *et al.* DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 19351–19356.
18. Garcia, M.M.; Gueant-Rodriguez, R.M.; Pooya, S.; Brachet, P.; Alberto, J.M.; Jeannesson, E.; Maskali, F.; Gueguen, N.; Marie, P.Y.; Lacolley, P.; *et al.* Methyl donor deficiency induces cardiomyopathy through altered methylation/acetylation of PGC-1alpha by PRMT1 and SIRT1. *J. Pathol.* **2011**, *225*, 324–335.
19. Kumar, K.A.; Lalitha, A.; Pavithra, D.; Padmavathi, I.J.; Ganeshan, M.; Rao, K.R.; Venu, L.; Balakrishna, N.; Shanker, N.H.; Reddy, S.U.; *et al.* Maternal dietary folate and/or vitamin B12 restrictions alter body composition (adiposity) and lipid metabolism in Wistar rat offspring. *J. Nutr. Biochem.* **2013**, *24*, 25–31.
20. Adaikalakoteswari, A.; Jayashri, R.; Sukumar, N.; Venkataraman, H.; Pradeepa, R.; Gokulakrishnan, K.; Anjana, R.; McTernan, P.G.; Tripathi, G.; Patel, V.; *et al.* Vitamin B12 deficiency is associated with adverse lipid profile in Europeans and Indians with type 2 diabetes. *Cardiovasc. Diabetol.* **2014**, *13*, doi:10.1186/s12933-014-0129-4.

21. Saravanan, P.; Maheshwaran, H.; Stranges, S.; Thorogood, M. Population levels of B12 and folic acid: Do we need to consider B12 fortification to prevent gestational diabetes and cardiovascular risk? *Diabet. Med.* **2010**, *27* (Suppl. 1), 37–188.
22. Saravanan, P.; Wood, C.; Andersen, N. B12 deficiency is more common than folate deficiency in early pregnancy: Do we need to consider B12 fortification? *Diabetologia* **2010**, *53*, S151–S152.
23. Milman, N.; Byg, K.E.; Bergholt, T.; Eriksen, L.; Hvas, A.M. Cobalamin status during normal pregnancy and postpartum: A longitudinal study comprising 406 Danish women. *Eur. J. Haematol.* **2006**, *76*, 521–525.
24. Milman, N.; Byg, K.E.; Hvas, A.M.; Bergholt, T.; Eriksen, L. Erythrocyte folate, plasma folate and plasma homocysteine during normal pregnancy and postpartum: A longitudinal study comprising 404 Danish women. *Eur. J. Haematol.* **2006**, *76*, 200–205.
25. Magera, M.J.; Lacey, J.M.; Casetta, B.; Rinaldo, P. Method for the determination of total homocysteine in plasma and urine by stable isotope dilution and electrospray tandem mass spectrometry. *Clin. Chem.* **1999**, *45*, 1517–1522.
26. Finer, S.; Saravanan, P.; Hitman, G.; Yajnik, C. The role of the one-carbon cycle in the developmental origins of type 2 diabetes and obesity. *Diabet. Med.* **2014**, *31*, 263–272.
27. Wallace, J.M.; Bonham, M.P.; Strain, J.; Duffy, E.M.; Robson, P.J.; Ward, M.; McNulty, H.; Davidson, P.W.; Myers, G.J.; Shamlaye, C.F.; *et al.* Homocysteine concentration, related B vitamins, and betaine in pregnant women recruited to the Seychelles child development study. *Am. J. Clin. Nutr.* **2008**, *87*, 391–397.
28. United States Department of Agriculture. USDA Table of Nutrient Retention Factors, Release 5. 2003. Available online: http://www.ars.usda.gov/Main/docs.htm?docid=9448 (accessed on 27 March 2015).
29. Adaikalakoteswari, A.; Finer, S.; Voyias, P.D.; McCarthy, C.M.; Vatish, M.; Moore, J.; Smart-Halajko, M.; Bawazeer, N.; Al-Daghri, N.M.; McTernan, P.G.; *et al.* Vitamin B12 insufficiency induces cholesterol biosynthesis by limiting *S*-adenosylmethionine and modulating the methylation of SREBF1 and LDLR genes. *Clin. Epigenetics* **2015**, *7*, doi:10.1186/s13148-015-0046-8.
30. Schaefer-Graf, U.M.; Graf, K.; Kulbacka, I.; Kjos, S.L.; Dudenhausen, J.; Vetter, K.; Herrera, E. Maternal lipids as strong determinants of fetal environment and growth in pregnancies with gestational diabetes mellitus. *Diabetes Care* **2008**, *31*, 1858–1863.
31. Schaefer-Graf, U.M.; Meitzner, K.; Ortega-Senovilla, H.; Graf, K.; Vetter, K.; Abou-Dakn, M.; Herrera, E. Differences in the implications of maternal lipids on fetal metabolism and growth between gestational diabetes mellitus and control pregnancies. *Diabet. Med.* **2011**, *28*, 1053–1059.
32. Molloy, A.M.; Mills, J.L.; Cox, C.; Daly, S.F.; Conley, M.; Brody, L.C.; Kirke, P.N.; Scott, J.M.; Ueland, P.M. Choline and homocysteine interrelations in umbilical cord and maternal plasma at delivery. *Am. J. Clin. Nutr.* **2005**, *82*, 836–842.
33. Wald, D.S.; Law, M.; Morris, J.K. Homocysteine and cardiovascular disease: Evidence on causality from a meta-analysis. *BMJ* **2002**, *325*, 1202.
34. Dominguez-Salas, P.; Cox, S.E.; Prentice, A.M.; Hennig, B.J.; Moore, S.E. Maternal nutritional status, C(1) metabolism and offspring DNA methylation: A review of current evidence in human subjects. *Proc. Nutr. Soc.* **2012**, *71*, 154–165.

35. Brindle, N.P.; Zammit, V.A.; Pogson, C.I. Regulation of carnitine palmitoyltransferase activity by malonyl-CoA in mitochondria from sheep liver, a tissue with a low capacity for fatty acid synthesis. *Biochem. J.* **1985**, *232*, 177–182.
36. Cikot, R.J.; Steegers-Theunissen, R.P.; Thomas, C.M.; de Boo, T.M.; Merkus, H.M.; Steegers, E.A. Longitudinal vitamin and homocysteine levels in normal pregnancy. *Br. J. Nutr.* **2001**, *85*, 49–58.
37. Carmel, R.; Agrawal, Y.P. Failures of cobalamin assays in pernicious anemia. *N. Engl. J. Med.* **2012**, *367*, 385–386.

# Lipaemia: an overrated interference?

N.R. ANDERSON, S. SLIM, R. GAMA and M.R. HOLLAND
*Department of Clinical Chemistry, New Cross Hospital, Wolverhampton WV10 0QP, UK*

*Accepted: 23 July 2003*

**ABSTRACT**

Reagent method sheets for analysis of common serum analytes often highlight the possibility of interference from lipaemia but the information given is often brief and may not be instrument-specific. Thus study assesses the degree of interference from lipaemia in a range of common serum analytes on the Bayer Opera (with a serum blank) using a commercial polymer, LipoClear, as a lipid-clearing agent. Serum samples (mean serum triglyceride 6.89 [range 0.58–28.4] mmol/L) are analysed for 14 common chemistry analytes and the results compared before and after treatment with LipoClear. Results showed no significant critical differences in analyte values before and after treatment, except for an expected fall in total protein, phosphate, cholesterol and triglyceride concentrations. Most of the common analytes in use on the Bayer Opera are not subject to interference from lipaemia; however, we recommend that where method sheets indicate interference from lipaemia then this should be quantified for the analyte in question.

KEY WORDS: Chemistry. Lipids and antilipemic agents. Triglycerides.

## Introduction

Lipaemia is reported to interfere in many routine assays. Many reagent suppliers provide information on the effect of lipaemia in their assays, but this is often vague, not quantified and may not be instrument-specific.[1,2]

Lipaemia, like haemolysis and icterus, causes chromophoric interference in photometric analyses due to high background readings, interference at the measured wavelength and light scattering caused by the interfering substance.[2,3] The interference may be dose-dependent for some analytes but not for all.[1]

The interference from lipaemia can be minimised in a number of ways, including the use of a sample blank reading, kinetic analysis, changing the wavelength at which the reaction is read to one at which there is minimal absorbance from the interferant,[4,5] and the use of commercial preparations that clear the lipid content from serum.[6]

In the laboratory setting, staff use different methods – such as visual inspection, lipaemia index, serum indices and triglyceride concentration – to determine the degree of turbidity from lipaemia. These assessments, however, may be inaccurate as the degree of interference from lipaemia is method- and instrument-dependent.[1-3]

The aim of this study, therefore, is to evaluate the effects of lipaemia and LipoClear, a non-toxic polymer for serum lipid clearance, on 14 tests commonly analysed on the Bayer Opera analyser, prior to the introduction of LipoClear into our routine laboratory repertoire.

