A Proteomic Approach to Determining Cause of Death in Sudden Unexpected Death in Infancy (SUDI)

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Declaration

I, Andrew Richard Bamber, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Introduction: Despite improvements in the understanding of infant death over recent years, many infants die each year in whom no cause of death is identified. There is evidence to suggest that a proportion of these unexplained deaths are the consequence of infection, either by a classical mechanism or as a consequence of the action of bacterial toxins. Post mortem tests for bacteria are robust, but there is a lack of effective post mortem tests for inflammatory markers which might assist in the interpretation of bacteriological results, and for identification of bacterial toxins.

Methods: Proteomic techniques including biomarker discovery techniques using liquid chromatography mass spectrometry, and targeted techniques using multiple reaction monitoring tandem mass spectrometry, were used to identify potential biomarkers for infection and identify bacterial organisms and toxins, with a view to creating clinically-useful tests.

Results: First, a rapid test for three biomarkers was developed which allows identification of infection and sepsis with high sensitivity and specificity in post mortem liver samples; this may be rapidly translated for clinical use. Second, a highly specific and sensitive test for *Staphylococcus aureus* and seven Staphylococcal exotoxins was developed which may be used to study the significance of Staphylococcal toxins in infant deaths. Furthermore this technique may adapted to identify other organisms; allowing potential use as a rapid diagnostic test in clinical practice in the living. Thirdly, the tests developed have identified inflammatory markers which are decreased in infants dying of infection; raising the possibility that acquired immune paresis may contribute to these deaths. This finding contributes to the understanding of mechanisms of fatal infection in infants, and in their prevention and management. Finally, a number of mitochondrial proteins have found to be raised in SIDS cases, which may provide additional insight into the mechanism of death in some of these cases.

Contents

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Publications Arising From This Thesis

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Bamber AR, Mifsud W, Wolfe I, Cass H, Pryce J, Malone M, Sebire NJ. Potentially preventable infant and child deaths identified at autopsy: findings and implications. Forensic Science, Medicine and Pathology 2015;11(3):358-64

Bamber AR, Pryce JW, Ashworth MT, Sebire NJ. Immersion-related deaths in infants and children: autopsy experience from a specialist center. Forensic Science, Medicine and Pathology 2014;10(3):363-70

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Aims

The aim of this work is to utilise proteomic techniques to allow the development of:

- A specific and sensitive direct test for Staphylococcus aureus and its major exotoxins in order to allow:
    - Proof of principle that such a test can be created
    - Use of the test to investigate further the role of bacterial toxins in SIDS
    - Use of the method for the development of clinical tests to allow rapid identification of bacteria and bacterial toxins in the living

- A sensitive and specific multiplex test for inflammatory markers in order to allow:
    - Accurate identification of infection and sepsis as causes of death in infants in clinical post mortem practice
    - Determination of what proportion of unexplained infant deaths, if any, is attributable to infective causes

- Reliable tests for biomarkers which may be used to enhance the understanding of the mechanisms underlying unexplained infant deaths and deaths arising as a result of infection and sepsis

Part One

Introduction

Chapter 1 – Infant Death, Infection and Post Mortem Investigations

1.1 Introduction

Over the last 50 years, thanks to education and research efforts, the number of infant deaths in the United Kingdom has dropped dramatically. In 2014 there were 2,517 deaths in children under one year of age in England and Wales, compared with 6,037 similar deaths in 1984 [ONS 2016]. While this improvement is clearly to be welcomed, any child death is a tragedy both for society and for the parents. The distress caused to parents is often compounded by the fact that in many cases the cause of death in these infants cannot be identified (212 cases in 2014 [ONS 2016a]).

Guidance from organisations such as the Royal College of Pathologists [RCPath, 2005] and the Royal College of Paediatrics and Child Health [RCPath and RCPCH 2016], has helped to ensure a minimum standard to the conduct of post mortem examinations in cases of infant death occurring in the United Kingdom. This means that wherever an infant dies in the UK, the conduct of their autopsy should, in general terms, follow the same procedure. However, despite such guidance, there is still a significant variation in the detail of how these autopsies are conducted and what further tests are carried out. Furthermore, and more significantly, there is marked variation in the UK and worldwide with regard to interpreting the results of such investigations [Pryce et al 2011]. Due to the poor understanding of the aetiology of these deaths and a lack of tests to assist in interpretation, these tests are often interpreted by the pathologist conducting the examination on the basis of a combination of educated guesswork, precedent and instinct.

The practical result of this is that the geographical location of the autopsy and the person performing it can make a significant difference to the outcome of the autopsy and the information given to parents. For example, if an infant were to die and only a single microscopic focus of bronchopneumonia were found at post mortem examination, depending on where in the UK the examination were performed and who performed it, the parents may be told either that the cause of death is infection, or that the cause of death remains unexplained.

This situation is confusing and distressing for parents, and can potentially skew infant death statistics such that some areas appear to have less favourable unexplained infant death rates than others. This may in turn have an effect on Public Health efforts to tackle the causes of infant death. Furthermore, this variability adversely affects research efforts as even if definitions of infant death which include post mortem findings as a factor are agreed between jurisdictions, the group of cases may differ due to post mortem practice variation.

The reason for this variation is not usually poor practice, but rather a lack of robust investigations to aid in the understanding of the mechanisms underlying infant deaths and to assist in the interpretation of post mortem findings. Indeed, that part of the second edition of the Kennedy Report dedicated to the post mortem examination is essentially unchanged from the first edition published over a decade ago [RCPath and RCPCH 2004, RCPath and RCPCH 2016]. This is because there has been very little practical change in the investigation of these deaths during that period. The overall aim of the research conducted as part of this thesis is to create robust clinical tests which can be affordably applied in practice nationally to improve the interpretation and understanding of the mechanism underlying infant deaths and post mortem findings.

1.2 Definitions

In any study, accurate definitions are essential for the generation of robust and reproducible results. This is particularly important in the case of infant deaths, because the relative rarity of the cases means that effective research mandates the combination of cases from several centres, sometimes over National or International borders. Unfortunately, there is a lack of consensus with regard to the definitions used in infant deaths. Furthermore there is also a marked variation both temporally and geographically with regard to the application of terms [Beckwith 1970, Willinger, James and Catz 1991, Limerick and Bacon 2004, Fleming et al 2000, Green 1999, Bajanowski, Brinkmann and Vennemann 2006].

For the purposes of this thesis, terms and abbreviations used will be defined as detailed in Figure 1.1. This is generally in line with the use of these terms in the United Kingdom and Europe, but may be significantly different from those used in other jurisdictions.

Entity	Abbreviation	Definition
Sudden Unexpected Death in Infancy	SUDI	The death of an infant between the ages of 7 days and one year, which is both sudden and unexpected
Explained Sudden Unexpected Death in Infancy	-	SUDI cases in which a cause of death is identified following a thorough review of the circumstances of death and a post mortem examination
Sudden Infant Death Syndrome	SIDS	SUDI cases in which no cause of death is identified following a thorough review of the circumstances of death and a post mortem examination

Figure 1.1 Definitions of infant death adopted in this thesis

1.3 Explained Sudden Unexpected Death in Infancy

Using currently available routine tests, less than half of sudden unexpected deaths in infancy are explained following post mortem examination and full review of the circumstances of death. For example, in a study of infant deaths in London between 2005 and 2010 using Metropolitan Police data, 57% of 477 sudden unexpected infant deaths remained unexplained [Bamber et al 2016]. In a study of 546 post mortems carried out in cases of sudden unexpected infant death in one specialist centre, 63% remained unexplained [Weber et al 2008A].

Where the cause of death is explained, the majority of deaths are the result of infection with smaller numbers due to congenital abnormalities, cardiovascular disease, respiratory disease and other causes. In a study of paediatric post mortem examinations conducted in cases of sudden and unexpected infant death in one specialist centre, 58% of explained deaths were due to infection [Weber et al 2008B].

Evidence gained from analysis of post mortem data in explained infant deaths can be of great use in improving the understanding and investigation of some

specific causes or circumstances of death, for example those dying of myocardial disease [Bamber et al 2013], drowning [Bamber et al 2014a], and aspiration of foreign bodies [Bamber et al 2014b].

1.4 Unexplained SUDI (SIDS)

Using predominantly epidemiological studies, a number of risk factors for SIDS have been identified and are described in Figure 1.2.