## Materials and methods

A total of 14 analytes were measured in up to 44 serum samples (Table 1) with either no lipaemia or varying degrees of lipaemia (mean serum triglyceride 6.89 [range 0.58–28.4] mmol/L) using methods recommended for use by the instrument manufacturer. Twelve samples had serum triglyceride ≤2 mmol/L; 20 samples had a serum triglyceride >2 and ≤10 mmol/L; and 12 samples had a serum triglyceride >10 mmol/L. Each analyte was determined before and after treatment with LipoClear. (*phi*Tec International, UK) on a Bayer Opera analyser (Bayer AG, Germany).

A 0.5 mL serum sample was added to 0.1 mL LipoClear, mixed and left to stand for 5 min. The mixture was centrifuged and the supernatant analysed. Results were multiplied by 1.2 to correct for the initial dilution.

Significance of differences before and after the use of LipoClear were calculated using the paired *t*-test and Wilcoxon matched pairs for parametric and non-parametric data, respectively. Changes in results were assessed as being significant if the difference between results was greater than 2.8 times the analytical coefficient of variation (CV).[7] This is derived from an application of the *t*-test, and indicates (if the difference between results is greater than 2.8 times the analytical CV) that there is a less than 5% chance of that being due to random variation.

## Results

With the exception of alanine transaminase (ALT), amylase and bicarbonate, significant differences in the other analyte values before and after treatment with LipoClear were seen using standard statistical techniques (Table 2).

When analytical CV was taken into account, only phosphate, total protein, cholesterol and triglyceride showed significant analytical change (Table 3).

## Discussion

Most methodologies used on the Bayer Opera appeared to be subject to statistically significant interference from lipaemia when evaluated by standard statistical methods,

*Correspondence to: Mr Neil Anderson*
*Email: neil.anderson@rwh-tr.nhs.uk*

**Table 1.** Method details for analytes in the study

| Analyte | Reagent supplier | Method | Package insert details on interference from lipaemia |
|---|---|---|---|
| Urea | Bayer | Kinetic, urease | No significant interference |
| Creatinine | Bayer | Kinetic, Jaffe | No information given |
| Albumin | Bayer | BCG succinate | No significant interference |
| Alkaline phosphatase | Bayer | DEA-optimised, 37°C | No significant interference |
| Alanine transaminase | Bayer | IFCC-optimised | Positive interference |
| Amylase | Bayer | CNPG3, 37°C | No interference up to triglyceride of 10.5 mmol/L |
| Phosphate | Bayer | Phosphomolybdate, | UV No information given |
| Glucose | Bayer | Glucose oxidase | Positive interference |
| Bicarbonate | Trace | Phosphoenol pyruvate carboxylase | Positive interference |
| Total protein | Bayer | Biuret | Positive interference |
| Total bilirubin | Bayer | Diazo | No significant interference |
| Calcium | Randox | Arsenazo | Positive interference |
| Triglyceride | Bio-Stat | Enzymic endpoint | Positive interference |
| Cholesterol | Bio-Stat | Cholesterol oxidase | Positive interference |

**Table 2.** Mean or median analyte results before and after lipid extraction

| Analyte | Number tested | Analyte range | Mean or median before lipid extraction | Mean or median after lipid extraction |
|---|---|---|---|---|
| Urea (mmol/L) | 44 | 1.6–32.9 | 6.8 (4.8–8.6) | 7.1 (5.1–9.0)*** |
| Creatinine (μmol/L) | 44 | 75–854 | 146 (104–187) | 149 (106–191)* |
| Albumin (g/L) | 44 | 21–60 | 42 (39–44) | 41 (38–43)* |
| Alkaline phosphatase (iu/L) | 37 | 92–546 | 211 (174–247) | 209 (175–242)* |
| Alanine transaminase (iu/L) | 38 | 5–150 | 28 (20–35) | 28 (19–35) |
| Amylase (iu/L) | 44 | 3–96 | 47 (20) | 47 (21) |
| Phosphate (mmol/L) | 39 | 0.77–2.38 | 1.37 (0.40) | 1.24 (0.22) * |
| Glucose (mmol/L) | 43 | 3.4–33.4 | 9.0 (7.1–10.8) | 9.3 (7.3–11.2)*** |
| Bicarbonate (mmol/L) | 30 | 9–29 | 20.7 (5.2) | 21.7 (4.5) |
| Total protein (g/L) | 44 | 59–138 | 76 (14.0) | 65 (6.0)*** |
| Total bilirubin (μmol/L) | 43 | 2–233 | 23 (8.1–38) | 22 (7.4–37)*** |
| Calcium (mmol/L) | 44 | 1.24–2.94 | 2.35 (2.26–2.44) | 2.26 (2.18–2.35)*** |
| Triglyceride (mmol/L) | 44 | 0.58–28 | 5.28 (4.73–9.07) | 1.71 (1.41–2.25)*** |
| Cholesterol (mmol/L) | 44 | 1.7–9.3 | 5.7 (1.7) | 2.6 (1.2)*** |

Results reported as: mean (standard deviation) for parametric distribution, median (95% confidence intervals) for non-parametric distribution.
* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

but these do not consider the analytical imprecision of assays. When the analytical CV was taken into account, most of the differences failed to achieve critical significance. Only phosphate, protein, cholesterol and triglyceride values remained critically different after the addition of LipoClear.

These findings support a previous study of LipoClear[6] and were expected as LipoClear, a non-ionic polymer, precipitates lipoproteins and phospholipids. Lipaemia did not critically affect measurement of other analytes, probably because the Bayer Opera performs an initial blank reading at the start of the reaction, supporting previous reports recommending the use of serum blanks in minimising lipaemic interference.[1,2]

However, these results are at variance with the manufacturer's method sheets, which indicate lipaemia interference with ALT, amylase, glucose, bicarbonate and calcium methods (Table 1).

It is possible that the manufacturer does not take into account assay imprecision in assessing lipaemic interference. The difference in the results for the tests for significance used is that, for the Mann Whitney and *t*-tests, individual paired points are compared which are considered to have no imprecision. When the calculation of significant change in test results is undertaken, however, the imprecision of the test results is also taken into account. Hence, the apparent significant differences.

**Table 3.** Comparison of analyte coefficients of variation with % difference in analyte result before and after lipid extraction

| Analyte | Analytical CV | 2.8x CV | % Difference | Significance |
|---|---|---|---|---|
| Urea | 2.5 | 7.0 | +4.4 | Not significant |
| Creatinine | 2.6 | 7.3 | +2.1 | Not significant |
| Albumin | 1.3 | 3.6 | –2.4 | Not significant |
| Alkaline phosphatase | 4.9 | 13.7 | –1.0 | Not significant |
| Alanine transaminase | 3.0 | 8.4 | 0 | Not significant |
| Amylase | 3.5 | 9.8 | 0 | Not significant |
| Phosphate | 1.6 | 4.5 | –9.5 | Significant |
| Glucose | 2.6 | 7.3 | +3.3 | Not significant |
| Bicarbonate | 4.6 | 12.9 | +4.8 | Not significant |
| Total protein | 2.3 | 5.6 | –14.5 | Significant |
| Total bilirubin | 3.7 | 10.4 | +4.5 | Not significant |
| Calcium | 2.1 | 5.9 | –3.9 | Not significant |
| Triglyceride | 1.2 | 3.4 | –74 | Significant |
| Cholesterol | 2.5 | 7.0 | –54 | Significant |

In conclusion, LipoClear does reduce lipaemia but most methodologies are often sufficiently robust to avoid interference from lipaemia. Therefore, we recommend that individual laboratories quantify interference from lipaemia for their specific methods and instruments, as the interference could be analyser- and/or reagent-specific. Only if there is significant interference should the use of lipid clearing agents be considered. □

## References


1 Glick MR, Ryder KW. Analytical systems ranked by freedom from interferences. *Clin Chem* 1987; **33**: 1453–8.

2 Glick MR, Ryder KW, Jackson SA. Graphical comparisons of interferences in clinical chemistry instrumentation. *Clin Chem* 1986; **32**: 470–5.

3 Brady J, O'Leary N. Interference due to lipaemia in routine photometric analysis – survey of an underrated problem. *Ann Clin Biochem* 1994; **31**: 281–8.

4 O'Leary N, Pembrooke A, Duggan PF. Improving accuracy of glucose oxidase procedure for glucose determinations on discrete analysers. *Clin Chem* 1992; **38**: 298–302.

5 Randall AG, Garcia-Webb P, Beilby JP. Interference by haemolysis, icterus and lipaemia in the assays on the Beckman Synchron CX5 and methods for correction. *Ann Clin Biochem* 1990; **27**: 345–52.

6 Rogers LC, Smith FA. Evaluation of LipoClear, a non-ionic polymer system, for the removal of interfering lipaemia. *Clin Chem* 1989; **35**: 1103.

7 Jones R, Payne B. Data for diagnosis and monitoring. In: McCreary RG, Sherwood R, eds. *Clinical investigation and statistics*. ACB Venture Publications, 1997: 66–88.

Rheumatology Advance Access published July 20, 2004

 doi:10.1093/rheumatology/keh307

## Concise report

# Raised serum prolactin in rheumatoid arthritis: genuine or laboratory artefact?

S. Ram, D. Blumberg[1], P. Newton[1], N. R. Anderson and R. Gama

***Objectives*. Serum prolactin concentrations have been reported as higher, similar or lower in patients with rheumatoid arthritis (RA) compared with control subjects. We investigated whether low biological activity macroprolactin (a prolactin antibody complex), which is detected variably in different prolactin immunoassays, could account for the discrepant total prolactin results reported in RA.**

***Methods*. We compared serum total prolactin and free prolactin in 60 women with RA and 31 female controls.**

***Results*. No subject had hyperprolactinaemia or macroprolactinaemia. Serum concentrations of total and free (monomeric) prolactin were higher ($P < 0.05$) in women with RA [mean (S.D.), 225.6 (104.6) and 201.6 (95.4) mU/l respectively] compared with controls [175.0 (68.5) and 154.0 (60.9) mU/l respectively].**

***Conclusions*. We report higher serum free prolactin concentrations in women with RA compared with control subjects. This result indicates that the higher serum total prolactin levels in patients with RA are the consequence of increased free prolactin concentrations and are not due to macroprolactin.**

KEY WORDS: Prolactin, Macroprolactin, Big prolactin, Big big prolactin, Rheumatoid arthritis.

It has been suggested that prolactin may have a pathogenetic role in rheumatoid arthritis (RA), since *in vitro* studies have shown that prolactin enhances inflammatory responses [1–4]. Indeed, increased serum prolactin concentrations have been reported in patients with RA [5–10]. Furthermore, dopamine agonists, which suppress pituitary secretion of prolactin, may be a useful adjunct to treatment in patients with RA [11–13]. Prolactin circulates in several different molecular forms, predominantly monomeric prolactin but also as small but variable amounts of 'big prolactin' and 'big, big prolactin' (macroprolactin). The binding of prolactin to an immunoglobulin forms macroprolactin. Since macroprolactin is less physiologically active and is less effectively cleared than free unbound prolactin, the total concentration of serum prolactin increases. Depending on the immunoassay used, macroprolactin may account for 4–5% of cases of hyperprolactinaemia [14, 15] and has also been reported in normoprolactinaemic samples [16].

Systemic lupus erythematosus (SLE), an autoimmune disease with an increased prevalence of serum autoantibodies, is associated with macroprolactinaemia [16, 17]. RA is also associated with an increased frequency of circulating antibodies and macroprolactin has been reported in patients with RA [18]. It is therefore possible that macroprolactin could account for the hyperprolactinaemia observed in RA, particularly since increased serum prolactin in RA has been reported in most [5–10] but not all [19–23] studies, possibly due to the use of different prolactin assays with varying macroprolactin cross-reactivity [24, 25].

This is the first report investigating the contribution of low biological activity macroprolactin to circulating prolactin in RA by comparing total and free (monomeric) prolactin concentrations in women with and without RA.

## Material and methods

### *Patients*

Sixty women with RA and 31 women with osteoarthritis, who served as controls, were studied. A blood sample for serum prolactin was collected at least 2 h after awakening, between 09.30 and 12.00 h. Exclusion criteria included endocrine and any other disease or medication known to affect serum prolactin concentrations. All women gave informed written consent according to the Declaration of Helsinki to participate in this study, which was approved by the Wolverhampton District Local Research Ethics Committee.

### *Analytical methods*

Polyethylene glycol (PEG) was used to precipitate and remove big prolactin and macroprolactin from serum samples [26, 27]. Serum prolactin was therefore measured before (total prolactin) and after (free or monomeric prolactin) precipitation with PEG 6000 [27] using the Architect prolactin assay (Abbott Laboratories, Diagnostics Division, IL, USA). Respective inter-assay and intra-assay coefficients of variation for serum prolactin were 4.7 and 3.8%. Prolactin recovery <40% is indicative of macroprolactin [27].

### *Statistical analysis*

Data were normally distributed. Unpaired and paired Student's *t* tests were therefore used to assess differences in variables between groups and within groups respectively. Results are expressed as

**Departments of Clinical Chemistry and [1]Rheumatology, New Cross Hospital, Wolverhampton, West Midlands WV10 0QP, UK.**

Submitted 8 March 2004; revised version accepted 15 June 2004.

Correspondence to: R. Gama, Clinical Chemistry, New Cross Hospital, Wolverhampton, West Midlands. WV10 0QP, UK. E-mail: rousseau.gama@rwh-tr.nhs.uk

TABLE 1. Clinical and biochemical characteristics of women with rheumatoid arthritis and their controls

| | RA | Controls | *P* |
|---|---|---|---|
| Number of subjects | 60 | 31 | |
| Age (yr) | 56.7 (12.9) | 53.1 (16.1) | 0.2987 |
| ESR (mm/h) | 27.7 (19.7) | – | – |
| Total prolactin (mU/l) | 225.6 (104.6) | 175.0 (68.5) | 0.0387 |
| Free prolactin (mU/l) | 201.6 (95.4) | 154.0 (60.9) | 0.0127 |

Data are mean (S.D.).

mean (S.D.). Pearson's linear correlation was used to measure the significance of association between variables.

## Results

Demographic, clinical and biochemical data are shown in Table 1. No subjects had hyperprolactinaemia or macroprolactinaemia. Serum concentrations of total and free (monomeric) prolactin were higher ($P < 0.05$) in women with RA [225.6 (104.6) and 201.6 (95.4) mU/l respectively] than in controls [175.0 (68.5) and 154.0 (60.9) mU/l respectively]. Although serum prolactin fell following PEG precipitation in women with RA and controls, this was not significant ($P = 0.1936$ and $P = 0.2877$ respectively).

In women with RA, there were no correlations between ESR and total prolactin ($r = 0.1163$; $P = 0.3762$) or free prolactin ($r = 0.07725$; $P = 0.5574$), but ESR inversely correlated with the percentage of free prolactin of the total prolactin ($r = -0.3278$; $P = 0.0106$).

## Discussion

Although no subjects in this study had hyperprolactinaemia, we report higher serum prolactin concentrations in women with RA compared with controls. This result is consistent with other studies similarly reporting higher total prolactin concentrations in patients with RA than in controls [5–10], but differs from studies reporting similar [19, 20] or lower [21, 22] prolactin concentrations in patients with RA. In addition we report for the first time higher free prolactin concentrations in subjects with RA. It is therefore unlikely that the different total prolactin concentrations reported in various studies are solely due to varying macroprolactin cross-reactivity in different prolactin assays. The difference in these studies, however, could be due to selection of patient groups and type 1 and 2 statistical errors.

Macroprolactinaemia is well recognized in SLE [16, 17] and the difference in the prevalence of macroprolactinaemia between SLE and RA may be related to the type of circulating immunoglobulin (Ig). Macroprolactin is usually formed by the binding of prolactin to an IgG antibody. In RA the predominant circulating immunoglobulin is an IgM antibody, which may not bind to prolactin.

Prolactin has a role in immunomodulation and it has been proposed that prolactin is a risk factor for the development of autoimmunity [1–4]. However, it remains unclear whether the higher prolactin concentrations are the cause or consequence of RA. There is increased risk of developing RA post-partum and this further increases five-fold if breastfeeding [28–30] and disease activity in RA improve with dopamine agonists [11–13]. This suggests that prolactin may have a role in the pathogenesis, or at least the modulation, of disease activity in RA. However, stress increases serum prolactin concentration. It is therefore possible that stress associated with RA may be responsible for the increased prolactin concentrations in RA, but this is unlikely because cortisol, which also increases with stress, has been reported to be similar or lower in subjects with RA than in controls [10, 20, 21].

In summary, we confirm higher free prolactin concentrations in subjects with RA. We have found no evidence to support the notion that low biological activity macroprolactin contributes to the elevated prolactin concentrations observed in RA.

The authors have declared no conflicts of interest.