There are a number of factors which are considered controversial with regard to their influence over SIDS risk. First, deprivation and a "chaotic family environment" have been linked with an increased rate of SIDS. In a study of 477 deaths in infants aged less than two years in London between 2005 and 2010, there was a significant association between unexplained death rate and deprivation score (Figure 1.3). Interpretation of such data is difficult; there are numerous confounding factors such as smoking rates and practical ability to follow safe sleep guidance [Bamber et al 2016]. It is therefore important that social class or level of deprivation alone is not used to victimize parents [Emery and Waite 2000]. Rather, the connection between deprivation and SUDI is evidence that public health measures should be targeted towards deprived groups, for example by the provision of "baby boxes" to improve sleep environment.

Second, swaddling is a somewhat controversial area and the practice appears to be increasing in popularity. Published data would tend to suggest that the reported increased risk of infant death in swaddling relates to the interaction between swaddling and other risk factors, i.e. infants who are swaddled and placed prone are at greater risk than infants who are swaddled and placed supine [Pease et al 2016].

Finally, while the evidence with respect to SIDS risk is relatively clear cut, vaccination is still an area of some uncertainty amongst parents [Moon et al 2016]. Unfortunately, the issue of vaccination and SIDS has become caught up in the (unfounded) concerns regarding vaccinations and autism. It is not uncommon for infants to die in the days after their routine childhood vaccinations, but this is likely to be coincidence of the childhood vaccination

schedule and the peak age for SIDS. Robust studies have continued to show that there is no increased risk of SIDS following vaccination [Miller et al 2015, Moro et al 2015a, Moro et al 2015b]. There is some evidence that vaccination may have a protective effect [Mitchell, Stewart and Clements 1995, Jonville-Bera et al 2001, Fleming et al 2001, Vennemann et al 2007], but this is less clear cut and it has been theorized that this apparent protective effect may be caused by a bias towards vaccinating healthy children [Virtanen et al 2000].

Modifiable Risk Factors
Prone sleeping [De Jonge et al 1989]
Bed-sharing in the presence of hazardous circumstances (alcohol use, parental smoking, on a sofa, with premature infants) [Blair et al 2014, Blair et al 1999, Scheers, Rutherford and Kemp 2003, Scragg, Mitchell and Taylor 1993]
Unsuitable sleep environment e.g. car safety seats [Bamber et al 2014c]
Overheating [Burke and Hanani 2012, Rubens and Sarnat 2013]
Use of excessive soft coverings [Kemp et al 1993, Ponsonby et al 1998]
Parental smoking, including during pregnancy [Fleming et al 2000]
Re-use of mattresses

Non-modifiable Risk Factors
Age [Bamber et al 2016]
Male sex [Bamber et al 2016]
Winter months [Bamber et al 2016]
Prematurity and low birth weight [Blair et al 2014]

Protective Factors
Pacifier use [Alm et al 2016, Hauck, Hauck and Siadaty 2005, Franco et al 2000]
Breastfeeding [Alm et al 2016]

Unclear or controversial factors (see text)
Deprivation and chaotic family environment
Vaccination
Swaddling

Figure 1.2 Summary of the known factors affecting the risk of Sudden Infant Death Syndrome

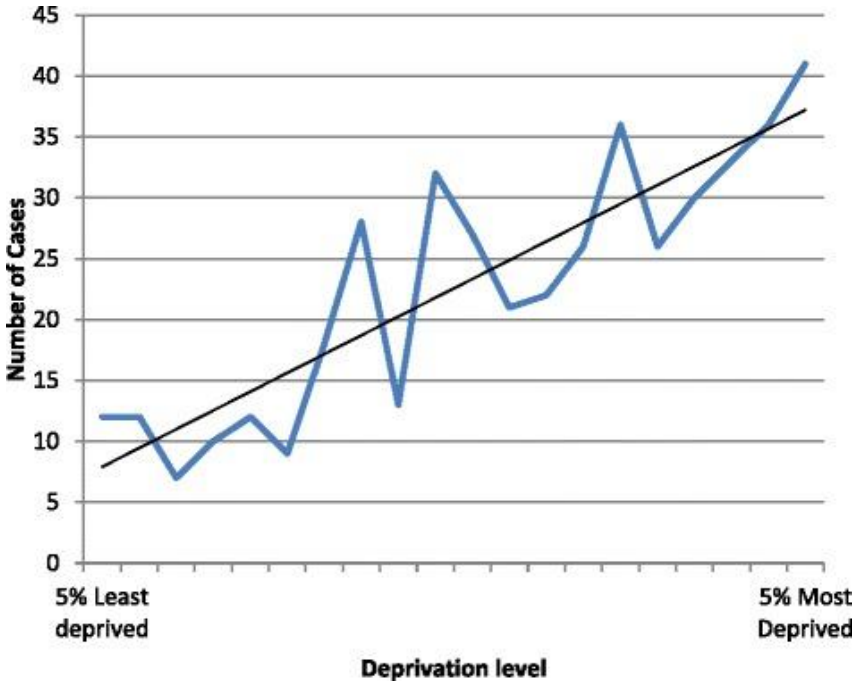

Figure 1.3 Deaths amongst 477 individuals aged less than two years dying in the Metropolitan Police area between 2005 and 2010 arranged by deprivation level (Indices of Multiple Deprivation Score Rank for Lower Layer Super Output Areas (LSOAs)) [Bamber et al 2016]

1.5 Aetiology of SIDS

Despite the identification of risk factors using population-based studies, the introduction of guidance and interventions to target them, and the resulting improvements in infant death rates, the aetiology of SIDS remains poorly understood. Over past decades, there have been numerous theories that have attempted to explain the phenomenon of unexplained infant death [Goldwater 2017], including brainstem control of cardio-respiratory function, respiratory obstruction (inflicted and accidental), cardiac channelopathies, infection, anaphylaxis, thermal stress, diaphragmatic failure, carbon dioxide re-breathing, inborn errors of metabolism, toxic gases from mattresses and abuse.

Some of these theories, such as the theory that toxic gases released from mattresses cause SIDS, have been comprehensively disproven [Warnock et al 1995, Jenkins et al 2000]. However, many theories have accumulated a body of literature which provides support. In many cases, even where an individual theory lacks sufficient evidence to conclude that it is ‘the cause’ of SIDS, it may be possible that a given mechanism or group of mechanisms may account for a

proportion of SIDS cases when linked together. The best developed theory regarding multifactorial SIDS causation is the “triple risk hypothesis”, which suggests that SIDS occurs as a consequence of an external trigger, at a vulnerable stage of development, in a susceptible infant [Guntheroth and Spiers 2002].

Despite the relatively widespread recognition of the triple risk hypothesis it is relatively common, even in the infant death literature, for experts to discuss SIDS cases in terms which suggest that they are a consequence of a single mechanism. For example, even in the most recent literature experts have suggested that a single hypothesis is the ‘key’ to SIDS and that only this factor satisfactorily links the other known risk factors [Goldwater 2017, Siren 2017]. Furthermore these authors also suggest that other avenues of investigation in SIDS be abandoned in favour of their favoured mechanism. Siren suggests that as genetic/congenital factors are not part of their theory they are not likely to be useful avenues of research in SIDS [Siren 2017], whilst Goldwater criticizes the approach of identifying a specific possible cause of SIDS and investigating it further [Goldwater 2017].

There could be considered a flaw in this view of SIDS investigation and causation as it relies on a premise that SIDS is a ‘disease’, i.e. all, or the majority of, deaths categorized as SIDS are the consequence of a single underlying cause or mechanism. As can be established from a review of the SIDS definition given above, SIDS is in fact an ‘umbrella’ term for all deaths which are otherwise unexplained occurring in similar general circumstances in children of a similar age. While some of these cases may share an underlying mechanism, it seems inevitable that previously unidentified causes of death will be discovered which remove deaths from this group. A good example of this is Medium Chain Acyl-Co-A Dehydrogenase Deficiency (MCADD); a metabolic disease which was defined relatively recently. Deaths due to this disease in the past would have been given a diagnosis of SIDS. However, since the inclusion of MCADD in newborn screening tests, it has become extremely rare to die of the condition, and even where deaths do occur the disease will be picked up on routine post mortem investigations. Over time, it seems likely that the group of SIDS cases will be gradually reduced in size as improved post mortem tests and the recognition of new disease entities moves increasing numbers of cases

into the ‘explained’ category. It may be that a small subgroup emerges with a common mechanism, but until that time it makes most sense to scientifically explore all possible causes.

1.6 Infection and infant death

While the above comments regarding aetiology of SIDS should be borne in mind, there is a significant body of evidence, both in terms of laboratory research and risk factor analysis, which suggests that a proportion of SIDS cases may be the consequence of infection.

A number of the risk factors discussed above could be explained by an infective aetiology. SIDS cases are more common in the winter months, when viral infections are more common. There is some evidence that vaccination may have a protective effect in respect to SIDS [Fleming et al 2001, Muller-Nordhorn et al 2015]. It is common in post mortem examination in SIDS cases to find evidence of non-specific inflammatory changes, such as peribronchial and perivascular lymphoid aggregates in the lungs. A common feature in many SIDS cases is the history of preceding coryza or upper respiratory tract infection, and there is some evidence to suggest that when compared with controls, SIDS cases have increased inflammatory changes in the laryngeal epithelium and sub-epithelium [Scadding et al 2014]. In addition to these factors, it must be considered that infection is by far the most common cause of explained SUDI and post mortem tests have significant limitations; it is therefore likely that some cases of infection are missed and then incorrectly labelled as SIDS cases.

A number of genetic variants of common pro- and anti-inflammatory cytokines have been reported to be increased in SIDS cases compared with controls, most notably Interleukin-1 (IL-1), Interleukin-1 receptor antagonist, Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-10 promoter, Tumour Necrosis Factor alpha (TNFα), and Vascular Endothelial Growth Factor (VEGF) [Summers et al 2000, Opdal 2004, Dashash et al 2006, Highet, Gibson and Goldwater 2010a, Ferrante and Opdal 2015, Ferrante et al 2013, Moscovis et al 2006]. However in

other studies the reported increase in SIDS groups has failed to be reproduced (e.g. Interleukin-10 promoter) [Courts and Madea 2011].

The colonization of the gut with bacteria in early infancy is thought to play a major role in the development of a healthy immune system. It has been reported that the gut microbiome in infants dying of SIDS differs significantly from that in control babies, and it has been theorized that this may reflect a degree of immune compromise. It has also been suggested that this may explain the increased risk of SIDS in babies who sleep prone [Highet et al 2014, Goldwater 2015].

Distinct patterns of C-reactive protein (CRP) and Intercellular Adhesion Molecule 1 (ICAM-1) have been reported in SUDI cases depending on cause of death, but were not found to be raised in SIDS cases [Pryce et al 2014]. This raises the possibility that death in SIDS cases may be unrelated to infection, or possibly occurring via an alternative mechanism to classical sepsis. One possible alternative mechanism is the development of the so-called 'cytokine storm'. This has been raised as a possibility in a small number of SIDS infants following the identification of extremely high levels of Interleukin-6 (IL-6) [Vennemann et al 2012].

1.7 Post mortem inflammatory markers in sepsis

A number of inflammatory markers have been hypothesized as being of use in post mortem samples from deaths arising as a consequence of infection or sepsis. These markers have been studied in a number of bodily fluids, and in specific solid tissues by means of immunocytochemistry, most notably the lung.

In a study of perinatal deaths where there was evidence of Fetal Inflammatory Response Syndrome (FIRS), there was an increase in various inflammatory markers which was linked to the underlying cause of death. Interleukin 6 was increased in FIRS arising in the context of congenital malformation, C-reactive protein was increased in FIRS arising as a consequence of ascending infection, and Tumour Necrosis Factor alpha was increased in cases of FIRS caused by perinatal anoxia [Pereira et al 2014].

A number of markers have been studied in pericardial and pleural fluid; Procalcitonin, C-reactive protein, Soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), soluble interleukin-2 receptor (sIL-2R), and Interleukin-6 (although IL-6 was not found to be a specific marker of sepsis, but rather of a more generalised inflammatory response) [Tsokos et al 2001, Palmiere and Augsberger 2014, Palmiere and Egger 2014].

Serum and vitreous procalcitonin has also been shown to be increased in sepsis [Bode-Janisch et al 2013, Schrag et al 2012], although other studies have called into question the increase in serum procalcitonin in sepsis [Augsbuerger et al 2013].

A number of markers have been suggested for identification of sepsis-related lung injury; Integrin alpha-4 beta-1 (VLA-4, CD49d/CD29), Intercellular adhesion molecule-1 (CD54), lactoferrin, vascular endothelial growth factor (VEGF), Pulmonary Angiotensin-converting Enzyme (ACE), and E-selectin [Tsokos and Fehlauer 2001, Tsokos 2003, Tsokos, Fehlauer and Puschel 2000, Muller et al 2008]

Despite a number of reports highlighting their potential utility in post mortem practice, none of these markers are currently in routine diagnostic use in the United Kingdom. This may relate to the cost and complexity of setting up assays for individual markers in units where they would only be infrequently used.

1.8 Bacterial toxins and SIDS

The possibility that common bacterial toxins may account for SIDS cases was first hypothesized in 1987, when it was suggested that bacteria proliferating in the upper respiratory tract might produce toxins resulting in the death of the infant. This, it was theorized, would account for the minimal changes at autopsy, the common feature of preceding viral infection, and the age distribution in SIDS cases [Morris, Haran and Smith 1987].

In the decades since this hypothesis was first published, the theory has been further investigated by a number of methods. A number of Staphylococcal

toxins (Enterotoxins A, B, C, D, Toxic Shock Syndrome Toxin 1 (TSST-1), and Alpha-haemolysin), were identified more commonly in SIDS cases than controls using immunohistochemical staining and gel-based techniques [Newbould et al 1989, Malam et al 1992].

In other studies, bacterial isolates from nasopharyngeal samples were used to create crude toxin preparations which were applied to early chick embryos. It was determined that infants dying of SIDS had a significantly higher probability of having lethally toxigenic bacteria in their nasal flora than controls [McKendrick et al 1992]. Further studies of lethality in chick embryos by the same group also demonstrated a synergistic effect when toxins from *Staphylococcus aureus* and *Escherichia coli* were administered to chick embryos together [Drucker et al 1992], and where toxins were administered with very low doses of nicotine [Sayers et al 1995]; this latter finding providing a possible explanation for the association between SIDS and smoking.

Results of examination of antibodies to common bacterial toxins in SIDS infants and controls suggested that many infants are exposed to bacterial toxins and develop immunity. However in SIDS infants there is evidence of a lower Immunoglobulin A response to Staphylococcal toxins than controls [Siarakas, Brown, and Murrell 1999]. It has also been suggested that variation in the genes encoding common inflammatory cytokines may negatively impact the physiological response to some bacterial toxins. For example, single nucleotide polymorphisms in the gene encoding Interleukin-1 beta (IL1β) are hypothesized to contribute to an attenuated response to TSST-1, particularly in the context of cigarette smoking [Moscovis et al 2004]. However, a study examining the role of polymorphisms of T cell receptor BV3 recombination signal sequence (a Staphylococcal enterotoxin receptor gene) showed no significant difference between SIDS cases and controls [Highet, Gibson, and Goldwater 2010b].

A number of studies have demonstrated that toxigenic bacterial strains of *Staphylococcus aureus* and *Escherichia coli* are commonly isolated in infant deaths of all causes, with no significant difference in toxigenic strains isolated from infants dying of explained causes and SIDS [Highet et al 2009, Highet and Goldwater 2009, Weber et al 2011].

1.9 Conclusion

While there is a substantial body of evidence that raises the possibility that at least some cases of SIDS are the consequence of infection or inflammatory mechanisms, there is a lack of widely available tests which could be applied in practice to investigate this possibility further. The body of research in this piece of work will comprise the use of proteomic techniques to further investigate the role of infection in infant death, and develop novel markers for clinical use.

Chapter 2 - Application of Proteomic Techniques to Post Mortem Tissue

2.1 Introduction

The term ‘proteome’ was first used to describe the “total protein complement able to be encoded by a given genome” [Wasinger et al 1995]. Proteomics can therefore be defined as the study of the proteome (i.e. the complete set of proteins expressed by an organism, tissue, or cell). It includes the study of changes in protein expression patterns as related to diseases and environmental conditions. By comparing proteomes from different disease states (comparative proteomics), candidate biomarkers can be identified which may form the basis of tests for the disease of interest. These techniques have dramatically altered the approach to determination of disease aetiologies and mechanisms in life and have the potential to do the same for investigation after death. Specifically, such novel tests may in future allow more accurate determination of the cause, mode and timing of death and may alter the overall strategy for investigation after death. In the perinatal and paediatric setting, such approaches have particular application since in this population, specific genetic disorders are more common, and disease processes are likely to be more detectable due to the relative absence of co-morbidities and environmental influences. Background to the specifics of the technical aspects of proteomics is given in Chapter 3. Here follows an introductory discussion of the ways in which proteomic techniques have been applied to the investigation of infant deaths to date.