## References


1. Neidhart M. Prolactin in autoimmune diseases. Proc Soc Exp Biol Med 1998;217:408–19.
2. Matera L, Mori M, Geuna M, Buttiglieri S, Palaestro G. Prolactin in autoimmunity and antitumor defence. J Neuroimmunol 2000;109: 47–55.
3. Neidhart M, Gay RE, Gay S. Prolactin and prolactin-like polypeptides in rheumatoid arthritis. Biomed Pharmacother 1999;53:218–22.
4. Jara LJ, Lavalle C, Fraga A *et al*. Prolactin, immunoregulation, and autoimmune diseases. Semin Arthritis Rheum 1991;20:273–84.
5. Walker SE, Jacobson JD. Roles of prolactin and gonadotropin-releasing hormone in rheumatic diseases. Rheum Dis Clin North Am 2000;26:713–36.
6. Seriolo B, Ferretti V, Sulli A, Fasciolo D, Cutolo M. Serum prolactin concentrations in male patients with rheumatoid arthritis. Ann N Y Acad Sci 2002;966:258–62.
7. Jorgensen C, Maziad H, Bologna C, Sany J. Kinetics of prolactin release in rheumatoid arthritis. Clin Exp Rheumatol 1995;13:705–9.
8. Mateo L, Nolla JM, Bonnin MR, Navarro MA, Roig-Escofet D. High serum prolactin levels in men with rheumatoid arthritis. J Rheumatol 1998;25:2077–82.
9. Mateo L, Nolla JM. Prolactin in rheumatoid arthritis. Lupus 1999;8:251.
10. Zoli A, Lizzio MM, Ferlisi EM *et al*. ACTH, cortisol and prolactin in active rheumatoid arthritis. Clin Rheumatol 2002;21:289–93.
11. McMurray RW. Bromocriptine in rheumatic and autoimmune disease. Semin Arthritis Rheum 2001;31:21–32.
12. Erb N, Pace AV, Delamere JP, Kitas GD. Control of unremitting rheumatoid arthritis by the prolactin antagonist cabergoline. Rheumatology 2001;40:237–9.
13. Eijsbouts A, van den Hoogen F, Laan RF, Hermus RM, Sweep FC, van de Putte L. Treatment of rheumatoid arthritis with the dopamine agonist quinagolide. J Rheumatol 1999;26:2284–5.
14. Miyai K, Ichihara K, Kondo K, Mori S. Asymptomatic hyperprolactinaemia and prolactinoma in general population—mass screening by paired assays of serum prolactins. Clin Endocrinol 1986;25:549–54.
15. Fahie-Wilson M. In hyperprolactinaemia, testing for macroprolactin is essential. Clin Chem 2003;49:1434–6
16. Ram S, Anderson NR, Elfatih A, Gama R. Do normoprolactinaemic samples contain macroprolactin? In: Martin SM, ed. Proceedings of the ACB National Meeting. London: Association of Clinical Biochemists (UK), 2003: 80.
17. Leanos-Miranda A, Pascoe-Lira D, Chavez-Rueda KA, Blanco-Favela F. Antiprolactin autoantibodies in systemic lupus erythematosus: frequency and correlation with prolactinemia and disease activity. J Rheumatol 2001;28:1546–53
18. Liederman S, Garfinkiel M, Damilano S *et al*. Prolactin and IgG–prolactin complex levels in patients with rheumatic arthritis. Ann N Y Acad Sci 2002;966:252–7.
19. Leanos A, Pascoe D, Fraga A, Blanco-Favela F. Anti-prolactin autoantibodies in systemic lupus erythematosus patients with associated hyperprolactinemia. Lupus 1998;7:398–403.
20. Gutierrez MA, Garcia ME, Rodriguez JA, Mardonez G, Jacobelli S, Rivero S. Hypothalamic–pituitary–adrenal axis function in patients with active rheumatoid arthritis: a controlled study using insulin hypoglycemia stress test and prolactin stimulation. J Rheumatol 1999;26:277–81.
21. Rovensky J, Bakosova J, Koska J, Ksinantova L, Jezova D, Vigas M. Somatotropic, lactotropic and adrenocortical responses to

insulin-induced hypoglycemia in patients with rheumatoid arthritis. Ann N Y Acad Sci 2002;966:263–70.
22. Berczi I, Cosby H, Hunter T, Baragar F, McNeilly AS, Friesen HG. Decreased bioactivity of circulating prolactin in patients with rheumatoid arthritis. Br J Rheumatol 1987;26:433–6.
23. Nagy E, Chalmers IM, Baragar FD, Friesen HG, Berczi I. Prolactin deficiency in rheumatoid arthritis. J Rheumatol 1991;18:1662–8.
24. Schneider W, Marcovitz S, Al-Shammari S, Yago S, Chevalier S. Reactivity of macroprolactin in common automated immunoassays. Clin Biochem 2001;34:469–73.
25. Smith TP, Suliman AM, Fahie-Wilson MN, McKenna TJ. Gross variability in the detection of prolactin in sera containing big big prolactin (macroprolactin) by commercial immunoassays. J Clin Endocrinol Metab 2002;87:5410–15.
26. Suliman AM, Smith TP, Gibney J, McKenna TJ. Frequent misdiagnosis and mismanagement of hyperprolactinemic patients before the introduction of macroprolactin screening: application of a new strict definition of macroprolactinemia. Clin Chem 2003;49:1504–9.
27. Fahie-Wilson MN. Polyethylene glycol precipitation as a screening method for macroprolactinaemia. Clin Chem 1999;45:436–9.
28. Hampl JS, Papa DJ. Breastfeeding-related onset, flare, and relapse of rheumatoid arthritis. Nutr Rev 2001;59:264–8.
29. Ostensen M. Sex hormones and pregnancy in rheumatoid arthritis and systemic lupus erythematosus. Ann N Y Acad Sci 1999;876:131–43.
30. Brennan P, Ollier B, Worthington J, Hajeer A, Silman A. Are both genetic and reproductive associations with rheumatoid arthritis linked to prolactin? Lancet 1996;348:106–9.

# Pseudo-pseudohypercalcaemia, apparent primary hyperparathyroidism and Waldenström's macroglobulinaemia

**A Elfatih, N R Anderson, M N Fahie-Wilson, R Gama**

*J Clin Pathol* 2007;**60**:436–437. doi: 10.1136/jcp.2006.036756

An apparent primary hyperparathyroidism was reported due to pseudo-pseudohypercalcaemia in a 74–year-old man with Waldenström's macroglobulinaemia. It is important to recognise artificially elevated serum calcium levels so as to avoid erroneous diagnosis, unnecessary investigations and potentially inappropriate treatment.

Waldenström's macroglobulinaemia is a malignant disorder of B lymphocytes characterised by high serum concentrations of monoclonal IgM, a lymphoplasmacytic infiltrate of the bone marrow and an elevated serum viscosity.[1 2] Hypercalcaemia is unusual in Waldenström's macroglobulinaemia[2] but a characteristic feature of primary hyperparathyroidism. Although Waldenström's macroglobulinaemia is uncommon, primary hyperparathyroidism is relatively common. It is, therefore, possible that the two conditions may coexist. We describe a case of apparent primary hyperparathyroidism due to pseudo-pseudohypercalcaemia in association with Waldenström's macroglobulinaemia.

## CASE REPORT

A 74-year-old man was diagnosed with Waldenström's macroglobulinaemia when he presented in May 2002 with lethargy, weight loss and nasal bleeds, and an IgM κ paraprotein of 31.5 g/l (IgM reference range: 0.5–2 g/l). He received chemotherapy courses and in February 2003 was referred to the urologists with prostatism. His rectal examination was normal; however, his serum prostate-specific antigen (PSA) was raised (17 μg/l; reference range 0.1–6.5 μg/l; age ⩾70 years). A four-quadrant prostatic biopsy followed, but did not show malignant infiltration of the prostate. At an oncology review in April 2003, it was noticed that he had asymptomatic hypercalcaemia (serum adjusted calcium level 2.86 mmol/l; reference range 2.17–2.66 mmol/l; Arsenazo III dye binding method). A persistently elevated serum PSA of 52.2 μg/l measured in May 2003 had instigated a second four-quadrant prostatic biopsy, which was inconclusive of a diagnosis of prostatic carcinoma. Serum calcium levels remained elevated at 2.89 mmol/l. The possibility of hypercalcaemia due to metastatic bone disease, although uncommon in patients with prostate cancer despite the high frequency of skeletal metastases, prompted an isotope bone scan; however, it did not show any metastatic lesions. The diagnosis of primary hyperparathyroidism was then considered on the basis of the presence of a consistently elevated serum adjusted calcium level of 3.03 mmol/l with inappropriately non-suppressed two serum parathyroid hormone results (4 and 4.4 pmol/l; reference range 1.6–6.9 pmol/l, DPC immulite) and a normal serum creatinine level of 94 μmol/l. Subsequently, an ultrasound of the neck was performed, which suggested the presence of a parathyroid adenoma in the posteroinferior part of the left thyroid lobe. However, in July 2003, on metabolic review, the presence of a normal serum ionised calcium level of 1.10 mmol/l (reference range 1.11–1.3 mmol/l measured on a Radiometer ABL700) and a normal fasting urine calcium excretion of 0.012 mmol/l GF (reference range $Ca_E$ <0.045 mmol/l GF) has excluded genuine hypercalcaemia despite the repeatedly elevated serum adjusted calcium level of 3.1 mmol/l.

The diagnosis of pseudohypercalcaemia was made and was postulated to be due to an abnormal binding of calcium molecules to the IgM paraprotein.[3] This was, however, excluded when gel filtration chromatography showed a similar pattern between the patient's sample and that of a normal control. Both samples showed two peaks of calcium: the first peak associated with albumin and the second peak represented the complexed calcium; however, none was associated with the IgM paraprotein, thus excluding calcium–paraprotein binding as a cause of the falsely elevated serum calcium levels. Additional investigation with measurement of serum calcium level on the same patient's sample (serum adjusted calcium level of 3.1 mmol/l) with the o-cresolphthalein complexone (CPC) method gave a calcium level of 2.19 mmol/l (reference range 2.1–2.55 mmol/l), and with atomic absorption spectrophotometry gave a calcium level of 2.4 mmol/l (reference range 2.2–2.6 mmol/l). All these findings led to the conclusion of interference of the IgM paraprotein with the Arsenazo III dye binding calcium method.[4]

In November 2003, histological examination of repeat four-quadrant prostatic biopsies in the presence of a PSA of 79 μg/l (reference range 0.1–6.5 μg/l; age ⩾70 years) had confirmed the diagnosis of an adenocarcinoma for which he is currently receiving treatment.