2.2 Animal post mortem data

While the focus of this thesis is clearly deaths in humans, application of proteomics to other species has been of great use in establishing the effect of post-mortem changes on the proteome. Much of this data comes from studies in the meat industry, in which quantification of how muscle (meat) changes in the period from death is important for meat quality. Nevertheless, such data provide

useful general data regarding post-mortem proteomic applications and assessment of post mortem changes. Such data is based on both two-dimensional electrophoresis and mass spectrometry-based proteomics. Changes in relative values of specific muscle proteins are described during the post mortem interval including changes in actin fragments and myosin heavy chain fragments [Lametsch and Bendixen 2001, Lametsch et al 2003]. Of the proteins that change most during the initial PM period, these include metabolic enzymes, stress proteins, structural proteins, proteolytic enzymes, and otherwise unclassified proteins. Metabolic enzymes are predominantly associated with the glycolytic pathway or energy metabolism [Jia et al 2007].

Such changes demonstrate distinct patterns of temporal expression, with different groups of proteins changing at different points in the post mortem period, with association analysis suggesting potential biomarkers for features of interest [Te Pas et al 2009, Bjarnadottir et al 2010]. Similar data are available from fish storage, also demonstrating degradation of myosin heavy chains and glycolytic enzymes [Terova et al 2011]. Furthermore, principal component analysis demonstrates the effect of mode of slaughter, with asphyxial modes clustering together [Addis et al 2012, Grabez et al 2015].

2.3 Human post mortem data

Use of both two-dimensional difference gel electrophoresis (2-D DIGE) and mass spectrometry have also been well reported for proteomic investigation of human post mortem samples [Swatton et al 2004]. Analogous to the animal data, post mortem muscle specimens show upregulation of certain transcripts involved with protein biosynthesis, responses to oxidative stress, hypoxia, and ischemia. The data demonstrate that post mortem samples undergo an active transcriptional phase during the initial post mortem period [Sanoudou et al 2004]. In other tissues, such as brain, proteomic approaches can result in confident identification of proteins, which has been particularly useful in the investigation of the mechanisms underlying neurodegenerative disorders [Dumont et al 2006]. The successful use of post mortem fluids such as CSF for

proteomic analysis demonstrates a range of proteins with significant change in concentration in relation to post mortem interval [Finehout et al 2006].

Studies on post mortem muscle biopsies stored for varying periods up to 48 hours have demonstrated that at 4°C, the proteome profile is largely unchanged until 24 hours, after which there is a reduction in number of proteins identified, corresponding to 12 hours at room temperature [Tavichakorntrakool et al 2008]. This is similar to work with brain tissue reporting that 12 hours appears to be critical time point for preservation at room temperature, whereas after refrigeration at 4°C, most phosphoproteins are stable for at least 72 hours [Oka et al 2011]. Liquid chromatography mass spectrometry has also been used to identify specific elements for subsequent proteomic investigation [Liu et al 2011].

2.4 Use of formalin-fixed paraffin-embedded (FFPE) material

Most diagnostic pathology departments, including forensic pathology, have existing diagnostic archives of well characterised cases with FFPE tissue samples. The ability to use such retrospective resources if of great use in many rare diseases, and in the context of this study is vital in the investigation of SUDI; the relatively small number of cases in each centre per year meaning single centre prospective data collection might take many years. The nature of formalin-fixation and paraffin-embedding introduces complications which require adaption of the techniques used in fresh tissue; the specific difficulties are discussed in Chapter 3. Despite these complications FFPE material has now been successfully used for a number of ‘omic’ techniques.

Use of FFPE material has now been widely described including various methods for extraction of DNA and RNA [Okello et al 2010]. Post mortem FFPE tissue samples have also been used for polymerase chain reaction amplification, and DNA sequencing. Some genes were stable regardless of fixation time whereas others reduced in yield with increasing fixation time [Ferruelo et al 2011]. FFPE results in partially degraded RNA; the interpretation of which can be optimised with specific platforms [Chow et al 2011].

Formalin-fixed paraffin-embedded tissue taken at the time of post mortem examination has been successfully used for proteomic investigation and has resulted in the identification of a number of clinical biomarkers [Murray 2012]. This is of particular interest in rare conditions such as paediatric tumours and neurodegenerative disorders, where post mortem samples may be the only samples available for testing [Nazarian et al 2008, Drummond et al 2015]

2.5 Other techniques; metabolomics and metagenomics (microbiome)

Other laboratory investigations have recently started to become applied to the PM setting, although the complexity in their interpretation remains. For example, rat femoral muscles alter their metabolic profiles after death depending on the mode of death and post mortem interval [Hirakawa et al 2009]. Similarly, mass spectrometry for a range of metabolites could separate groups of animals based on the time since death; illustrating the potential for metabolic profiling to determine post mortem interval under certain conditions [Sato et al 2015].

Evaluation of the human microbiome has allowed determination of changes in gut bacterial populations with increasing post mortem interval and demonstrated for example, that *Bacteroides* and *Lactobacillus* relative abundances decline with increasing post mortem interval, and could be used as quantitative indicators of time since death [Hauther et al 2015].

Despite the proven utility of these techniques in post mortem tissue, such techniques are of limited application in formalin-fixed paraffin-embedded tissue, where the nature of processing means that proteins present at a low abundance may have been washed away during processing.

2.6 Conclusion

Proteomics has been successfully applied to post mortem material including the analysis of formalin-fixed paraffin embedded samples. Despite the well documented changes in protein structure and the proteome in the post mortem period in both animals and humans, useful data in disease groups has been

obtained. This has allowed the detection of biomarkers for rare tumours and neurodegenerative diseases. The use of formalin-fixed paraffin-embedded tissue for proteomics allows the inclusion of large number of cases in rare conditions, including infant deaths, where prospective tissue collection would take many years. However, the use of this tissue requires refinement of the conventional proteomic techniques, which are discussed in detail in Chapter 3.

Part Two

Methods

Chapter 3 - Methods and Method Development

3.1 Using formalin-fixed paraffin-embedded tissue for proteomic analysis

3.1.1 Introduction

Any study of causes of death necessitates the collection of large quantities of post mortem material for analysis. Infant deaths are relatively rare occurrences and arise as a consequence of a number of different aetiologies. Prospective collection of tissue samples from a sufficiently large number of cases in defined disease groups would therefore take many years and necessitate the involvement of a large number of centres. Since there is marked variation in autopsy practice between centres, there would be serious concerns regarding reliable comparison of cases investigated in different centres.

These problems may be avoided by the use of archived tissue taken over the course of many years at a single centre. Histopathology departments have large stores of such material stored as ‘blocks’ of tissue embedded in paraffin wax (Figure 3.1).

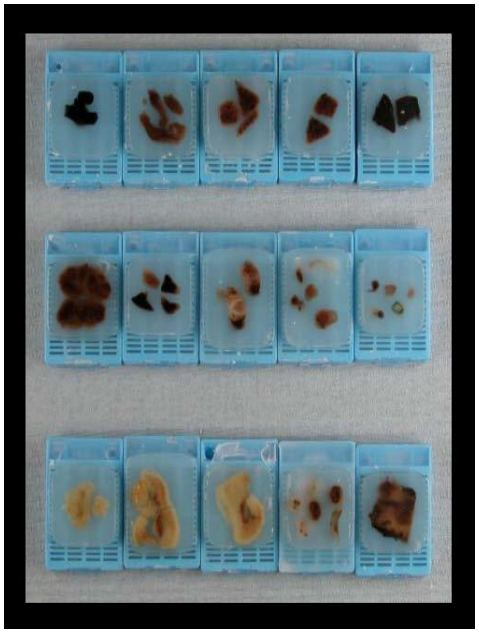

Figure 3.1 Formalin-fixed paraffin-embedded tissue stored in wax blocks

The use of such material circumvents the difficulties of sample collection and inter-department variation in practice [Ralton and Murray 2011]. The process of production of such blocks from post mortem material is as follows.

When a post-mortem specimen is received into the laboratory it is first “fixed” in a solution of 10% neutral buffered formalin (approximately 3.7% formaldehyde in phosphate buffered saline), for a variable period of time which depends on local turnaround time requirements, laboratory pressures, and tissue type. This preserves the tissue by means of a combination of reversible and irreversible protein cross linking of primary amino groups (principally the amino side chain of lysine) to nearby nitrogen atoms with a methylene bridge (-CH2-) (Figure 3.2) [Howat and Wilson 2014]. There is also evidence that cross linking can occur between aminomethylol groups and phenol, indole and imidazole side chains by a form of the Mannich reaction [Fraenkal-Conrat and Olcott 1948]. Cross links are formed both within individual proteins and between separate proteins; preventing, or significantly slowing, the process of protein breakdown.

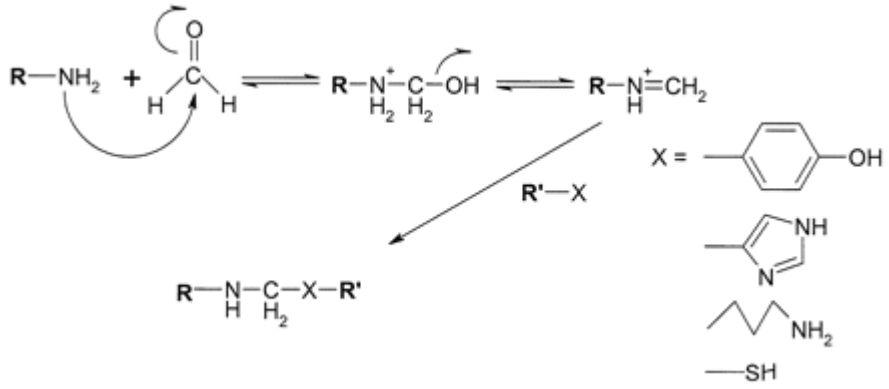

Figure 3.2 Chemical cross-linking of proteins by formaldehyde; the ammonium cation mechanism [Nadeau and Carlson 2007]

The fixed tissue is dehydrated by serial washes in increasing concentrations of ethanol (usually in the form of 99% industrial methylated spirit, IMS), typically ranging in concentration from 70% to 99%. As ethanol is non-miscible with paraffin wax, a clearing agent that is miscible with both the dehydrating agent

and with paraffin wax is required to form a bridge between these stages of the process. Xylene is the usual reagent used for this purpose.

Following clearing with xylene, the tissue is infiltrated with paraffin wax; a mixture of alkanes with the chemical formula C_nH_{2n+2}, where n falls within the range $20 \leq n \leq 40$. Finally, the tissue is embedded in a mould of paraffin wax to form a 'block' which provides mechanical support and allows mounting on a microtome for sectioning. Wax blocks can be stored at room temperature and remain stable for very long periods. A more detailed discussion of the variations in tissue processing commonly used is available from numerous specialised texts [for example Smith and Warfield 2005].

While the use of formalin-fixed paraffin-embedded tissue in infant death research has marked advantages, there are disadvantages to using this material for proteomic research. Paraffin wax is not compatible with the methods used in proteomics, and the protein is 'fixed', preventing its breakdown or digestion. For the tissue to be useful in proteomic research the paraffin wax must be removed; a modification of a previously adopted method was used for this purpose [Pryce 2012]. Furthermore, even if protein is effectively recovered from the tissue, there are changes in protein composition that occur with increasing post mortem interval which may affect the interpretation of results (see Chapter 2).

3.1.2 Tissue selection and preparation from wax block

Slides from appropriate tissues from selected cases were examined using light microscopy and suitable areas for sampling were marked on the slide (Figure 3.3). Using the marked slide as a guide, a 2 millimetre core of formalin-fixed paraffin-embedded tissue was removed from the block using a disposable biopsy punch (IntergraMiltex Disposable Biopsy punch, 2.0mm with plunger, 33-31-P/25, IntergraLifeSciences Holdings Corp; Plainsboro, NJ) (Figure 3.4). Each core was transferred to a labelled non-siliconised polypropylene microcentrifuge tube (Eppendorf) using the plunger system supplied with the biopsy punch. The same punch was used to collect each sample, but cleaned between each sample using proteomic grade ethanol.

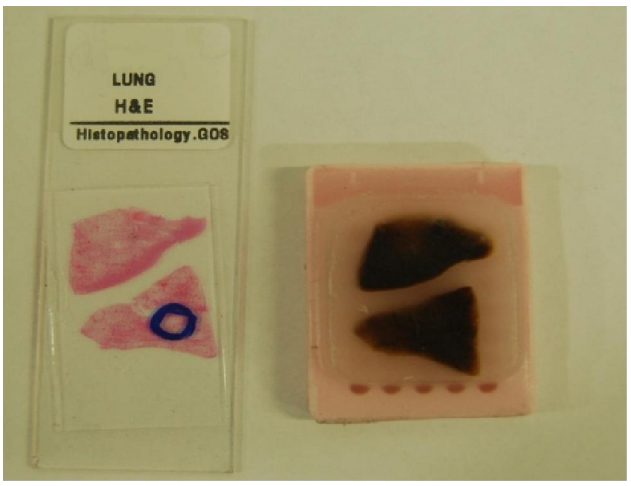

Figure 3.3 Marking of a suitable area for sampling on a glass slide

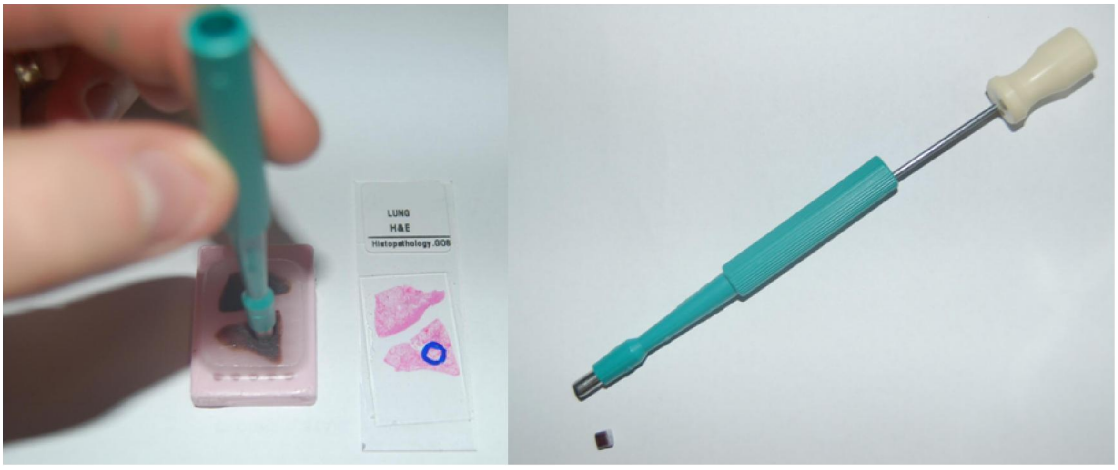

Figure 3.4 Block sampling of a pre-marked area using a punch biopsy system

3.1.3 Removal of alkanes/wax from tissue to allow proteomic analysis

The cores were initially heated at 60°C for 30 minutes to melt the paraffin wax, before being soaked in xylene for five minutes and undergoing three xylene washes. Rehydration was achieved by soaking in decreasing concentrations of proteomic grade ethanol (100%, 95%, 90%, 80%, and 70%), each for a period of five minutes, before immersion of the core in distilled water. This results in an identifiable change in colour and consistency which can be used as a proxy marker for successful deparaffinisation and rehydration (Figure 3.5). The rehydrated tissue cores are now suitable for processing in the proteomic laboratory.