Serum calcium level in January 2004, measured by a CPC method, was 2.21 mmol/l (reference range 2.1–2.55 mmol/l).

### Take-home messages

- It is important to investigate elevated serum calcium levels in association with paraproteinaemias, especially in patients with Waldenström's macroglobulinaemia where genuine hypercalcaemia is rare.
- True hypercalcaemia in these patients could be confirmed by measurement of plasma ionised calcium with or without urinary calcium excretion.
- Increased paraprotein concentrations in these patients interfere with the calcium Arsenazo *III* reagent, and elevated serum calcium level could be remeasured using the o-cresolphthalein complexone method.
- Confirmation of falsely elevated serum calcium levels will prevent erroneous diagnosis, unnecessary investigations and potentially inappropriate treatment.

**Abbreviations:** CPC, cresolphthalein complexone; PSA, prostate-specific antigen

## DISCUSSION

The presence of pseudo-pseudohypercalcaemia in association with Waldenström's macroglobulinaemia had resulted in an erroneous provisional diagnosis of primary hyperparathyroidism. In patients with Waldenström's macroglobulinaemia, genuine hypercalcaemia is rare[1 2] and pseudohypercalcaemia is even rarer.[3 4] Indeed, there were only two case reports of pseudohypercalcaemia in Waldenström's macroglobulinaemia due to either calcium binding to the IgM paraprotein[3] or paraprotein interference in the calcium Arsenazo *III* method.[4] The findings for this patient are consistent with the notion of IgM paraprotein interference with the calcium Arsenazo *III* method and the consequent pseudo-pseudohypercalcaemia.[4] The mechanism of the interference is likely to be increased turbidity produced by paraprotein interaction with the acidic medium of the calcium Arsenazo *III* reagent and not with the alkaline CPC method.[4]

It is possible that if the pseudo-pseudohypercalcaemia had not been recognised, this patient would have had an unnecessary neck exploration, since persistent hypercalcaemia (as in this case) is an indication for parathyroid surgery irrespective of symptoms or complications of primary hyperparathyroidism.[5]

## CONCLUSION

We report a case of apparent primary hyperparathyroidism due to pseudo-pseudohypercalcaemia in association with Waldenström's macroglobulinaemia.

We conclude that it is important to identify patients with falsely elevated serum calcium levels in patients with paraproteinaemias to prevent unnecessary investigations, erroneous diagnosis and potentially inappropriate treatment.

Since pseudo-pseudohypercalcaemia in association with Waldenström's macroglobulinaemia may be multifactorial in origin, we suggest that apparent hypercalcaemia in these patients should be confirmed by measurement of plasma ionised calcium with or without urinary calcium excretion.

**Authors' affiliations**
**A Elfatih,** Department of Clinical Biochemistry, Whipps Cross University Hospital, Leytonstone, London, UK
**N R Anderson,** Department of Clinical Biochemistry, George Elliott Hospital, Nuneaton, UK
**M N Fahie-Wilson,** Department of Clinical Biochemistry, Southend Hospital, Prittlewell Chase, Westcliffe-on Sea, UK
**R Gama,** Department of Clinical Chemistry, New Cross Hospital, Wolverhampton, UK; Department of Research Institute, Healthcare Sciences, Wolverhampton University, Wolverhampton, UK

Competing interests: None declared.

Informed consent was obtained from the patient described in this report.

Correspondence to: Dr A Elfatih, Department of Clinical Biochemistry, Whipps Cross University Hospital, Leytonstone, London E11 1NR, UK; abubaker.elfatih@whippsx.nhs.uk

Accepted 22 September 2006

## REFERENCES

1 **Dimopoulos MA**, Panayiotidis P, Kouropoulos LA, *et al*. Waldenstrom's macroglobulinaemia: clinical features, complications and management. *J Clin Oncol* 2000;**18**:214–26.
2 **Dimopoulos MA**, Galani E, Matsouka C. Waldenstrom's macroglobulinaemia. *Hematol Oncol Clin North Am* 1999;**13**:1351–66.
3 **Side L**, Fahie-Wilson MN, Mills MJ. Hypercalcaemia due to calcium binding IgM paraprotein in Waldenstrom's macroglobulinaemia. *J Clin Pathol* 1995;**48**:961–2.
4 **Rhys J**, Oleesky D, Issa B, *et al*. Pseudohypercalcaemia in two patients with IgM paraproteinaemia. *Ann Clin Biochem* 1997;**34**:694–6.
5 **Bilezikian JP**, Potts Jr JT, Fuleihan GE, *et al*. Summary statement from a workshop on asymptomatic primary hyperparathyroidism: a perspective for the 21st century. *J Clin Endocrinol Metab* 2002;**87**:5353–61.

# A distinctive vulval fibroma of so-called prepubertal type in a postmenopausal patient

**O O Ajibona, C J Richards, Q Davies**

*J Clin Pathol* 2007;**60**:437–438. doi: 10.1136/jcp.2006.040428

The case of a 65-year-old female patient who has had a large right vulval mass for over 30 years is reported here. The mass was excised, but the hypocellular mesenchymal tumour was difficult to classify histologically. On further specialist assessment, it was found to be identical to the recently recognised distinctive prepubertal vulval fibroma. These are very rarely reported in children, and till now, has never been reported in an adult.

## CASE REPORT

A 65-year-old postmenopausal woman was referred with a painless vulval swelling that the patient had had for over 30 years. It had gradually increased in size over the past few years, causing discomfort during sexual intercourse and when riding a bicycle. It also caused an embarrassing bulge in her leotard as she taught yoga. She remained fit and healthy. There was no previous medical history of note. Examination revealed a 9 cm×5 cm×5 cm soft, non-tender, non-reducible right vulval mass with normal skin fully mobile over it. It was partially fixed to underlying structures (fig 1).

MRI of the pelvis and perineum revealed a diffuse abnormality causing enlargement of the right side of the vulva and perineum extending from the infrapubic region to the anal margin. There was extensive signal change within the perineal fat. No discrete mass was seen and no lymphadenopathy was detected.

An initial incisional biopsy suggested that the unusual vulval mass was a benign smooth muscle harmatoma without atypia or malignancy. Surgical excision of the mass was performed with excellent cosmetic results followed by further histological assessment. This showed that the lesion was composed of bland fibroblastic cells associated with abundant stromal collagen, which seemed to be infiltrating and replacing large parts of the submucosal tissue, entrapping nerves, vessels and adipose tissue and extensively disrupting normal pre-existing smooth muscle (fig 2). The cellularity was increased above the appearances, normal at this site. The individual spindle cells had pale distinct cytoplasm with bland nuclei and no conspicuous mitotic activity. Immunostains showed CD34 positivity whereas S-100 protein and smooth muscle actin were negative. The tumour stained oestrogen and progesterone receptor negative. As the lesion was difficult to classify, a specialist histological opinion was sought.

# Letters

## Standardization of glycated haemoglobin

I read the UK Consensus Statement Group's letter[1] with some concern over its apparent emphasis.

At present, clinical laboratories do not measure $HbA_{1c}$; they provide only a method-dependent indicator of $HbA_{1c}$ content, harmonized by means of correlation factors with a non-specific (for $HbA_{1c}$) designated comparison method maintained by the National Glycohaemoglobin Standardization Program (NGSP) Core Laboratory.[2] The numbers produced are broadly comparable with the Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS) evidence bases, but they do not allow the science of $HbA_{1c}$ measurement to advance. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) reference measurement system for $HbA_{1c}$ (now defined in terms of a glycosylated hexapeptide)[3] has the potential to do this, which in turn, should improve understanding and clinical effectiveness of these measurements.

In my view it is impossible to have your cake ('a chemical specific standardization anchor for $HbA_{1c}$ measurement') and eat it ['expression of ($HbA_{1c}$) results (that) remain linked to DCCT/UKPDS values']. A linear correlation between a properly calibrated, specific method for a single chemical entity and an unstandardized non-specific method for a mixture of entities may be fortuitous and may not always be applicable to individual patients' specimens.

Think of it by analogy, where a total haemoglobin assay measures 'all apples', NGSP measures 'all green apples' and IFCC measures 'Granny Smiths'. If the proportion of 'Granny Smiths' is considered to be the marker of choice for a healthy apple cart, we should measure that properly. An assay for 'all green apples' will always be an imperfect substitute although it may have some value. During the development and application of 'Granny Smith' assays, specific assays for other kinds of apple may be found to be more useful in apple cart assessment in ways that are not yet perceived. 'Dumbing down' new assay systems to make them correlate with non-specific assays, rather than ensuring traceability to a specific assay with method-independent calibration, may inhibit exploitation of their potential.