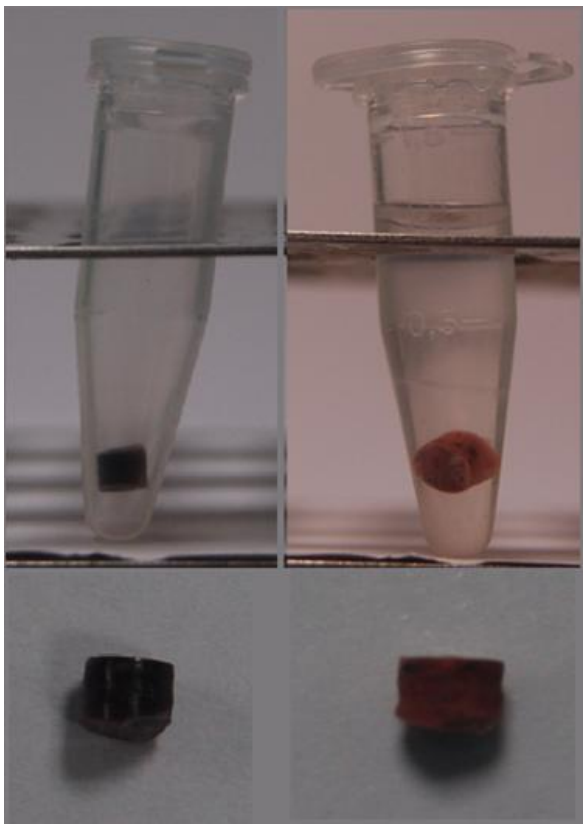

Figure 3.5 Tissue core before and after processing showing characteristic colour and size change with rehydration

3.2 Comparative Proteomics

3.2.1 Introduction

As discussed in Chapter 2, proteomics refers to the study of the proteome; the complete protein complement of a cell, tissue, or organism of interest, including post-translation modifications [Lawrie, Fothergill and Murray 2001, Lovric 2011]. In general terms, proteomic analysis can follow one of two approaches; top-down or bottom-up, with some authors advocating a combined approach ("middle-down") [Moradian et al 2014].

Top-down proteomics involves the separation of intact proteins into fractions by isoelectric focussing or size exclusion. The fractions are then digested and subjected to mass-spectroscopic analysis [Han et al 2006, Whitelegge 2013].

By contrast, bottom-up proteomics relies on inferred protein identification from the analysis of peptides released through a process of proteolysis of all the

proteins using one or more enzymes. The protein mixture is first homogenised and then digested 'in solution' without prior separation by any biochemical techniques. The complexity of the peptide solution is then reduced by separation using ultra performance liquid chromatography (UPLC), conducted prior to electrospray induction [McDonald and Yates 2002, Zhang et al 2013].

Both techniques have advantages and limitations that are outlined in Figure 3.6. In an effort to take advantage of the benefits of both techniques and minimise the limitations, hybrid techniques have been created and are referred to as "middle-down" proteomics. Such techniques usually involve different enzymes than those used in bottom-up techniques [Wu et al 2012].

Top-down proteomics	Bottom-up proteomics
Advantages	
The ability to simplify the proteome prior to mass spectral analysis by dividing the protein into groups (e.g. by size)	No losses and less experimental variability when compared with "top-down" techniques; this is better for quantitative and comparative analyses
Much greater proteome coverage can be attained compared with "bottom-up" techniques	Very quick and simple to perform
Disadvantages	
Many steps; this can lead to protein losses and greater variability which can affect comparative analyses	Requires complex liquid chromatography/chromatographic separation
Very time consuming	Technically challenging
	Does not give as many protein identifications as "top-down" techniques

Figure 3.6 Advantages and limitations of top-down and bottom-up proteomic techniques

While documentation of the protein complement in healthy tissues and disease states can greatly advance the understanding of physiological and pathological cellular mechanisms, the real power of these techniques is in the field of comparative proteomics. In this technique two or more tissues of interest are subjected to analysis (e.g. disease and healthy control). The protein

complements of both tissues are then compared and significant differences between samples can be investigated further as potential biomarkers for the disease or condition of interest.

Comparative proteomics can be performed using top-down techniques by means of differential labelling and separation of the whole proteins from the tissues of interest using gel electrophoresis (e.g. 2-D fluorescence difference gel electrophoresis (DIGE) [May et al 2012]). This is followed by selection and digestion of differentially expressed peptides, which are then submitted to sequencing by liquid chromatography mass spectrometry. Alternatively, the different tissues may be investigated using 'bottom-up' proteomics, by individually submitting both samples to digestion and liquid chromatography mass spectrometry, and performing a statistical comparison of the calculated protein complement of both samples during the data analysis stage.

For the purposes of this study, a 'bottom-up' strategy was used due to its high throughput, ability to easily compare more than two tissues, and less labour intensive experimental methods than gel-based techniques. In addition, given that the crosslinking induced by formalin fixation is unlikely to have been reversed, it is likely that the quality of protein separation using gel-based techniques would be poor. Finally, there is an advantage in the ability to create a validatory targeted proteome with greater ease due to compatibility within the laboratory in which this work was being conducted.

In general terms the 'bottom-up' approach involves homogenising the tissue, quantitation of the protein using a protein assay, protein precipitation, reduction, enzyme digestion, fractionation, liquid chromatography, mass spectrometry, and data analysis. In this instance, a modification of previously published methods was used [Bennett et al 2010, Pryce 2012]. These steps are described below with an explanation of their purpose in the sample preparation process, and any method development which was required to facilitate the use of formalin-fixed paraffin-embedded tissue.

3.2.2 Homogenisation

The tissue was removed from the wax block and prepared as described previously (Sections 3.1.2 and 3.1.3). The rehydrated tissue cores were immersed in 500µL of buffer (50mM ammonium bicarbonate containing 2% ASB-14, pH 8.2). Ammonium bicarbonate is a useful buffer as it entirely decomposes to volatile compounds on lyophilisation, which allows rapid recovery of 'salt free' protein by freeze drying. ASB 14 (3-[N,N-Dimethyl(3-myristoylaminopropyl)ammonio]propanesulfonate) is an amphiphillic zwitterionic detergent which allows the release of proteins from biomembranes, and facilitates the resolubilisation of proteins precipitated during the fixing process, thus making them soluble and accessible to proteases.

The cores were then homogenised for 30 seconds divided into three ten-second bursts with a ten-second pause between each (where the solution was cooled on ice), using an electronic homogeniser (Omni Tissue Homogeniser with Hard Tissue Tip, TH115-PCRH, Omni International Inc; Kennesaw, Georgia) (Figure 3.7). The use of the electronic homogeniser results in a significant time saving, and greater efficiency when compared to hand homogenisation, which typically requires more than fifteen minutes per sample.

The homogenates were then sonicated for six cycles of ten seconds, with ten second pauses between each, with care taken to avoid overheating and protein oxidation by cooling in an ice bath. The samples were then centrifuged at 10000 x g for five minutes to remove any residual cellular debris.

3.2.3 Protein assay

For accurate comparison of two or more samples, quantitation of the protein content of each sample is necessary in order that equal masses of protein are submitted to analysis. Quantitation is achieved using a protein assay; techniques based on a protein-dependant colour change in a solution which may be measured using a colorimeter.

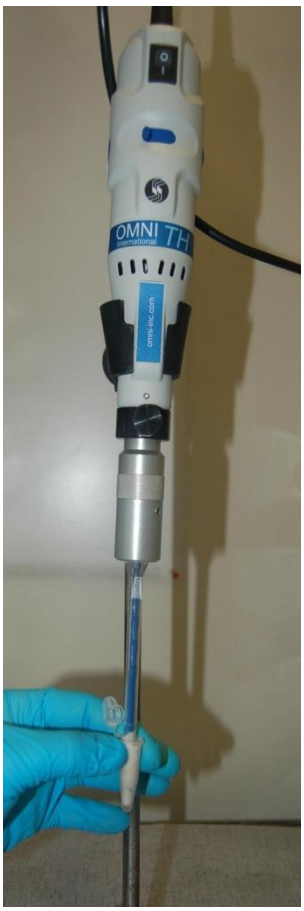

Figure 3.7 Homogenisation of tissue sample in buffer using an electronic homogeniser

A number of protein assay techniques have been used in the literature in formalin-fixed paraffin-embedded samples including the Smith/Pierce (bicinchoninic acid/copper sulphate) [Smith et al 1985], Bradford [Bradford 1976], and modified Lowry assays [Lowry et al 1951]. The Smith assay has been used most frequently in this sample type and, as several of the other assays cannot be used in the presence of detergents, this assay was selected for use in this study.

In the Smith assay, solutions of bicinchoninic acid and copper sulphate are added to a small aliquot of the protein homogenate. The peptide bonds in the protein facilitate the reduction of Cu^{2+} ions in Copper (II) sulphate to Cu^{+} ions (the Biuret reaction [Gornall, Bardawill and David 1949]). The reduction of Cu^{2+} ions is proportional to the amount of protein in solution. Two molecules of bicinchoninic acid chelate with each Cu^{+} ion; forming a coloured product which absorbs light strongly at a wavelength of 562nm. The absorption spectra of the homogenate and of protein samples of known concentrations (e.g. serial

dilutions of bovine serum albumin), may then be compared to allow quantitation of the protein content.