The apparent suggestion in the letter that the IFCC system has to remain always linked to NGSP, seems to perpetuate the myth that NGSP is somehow 'right' and the gold standard against which $HbA_{1c}$ reference measurement systems should be compared. It is like saying IDMS reference method values for cortisol should be 'anchored' to the Matingley fluorimetric method used before immunoassay became routine! Once the IFCC system for $HbA_{1c}$ is established, it will itself become the 'gold standard' against which $HbA_{1c}$ values in all previous studies will have to be re-assessed. Science must advance!

Clearly, the IFCC system must be evaluated and prove itself, but its concept and design is scientifically superior to that of NGSP (and other standardization systems), which it must replace as swiftly as practicable. This will enable an improved clinical knowledge base to grow and flourish rather than become 'fossilized' with the DCCT/UKPDS data. All involved should be promulgating this clear message, encouraging the IFCC to complete its work, stimulating manufacturers to embrace the new calibration and helping the diabetes community come to terms with changes in $HbA_{1c}$ values.

**Jonathan Middle**
UK NEQAS
PO Box 3909
Birmingham B15 2UE, UK

### References

1 Marshall SM, Home PD, Manley SE, Barth JH, John WG. Standardization of glycated haemoglobin. *Ann Clin Biochem* 2002; **39**: 78-9
2 Little Rohlfing CL, Wiedmeyer HM, Myers GL, Sacks DB, Goldstein DE. The National Glycohaemoglobin Standardization Program: a 5-year progress report. *Clin Chem* 2001; **47**: 1985-92
3 Jeppsson J-O, Kobold U, Barr J, Finke A, Hoelzel W, Hoshino T, *et al*. Approved IFCC reference method for the measurement of $HbA_{1c}$ in human blood. *Clin Chem Lab Med* 2002; **40**: 78-89

### Authors' reply

We thank Dr Middle for his comments on our letter. Like him, we recognize the inherent limitations of the NGSP and have no wish to stand in the way of scientific advancement. However, the current system has allowed a degree of harmony that has been immensely useful in clinical diabetes management. It would therefore seem imprudent to abandon this system until a more scientifically rigorous and properly validated replacement has been developed. At the time of writing our original letter, there was no peer-reviewed published information available on the relation of the IFCC system to DCCT values and thus no evidence on which to recommend a change.

The issue of changes in $HbA_{1c}$ values with a switch in reference system is not simply one of different numerical values or measuring different chemical entities. The DCCT and UKPDS have for the first time

allowed assessment of an individual's risk of developing microvascular and macrovascular disease. Validation of any new system must include measures to retain this relationship to $HbA_{1c}$, which is essential to delivering good diabetes care. This approach was recently endorsed in a working group meeting of all interested parties held under the auspices of the European Association for the Study of Diabetes.

Like Dr Middle, we would strongly encourage the proper development and evaluation of new standardization methods, with full peer review and open discussion of all relevant issues. We are committed to reviewing glycated haemoglobin standardization programmes annually and a meeting is already planned for June 2002. We very much hope there will then be sufficient data on the IFCC system in the public domain to allow for planning of the way forward.

**Sally M Marshall**[1]
**Philip D Home**[1]
**Garry John**[2]
**Susan E Manley**[3]
[1]Department of Medicine
University of Newcastle Upon Tyne
[2]Department of Clinical Biochemistry
Norfolk and Norwich University Hospital
[3]Diabetes Research Laboratories
University of Oxford, UK

## Audit of investigation of subclinical hypothyroidism in Scottish laboratories

We agree with Dr Berry that development of standards for interpretative comments for subclinical hypothyroidism is desirable.[1] We suggest, however, some modifications.

There was no attempt by the author to define either subclinical or primary hypothyroidism. Most studies define subclinical hypothyroidism as normal free thyroxine ($FT_4$) with thyroid stimulating hormone (TSH) above the reference range, but distinguish patients with a TSH above 10·0 mU/L, in whom thyroxine therapy is indicated.[2–4] Case 2 has a TSH above 10·0 mU/L, and we therefore suggest that it be reported as 'consistent with mild primary hypothyroidism, suggest repeat to confirm'.

We agree with Dr Berry that it is not, in general, appropriate for the laboratory to recommend thyroxine therapy on the basis of laboratory results alone,[1] but it can be useful if used cautiously.

While there was no case presented in the audit of a patient with a raised TSH that was antibody-negative, the standards should include this possibility. The Whickham survey found that the annual risk of developing hypothyroidism in women is 4·3% per annum if both a raised serum TSH and anti-thyroid antibodies are present, and 2·6% per annum if a raised TSH alone is present. The algorithms developed by both Weetman[3] and Ray *et al.*[4] advocate repeat testing in all patients with raised TSH, even if thyroid antibody-negative.

It is not necessary to delay measuring thyroid antibodies. In most cases it is convenient to do this at the same time and this knowledge will be useful to the clinician for future patient management.

We propose the following scheme as a standard for reporting results in ambulant patients, free from any acute illness and not receiving thyroid-related therapy:

1. If TSH above the reference range but below 10·0 mU/L:
   a. Estimation of thyroid antibodies.
   b. Annual review, particularly in the presence of positive thyroid antibodies which indicates the increased risk of developing hypothyroidism in such patients.
2. If TSH is >10·0 mU/L when associated with a low normal $FT_4$:
   Results should be reported as 'consistent with mild primary hypothyroidism, suggest repeat to confirm'. Thyroxine therapy should be recommended cautiously, after excluding untreated primary and secondary hypoadrenalism and myocardial insufficiency.
3. Consider assay interference if the TSH is disproportionately raised in comparison to the $FT_4$ concentration and perform checks in the laboratory before reporting.
4. In all acutely ill patients the comment should include repeat testing after recovery from the illness.

**Annie Armston**
Regional Endocrine Laboratory
Level D South Path Block
Southampton University NHS Trust
Southampton SO16 6YD, UK

**Sudha Bulusu**
Dept of Chemical Pathology
Newham General Hospital
London E13 8RU, UK

### References

1 Berry P. Audit of investigation of subclinical hypothyroidism in Scottish laboratories. *Ann Clin Biochem* 2001; **39**: 26- 33
2 Vanderpump MPJ, Turnbridge WMG, French JM, Appleton D, Bates D, Clark F, *et al.* The incidence of thyroid disorders in the community: a twenty-year follow-up of the Whickham survey. *Clin Endocrinol* 1995; **43**: 55- 68

3 Weetman AP. Hypothyroidism: screening and subclinical disease. *BMJ* 1997; **314**: 1175-8
4 Rae P, Farrar J, Beckett G, Toft A. Assessment of thyroid status in elderly people. *BMJ* 1993; **307**: 177-80

### Author's reply

I thank Dr Armston for her letter and suggested modifications. It is recognized that the standards derived in the paper are preliminary and will be modified with time.

It is correct to say that most studies define subclinical hypothyroidism as normal $FT_4$ or total thyroxine ($TT_4$) and TSH above the reference range. This was the definition used in the study – it must have disappeared during a re-write. Case 2 falls within this definition and we think that a repeat (as suggested by the derived standard) is appropriate. There may be differences of opinion on exactly how long to wait before repeating. Our repeat times are based on results from an unpublished in-house audit which looked at (among other things) long-term changes in TSH levels. Our personal preference is to await the repeat results before 'labelling' the patient.

We agree that it is sometimes justifiable to recommend thyroxine therapy or at least suggest it may be appropriate if applied cautiously. We agree that all patients with a normal $FT_4$ and consistently raised TSH who do not have raised TPO antibodies should continue to have their thyroid function monitored. We suggest this should be done at least annually depending on the TSH level and clinical picture.

We agree that it may not be necessary to delay measuring thyroid antibodies but there is a cost penalty involved in performing this test on every patient with a raised TSH and there may be logistical problems for laboratories that send their samples to reference laboratories.

Setting standards for an audit project such as this is difficult. The derived standards must be applicable to all patients with subclinical hypothyroid results and must be acceptable to most people reporting thyroid results. The suggested standards were considered to be the minimum required. This means that the scheme set out for commenting on patients with subclinical hypothyroidism may appear somewhat simple. Many laboratories will have much more sophisticated reporting schemes. However, we believe the audit project served its purpose and demonstrated the lack of consistency of comments on subclinical hypothyroid results both within and between laboratories.

With regard to the proposed scheme, clinical details are rarely complete and it may not be possible to know whether a patient is ambulant and free from acute (or chronic) illness. We therefore believe a repeat is advisable after a suitable time to allow the resolution of any non-thyroid illness affecting the results.

The cut-off point of 10·0 mU/L is widely used but is suspiciously 'round'. We believe that thyroid antibody measurement can be useful in patients with a normal $FT_4$ ('low–normal' would need defining) and a TSH above 10·0 mU/L.