When this assay was utilised in this study, it was noted that there was an extremely wide variation in results when analysis of a single sample was repeated under identical conditions. It was hypothesised that some element of the process of formalin-fixation and/or paraffin embedding was interfering with the assay. It has been established that there are differences in protein structure and modification between fresh frozen and formalin-fixed paraffin-embedded samples [Sprung et al 2009]. To investigate this further, two sets of small samples of increasing volume were taken from fresh frozen and formalin-fixed paraffin-embedded liver from the same case. The samples were subjected to the Smith assay (as described above and in the appendix), the Pierce assay (Pierce 660nm Protein Assay Kit, Thermo Scientific, 22662SPLC), and the Lowry assay (Modified Lowry Protein Assay Kit, Thermo Scientific, 23240). A commercially available ionic detergent compatibility reagent (Thermo Scientific, 22663) was used according to manufacturers recommended methodology.

Despite the addition of the ionic detergent compatibility reagent, both the Modified Lowry and the Pierce assays resulted in a coagulated sample which could not be subjected to colorimetry.

The results of the Smith assay are given in Figure 3.8. The standard curve had an r^2 value of 0.982; demonstrating no evidence of error in the performance of the assay. The r^2 values for the study groups clearly demonstrate that there is poor correlation between the weight of tissue processed and the concentration of the protein solution as measured by the assay. The reason for this is unclear given that the fresh-frozen tissue has a similarly poor performance to the formalin-fixed paraffin-embedded tissue, but it is possible that the innate properties of the histological processing have contributed. Given the concerns regarding use of the assay, comparison was conducted using wet weight rather than assay-derived protein content.

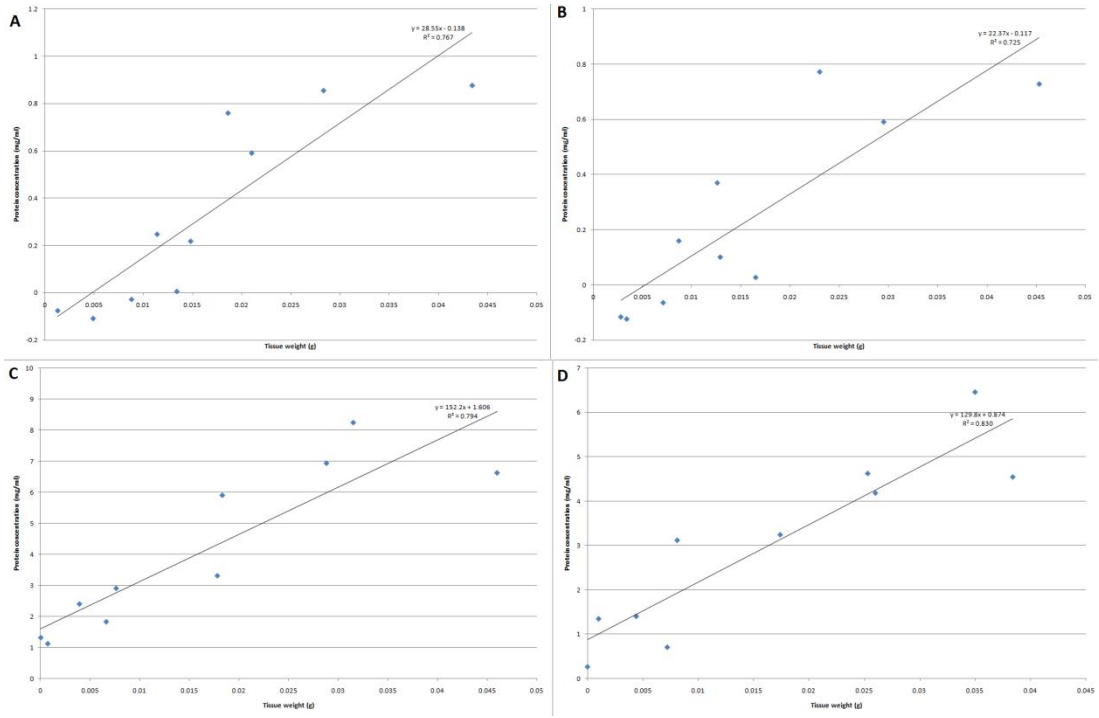

Figure 3.8 Graphs of tissue weight (g) vs. protein concentration (mg/ml) demonstrating poor correlation. Fresh frozen tissue (A, B) and formalin-fixed paraffin-embedded tissue (C, D)

3.2.4 Protein precipitation

Prior to digestion for mass spectral analysis, a process of precipitation is necessary to concentrate the protein present and to remove interfering substances used in the resolubilisation process e.g. salts. Solubility of proteins is dependent on the proportion of hydrophilic and hydrophobic amino acid residues at the surface of the protein and interactions with the solvent. Electrolytic solutions form a solvation layer (hydration layer) around the hydrophilic surface residues of a protein. This establishes a concentration gradient around the protein, with the highest concentration at the protein surface. This network has a dampening effect on the attractive forces between proteins, which prevents them from precipitating.

During precipitation a miscible solvent is added to the protein solution to disrupt the hydration layer around the protein. This results in gradual displacement of water from the protein surface; the water molecules then forming small hydration layers around the organic solvent molecules. Since the hydration

layers around the protein are significantly reduced, electrostatic and dipole forces between the protein molecules allow aggregation of the protein.

A number of solvents have been used for protein precipitation including acetone/trichloroacetic acid [Mechin, Damerval and Zivy 2007], sodium dodecyl sulphate/trichloroacetic acid [Schaffner and Weissmann 1973, Retz and Steele 1977] and chloroform-methanol [Wessel and Flugge 1984]. Because of its reliable performance as a general extraction solvent, with particular efficacy in removing lipids, and hence residual wax, the chloroform-methanol method was used in this study (Wessel and Flugge 1984). This method involves the addition of a mixture of chloroform and methanol, and then allows effective precipitation of the protein without the formation of a bilayer.

For this study, chloroform-methanol was prepared in a ratio of three parts methanol to one part chloroform, and added to the samples at a ratio of three parts chloroform-methanol to one part sample. The mixture was mixed thoroughly for 30 minutes and kept overnight at -20°C before centrifugation at 13,000 x g for two minutes. The supernatant was aspirated and discarded, and the protein pellet was freeze-dried.

3.2.5 Reduction and enzyme digestion

Prior to mass spectrometry, the protein pellets are resuspended and the protein digested into peptides. Digestion is facilitated by the addition of detergents (urea and ASB-14), which denature the tertiary structure of the proteins and increase their solubility and increases the accessibility of the proteins to the enzyme. The accessibility of proteins and efficiency of digestion is also facilitated by reduction of disulphide bridges by 1,4-dithioerythritol, and carbamidomethylation of free thiol groups by 2-iodoacetamide.

The protein pellets were reconstituted in 20 µL of 100mM Tris, pH 7.8, containing 6M urea and 1% ASB-14. The solution was vortexed well and left shaking at room temperature for one hour. 1.5 µL of 100mM Tris containing 0.195M 1,4-dithioerythritol, pH 7.8, was added and the sample vortexed again and left at room temperature for a further hour. 6 µL of 100mM Tris containing 0.195M 2-iodoacetamide, pH 7.8, was then added, before being vortexed again

and left at room temperature for thirty minutes. Enough distilled water was added to make a total volume of 200 µL.

Digestion was achieved with endoproteinase Lys-C and trypsin. 2µg of endoproteinase Lys-C was added to the sample and incubated for two hours at 37°C. Trypsin was provided in the form of lyophilised 1mg aliquots (Sigma-Aldrich Company Ltd, Gillingham; UK), which were each reconstituted with 11µl of 40mM ammonium bicarbonate, with 10% acetonitrile. The resuspended trypsin was added to the sample and vortexed, before incubation for at least 16 hours in a 37°C water bath. The resulting digested samples were then stored at -20°C.

3.2.6 Fractionation

In order to increase peptide identification, each sample can be split (fractionated) into a number of smaller samples (fractions) based on a peptide characteristic of interest, such as hydrophobicity, using a form of reverse phase chromatography. This is achieved using a column packed with a non-polar, hydrophobic stationary phase, such as silica modified with silyl ethers containing non-polar alkyl groups such as C4, C8, or C18. The peptides in the solution adsorb to the stationary phase in the column; those with high net hydrophobicity adsorbing most effectively. Peptides are then eluted from the column with increasingly hydrophobic solutions; allowing the lowest hydrophobicity peptides to be washed into solution at each stage of elution.