We prefer to leave the decision to treat with thyroxine to the clinician who knows the patient. We may occasionally mention that a patient with a consistently raised TSH (even if it is below 10·0 mU/L) and raised thyroid antibodies may benefit from a trial of thyroxine but this is usually done because general practitioners are relatively unfamiliar with the significance of raised thyroid antibodies. We do not do it routinely and do not feel it should be a reporting standard.

We agree with Dr Armston regarding acutely ill patients and with her comments for assay interference but would suggest that the checks include assaying the sample by a different method.

**P Berry**
Biochemistry Department
Monklands Hospital
Lanarkshire ML6 0JS, UK

## Limited clinical utility of high-sensitivity plasma C-reactive protein assays

We wish to make a further observation about the limited clinical utility of single hs-C-reactive protein (CRP) measurement in coronary heart disease (CHD) risk assessment.[1] It has been suggested that CRP values can be incorporated into CHD risk assessment to improve prediction of CHD morbidity.[2] Assessment of CHD risk usually involves opportunistic screening in primary care.[3] We measured hs-CRP by immunoassay (DPC, Los Angeles CA, USA) in 434 subjects from primary care in whom a laboratory-based CHD risk score calculation had been requested. Of these, 113 (26·0%) subjects had hs-CRP concentrations above 6·0 mg/L (detection limit of ordinary CRP assays) and 32 subjects (7·3%) had CRP values $>15$ mg/L, suggesting an active inflammatory process probably due to a minor illness. These results suggest that using CRP during opportunistic screening may falsely increase CHD risk score, leading to inappropriate patient management.

Furthermore, inflammatory processes, indicated by elevated CRP, may be associated with lowering of total cholesterol and high density cholesterol concentrations,[4] which may in turn influence calculated CHD risk scores. Our results have shown that a significant number of patients who have undertaken opportunistic screening may have minor or subclinical illness

that could affect their CHD risk scores and adversely influence their management.

Health care practitioners should be aware of the potential limitations of the use of CRP and opportunistic screening in CHD risk assessment.

**A Elfatih**
**K Chatha**
**NR Anderson**
**R Gama**
Department of Clinical Chemistry
New Cross Hospital
Wolverhampton WV10 0QP, UK

## References

1 Campbell B, Badrick T, Flatman R, Kanowski D. Limited utility of high sensitivity plasma C-reactive protein assays. *Ann Clin Biochem* 2002; **39**; 85- 8
2 Ridker PM. High sensitivity C-reactive protein: potential adjunct for global risk assessment in the primary prevention of cardiovascular disease. *Circulation* 2001; **103**: 1813- 27
3 The National Service framework of coronary heart disease. Modern standards and service models. London: Department of Health, 2000
4 Sammalkorpi KT, Valtonen VV, Maury CP. Lipoproteins and acute phase response during acute infection. Interrelationships between C-reactive protein and serum amyloid A and lipoproteins. *Ann Med* 1990; **22**: 397- 401

# 'Glucose meter hypoglycaemia': often a non-disease

R Gama[1], N R Anderson[1] and V Marks[2]
*From the [1]Department of Clinical Chemistry, New Cross Hospital, Wolverhampton, West Midlands WV10 0QP and the [2]European Institute of Health and Medical Sciences, University of Surrey, Guildford, Surrey, UK*

The diagnosis of hypoglycaemia depends upon the demonstration of a low blood glucose concentration during a spontaneous symptomatic episode. Glucose monitoring devices may misdiagnose many healthy individuals with non-specific symptoms as having hypoglycaemia. We present three illustrative cases of 'glucose meter non-hypoglycaemia'.

## CASE 1

A 31-year-old housewife gave a 20-year history of intermittent fatigue and 'shakes' unrelated to food or exercise. She attributed these symptoms to hypoglycaemia when a nurse recognized the 'symptoms of hypoglycaemia' and checked her capillary blood glucose on a meter, which gave a low reading (1·9 mmol/L) (unconfirmed however by laboratory measurement) during inpatient investigations for abdominal pain 1 year previously. Her GP then provided her with blood glucose reagent strips. She visually recorded her glucose being as low as 1–2 mmol/L when symptomatic, and was therefore referred for assessment.

During an exercise test following an 18-h fast her plasma venous glucose rose from 4·3 to 4·7 mmol/L and her insulin concentrations were low, at $<25$ pmol/L. During a prolonged (5-h) 75 g glucose load test her nadir plasma venous glucose was 5·5 mmol/L at 240 min, but she experienced 'shakes and tiredness' when her plasma venous glucose was 6·9 mmol/L at 180 min.

We explained that hypoglycaemia was unlikely to explain her symptoms. We suggested that she should have a blood sample collected when symptomatic and taught her how to collect capillary blood into a fluoride capillary tube. She was unhappy with our explanation and never reattended.

Correspondence: Dr R Gama.
E-mail: dr.gama@rwh-tr.wmids.nhs.uk

## CASE 2

A 29-year-old female nurse gave a 3-year history of intermittent disorientation, sweats and lethargy unrelated to food or exercise. While at work, during two of these attacks finger-prick blood samples gave low glucose meter readings ($<2·0$ mmol/L). She was referred for further assessment. An exercise test following an 18-h fast and a 5-h 75 g glucose load test, during which she was asymptomatic, provided no evidence for hypoglycaemia.

During one of her symptomatic episodes a glucose meter reading was 1·1 mmol/L, but simultaneously collected laboratory capillary blood glucose was 5·5 mmol/L. She accepted that her symptoms were not due to hypoglycaemia and that her previous readings were due either to faulty technique or to a faulty meter.

## CASE 3

While at work during a period of personal stress, a 24-year-old female nurse felt dizzy with sweats, nausea and 'shakes'. A nursing colleague using a glucose meter found her to have a reading of 1·6 mmol/L. She was therefore given oral glucose, which led to a resolution of the symptoms after 30 min. During a further similar attack 3 months later she was seen in an Accident and Emergency department with a label of hypoglycaemia and her glucose meter reading was confirmed as 'low'. She was given oral glucose and discharged with recommendations that she be investigated further. An exercise test following an 18-h fast and a 5-h 75 g glucose load test, during which she was asymptomatic, provided no evidence for hypoglycaemia. We explained that her symptoms were unlikely to be due to hypoglycaemia. As her personal stress resolved she had no further symptomatic episodes.

## DISCUSSION

Glucose meters have been justifiably popular for over 15 years and have advanced the management of diabetic patients. However, glucose meters, and especially visually read glucose test strips, are unsuitable for the diagnosis of spontaneous hypoglycaemia in the domestic environment, as many of the methods used may be unreliable in the hypoglycaemic range.[1–5] Faulty meters, faulty blood strips, faulty patient preparation and faulty analytical technique may also add to the considerable risk of erroneous results.[3] Glucose meters may however be useful in the clinical environment (e.g. in Accident and Emergency departments) as a rapid guide to the need for further blood collection (for confirmation and further investigation), followed immediately by the administration of glucose to relieve symptoms.

In the cases described here the subjects were initially 'investigated' in three different hospitals, suggesting that 'hypoglycaemia' was diagnosed using different types of meter. This suggests that any errors were probably due to faulty technique, but does not exclude instrument or reagent error. It also implies that the use of glucose meters to investigate vague symptoms for hypoglycaemia may be widespread.

It is likely that the simplicity and ready availability of glucose meters has led to their inappropriate use in the investigation of non-specific symptoms for the possibility of hypoglycaemia. The majority of 'patients' seen in our departments with 'glucose meter non-hypoglycaemia' have been mislabelled as having hypoglycaemia within the hospital environment without laboratory confirmation, even though suppliers of glucose meters generally recommend that meter readings $<2{\cdot}0$ mmol/L be verified by a laboratory glucose measurement. It is particularly important to obtain a laboratory glucose measurement, firstly to confirm or refute hypoglycaemia, and secondly, if hypoglycaemia is confirmed, as the ideal (and sometimes the only) opportunity to uncover its underlying aetiology. Also of concern, but difficult to identify, is that the indiscriminate use of glucose meters may misclassify subjects with genuine spontaneous hypoglycaemia as being normoglycaemic.

An important adverse consequence of mislabelling healthy people with a disease is a reduction in their quality of life, and in some individuals the adoption of a sick role ('the worried well'). The mislabel of 'hypoglycaemia' in these patients can however often be difficult to rectify, perhaps because patients who present with symptoms of hypoglycaemia may have abnormal psychological profiles.[6] Although a prolonged glucose load test is of limited value in the investigation of hypoglycaemia,[5–8] the occurrence of symptoms in the absence of biochemical hypoglycaemia may help convince patients that their symptoms are not due to hypoglycaemia.

The misdiagnosis of hypoglycaemia may also result in unnecessary and wasteful investigations, and may in part explain why 57% of samples received in a supraregional laboratory for the investigation of hypoglycaemia were inappropriate, having glucose values of greater than $3{\cdot}0$ mmol/L.[9]

In summary, we suggest that the use of glucose meters to diagnose spontaneous hypoglycaemia (without laboratory confirmation) is inappropriate, wasteful of resources and may adversely affect the health of the individual. Patients with symptoms suggestive of neuroglycopenia should be appropriately assessed and investigated.[9] We recommend that training of personnel in the use of glucose meters should include the inappropriateness of their routine use in investigating spontaneous hypoglycaemia in non-diabetic adults.

## REFERENCES

1 Trajanoski Z, Brunner GA, Gfrerer RJ, Wach P, Pieber TR. Accuracy of home glucose meters during hypoglycaemia. *Diabetes Care* 1996; **19**: 1412–5
2 Bergman M, Migake C, Felig P. Misdiagnosis of gestational diabetes and hypoglycaemia using portable blood glucose meters. *Arch Intern Med* 1989; **149**: 2602–3
3 Southgate HJ, Marks V. Measurement of hypoglycaemia by reflocheck. *Pract Diabetes* 1986; **3**: 206–7
4 Sandler M, Low-Beer T. Misleading capillary glucose measurements. *Pract Diabetes* 1990; **7**: 210
5 Burden ML, Jarvis J, Dromgoole P, Burden AC. Precision of glucose meters in the hypoglycaemic range and normoglycaemic range. *Pract Diab Int* 1998; **15**: 133–4
6 Johnson DD, Dorr KE, Swenson WM, Service FJ. Reactive hypoglycaemia. *JAMA* 1980; **243**: 1151–5
7 Lefebvre PJ, Andreani D, Marks V, Creutzfeldt W. Statement on postprandial hypoglycaemia. *Diabetes Care* 1988; **11**: 439–40
8 Yager J, Young RT. Sounding board: non-hypoglycaemia is an epidemic condition. *N Engl J Med* 1974; **291**: 907–8
9 Marks V, Teale JD. Investigation of hypoglycaemia. *Clin Endocrinol* 1996; **44**: 133–6

*Accepted for publication 11 April 2000*

# Appendix B. Confirmation from Principal Investigators of contribution made to papers where N R Anderson is not first or last Author

For the projects contained in this thesis I am first author or last author on 6 papers. For the remainder my contribution largely centred on the application of the analytical techniques and assessment of preanalytical variables for each paper. My contribution is detailed by the principal investigators in the following appendix.

**From:** GAMA, Rousseau (THE ROYAL WOLVERHAMPTON NHS TRUST) [mailto:rousseau.gama@nhs.net]
**Sent:** 14 May 2018 14:39
**To:** Anderson Neil (RKB) Director of Pathology Services
**Subject:** PHD papers

To whom it may concern.

N R Anderson PhD application by publication. Student number U1390491

Publication title: Chatha K, **Anderson NR**, Gama R. Ethnic variation in C-reactive protein: UK resident Indo-Asians compared with Caucasians. Journal of Cardiovascular Risk 2002;9;139-141.

Neil R Anderson was a significant contributor to this paper through his work with Ms Kam Chatha and Professor Gama. Neil Anderson helped design the study, supervised the laboratory analyses, helped in data analysis, and contributed to all stages of the manuscript. His work on the assay optimisation of the hsCRP assay with his understanding of its limitations in a clinical setting was crucial to this paper's publication.

Neil R Anderson MSc FRCPath CCHEM FRSC FRCP FRCPath

Professor Rousseau Gama BSc MSc MD CSci

GMC No. 2644789

Together Towards World Class

NHS University Hospitals Coventry and Warwickshire NHS Trust

**Prof P Saravanan PhD FRCP**
**Professor of Diabetes, Endocrinology & Metabolism**
Division of Health Sciences, Warwick Medical School
University of Warwick, Coventry, CV4 7AL, UK
Honorary Consultant Physician
George Eliot Hospital, Nuneaton, CV10 7DJ

14 Feb 2018

**Graduate School**
**University of Warwick**

Dear Colleagues

**Re:** N R Anderson PhD application by publication. Student Number U 1390491

Publication title: Adaikalakoteswari A, Vatish M, Lawson A, Wood C, Sivakumar K, McTernan PG, Webster C, **Anderson N**, Yajnik CS, Tripathi G, Saravanan P (2015). Low maternal vitamin B12 status is associated with lower cord blood HDL cholesterol in white Caucasians living in the UK. Nutrients;7(4):2401-14.

Neil R Anderson was a significant contributor to this paper through his work with my post-doctoral fellow Dr A Adaikalakoteswari. His work on the optimisation of the B12 and HDL assays, particularly his knowledge of the limitations of the sensitivity in a clinical setting, was key for this publication. Understandably, he also contributed in writing the analytical aspects and reviewed the whole manuscript prior to publication.

Please do not hesitate to contact me if you require any further information.

Yours sincerely,

Prof Ponnusamy SARAVANAN

**From:** GAMA, Rousseau (THE ROYAL WOLVERHAMPTON NHS TRUST) [mailto:rousseau.gama@nhs.net]
**Sent:** 14 May 2018 14:32
**To:** Anderson Neil (RKB) Director of Pathology Services
**Subject:** PHD

To whom it may concern.

N R Anderson PhD application by publication. Student Number U 1390491

Publication title: Ram S, Blumberg D, Newton P, **Anderson NR**, Gama R. Raised serum prolactin in rheumatoid arthritis: genuine or laboratory artefact? Rheumatology (Oxford). 2004:43(10):1272-4

Neil R Anderson was a significant contributor to this paper through his work with Professor Gama. Neil Anderson helped design the study, supervised the laboratory component of this project, helped analyse data and contributed to manuscript. His optimisation of the macroprolactin detection method, a pre-analytical interference in the prolactin assay, was critical in this peer-review publication.

Neil R Anderson MSc FRCPath CCHEM FRSC FRCP FRCPath

Professor Rousseau Gama BSc MSc MD CSci

GMC No. 2644789

**From:** GAMA, Rousseau (THE ROYAL WOLVERHAMPTON NHS TRUST) [mailto:rousseau.gama@nhs.net]
**Sent:** 14 May 2018 14:14
**To:** Anderson Neil (RKB) Director of Pathology Services
**Subject:** FW: PHD

To whom it may concern.

N R Anderson PhD application by publication. Student number U1390491

Publication title: Elfaith A, **Anderson NR**, Fahie-Wilson MN, Gama R. Pseudo-pseudohypercalcaemia, apparent primary hyperparathyroidism and Waldenstro"m's macroglobulinaemia. J Clin Pathol. 2007.

Neil R Anderson was a significant contributor to this paper through his work with Dr A Elfatlh and Professor Gama. Neil Anderson's knowledge on interferences in laboratory assays, in this case a paraprotein interference, was critical in respect to patient management and crucial to this letter being accepted for publication. In addition he contributed to manuscript review prior to publication.

Neil R Anderson MSc FRCPath CCHEM FRSC FRCP FRCPath

Professor Rousseau Gama BSc MSc MD CSci

GMC No. 2644789

Together Towards World Class

NHS University Hospitals Coventry and Warwickshire NHS Trust

**From:** GAMA, Rousseau (THE ROYAL WOLVERHAMPTON NHS TRUST) [mailto:rousseau.gama@nhs.net]
**Sent:** 14 May 2018 14:20
**To:** Anderson Neil (RKB) Director of Pathology Services
**Subject:** PHD

To whom it may concern.

N R Anderson PhD application by publication. Student number U1390491

Publication title: Elfatih A, Chatha K, **Anderson NR**, Gama R. Limited clinical utility of high sensitivity plasma C-reactive protein assays. Ann Clin Biochem. 2002;39:534-537.

Neil R Anderson was a significant contributor to this paper through his work with Dr A Elfatlh, Ms K Chatha and Professor Gama. His work on the assay optimisation of the hsCRP assay with his understanding of its pre-analytical limitations applied to cardiovascular risk assessment led to this letter being accepted for publication. In addition he contributed to experimental design and manuscript review prior to publication.

Neil R Anderson MSc FRCPath CCHEM FRSC FRCP FRCPath

Professor Rousseau Gama BSc MSc MD CSci

GMC No. 2644789

Together Towards World Class

NHS University Hospitals Coventry and Warwickshire NHS Trust

**From:** GAMA, Rousseau (THE ROYAL WOLVERHAMPTON NHS TRUST) [mailto:rousseau.gama@nhs.net]
**Sent:** 14 May 2018 14:21
**To:** Anderson Neil (RKB) Director of Pathology Services
**Subject:** PHD

To whom it may concern.

N R Anderson PhD application by publication. Student number U1390491

Publication title: Gama R, **Anderson NR**, Marks V. 'Glucose Meter Hypoglycaemia' : often a non-disease. Ann Clin Biochem 2000;37:731-732.

Neil R Anderson was a significant contributor to this paper through his work with Professor Gama in identification of relevant cases, literature review, commentary on the technical limitations of glucose meters and manuscript review prior to publication.

Neil R Anderson MSc FRCPath CCHEM FRSC FRCP FRCPath

Professor Rousseau Gama BSc MSc MD CSci

GMC No. 2644789


NHS
University Hospitals Coventry and Warwickshire
NHS Trust