For this study, high pH C18 fractionation was conducted using an Isolute 50mg C18 column (220-0005A). The column was primed with 1ml of 50% acetonitrile containing 0.1% ammonia. Equilibration of the column was achieved by the addition of 2ml of a solution of 0.1% ammonia.

The digested peptide sample was prepared by addition of 400µl of a solution of 0.2% ammonia to 100µl of the protein digest, before being vortexed and centrifuged at 13,000 x g for five minutes. This was then added to the column and allowed to flow through under gravity, caught, re-applied to the column, and washed through with 500µl of 0.1% ammonia.

For this study the mobile (liquid) phases used were 500µl of solutions of 3%, 5%, 8%, 10%, 15%, 25%, 35%, 50%, and 100% acetonitrile containing 0.1% ammonia, and 100% methanol. Each fraction was dried using a speed vac and reconstituted in 30µl of a solution of 3% acetonitrile containing 0.1% trifluoroacetic acid. A specified amount (5pmol) of marker peptide from yeast enolase *Saccharomyces cerevisiae* (ATCC 204508/S288c) was then added to allow quantitation.

3.2.7 Liquid chromatography

Ultra performance liquid chromatography (UPLC) is used for separation of the peptides prior to electrospray induction. This approach also allows visual examination of the chromatogram to assess the quality of the data being obtained both within and between runs. A nanoACQUITY UPLC system (Waters Corporation; Milford, MA) was utilised for UPLC. This consists of a column packed with C18-labelled silica particles ('stationary phase') through which the peptide-containing solution ('mobile phase') is injected. The interaction between the C18-labelled silica particles and the peptides results in adsorption of the peptides. Injection of different concentrations of organic washes results in a staged elution of the peptides. An example tabulated and diagrammatic representation of a typical elution method is given in Figure 3.9.

Given the use of formalin-fixed paraffin-embedded source material when compared to the usual fresh materials utilised in proteomics, and the need to maximise protein identifications, it was decided to optimise the elution process by varying the gradient and time period over which the elution was conducted. A total of 28 elution methods were designed and programmed. Each was run using the same digest from a core of formalin-fixed paraffin-embedded liver, which was processed using the above described method. The number of protein identifications for each sample is given in Figure 3.10. The most effective method tested comprised linear elution between 3% and 55% acetonitrile over thirty-four minutes of the forty-five minute cycle (Figure 3.11); the remainder of the time being given over to elution at 99% acetonitrile to remove contaminants from the column prior to the next run. Elution was

performed over a total period of forty-five minutes as a compromise between optimising the results obtained, and allowing efficient throughput of samples.

Step	Time	Flow	%A (Water)	%B (ACN)	Curve
1	I	0.3	97	3	I
2	1	0.3	97	3	6
3	30	0.3	60	40	6
4	32	0.4	1	99	6
5	37	0.4	1	99	6
6	44.9	0.35	97	3	11
7	45	0.3	97	3	11

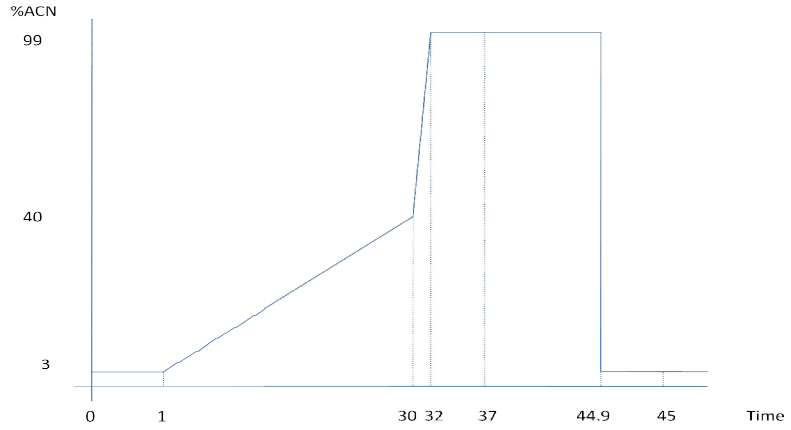

Figure 3.9 Tabulated and graphical representation of a typical 45 minute liquid chromatography inlet method

The improvement resulting from this method development can be observed clearly in the chromatography elution profiles shown in Figures 3.12 and 3.13 using the original and optimised methods. There is a significant increase in protein identifications and greater chromatographic separation over the time period of the method (Figures 3.12 and 3.13). This results in the mass spectrometer being able to sequence more peptides and hence increase the number of 'hits' attained.

Method	Protein hits
New_LP_LC45min_Andy_Bamber_a	0
New_LP_LC45min_Andy_Bamber_b	30
New_LP_LC45min_Andy_Bamber_c	64
New_LP_LC45min_Andy_Bamber_c_new	97
New_LP_LC45min_Andy_Bamber_d	55
New_LP_LC45min_Andy_Bamber_e	84
New_LP_LC45min_Andy_Bamber_f	69
New_LP_LC45min_Andy_Bamber_g	48
New_LP_LC45min_Andy_Bamber_h	43
New_LP_LC45min_Andy_Bamber_i	41
New_LP_LC45min_Andy_Bamber_j	70
New_LP_LC45min_Andy_Bamber_k	0
New_LP_LC45min_Andy_Bamber_l	34
New_LP_LC45min_Andy_Bamber_m	62
New_LP_LC45min_Andy_Bamber_n	49
New_LP_LC45min_Andy_Bamber_o	40
New_LP_LC45min_Andy_Bamber_p	45
New_LP_LC45min_Andy_Bamber_q	1
New_LP_LC45min_Andy_Bamber_r	22
New_LP_LC45min_Andy_Bamber_s	43
New_LP_LC45min_Andy_Bamber_t	28
New_LP_LC45min_Andy_Bamber_u	21
New_LP_LC45min_Andy_Bamber_v	25
New_LP_LC45min_Andy_Bamber_w	39
New_LP_LC45min_Andy_Bamber_x	40
New_LP_LC45min_Andy_Bamber_y	50
New_LP_LC45min_Andy_Bamber_z	18

Figure 3.10 Protein identifications achieved with different liquid chromatography inlet methods in the same clinical sample

The complete liquid chromatography method using the optimised inlet method was therefore as follows: peptides were trapped and de-salted before reverse phase separation using the UPLC pre-column (Symmetry C18 5µm, 5mm x 300 µm) at flow rate of 4µl/min in 0.1% formic acid for a total time of four minutes. Peptides were then eluted off the pre-column and separated on the reverse phase analytical column (Symmetry C18, 15 cm x 75µm) using the optimised gradient of acetonitrile with 0.1% formic acid.

Step	Time	Flow	%A (Water)	%B (ACN)	Curve
1	I	0.3	97	3	I
2	1	0.3	97	3	6
3	35	0.3	50	50	6
4	35.1	0.5	1	99	11
5	40	0.5	1	99	6
6	40.1	0.35	97	3	6
7	45	0.35	97	3	6

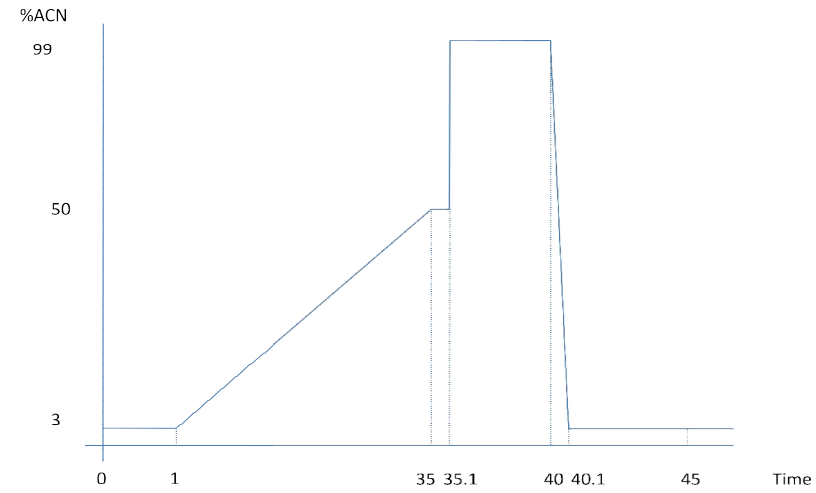

Figure 3.11 Tabulated and graphical representation of the optimised liquid chromatography inlet method

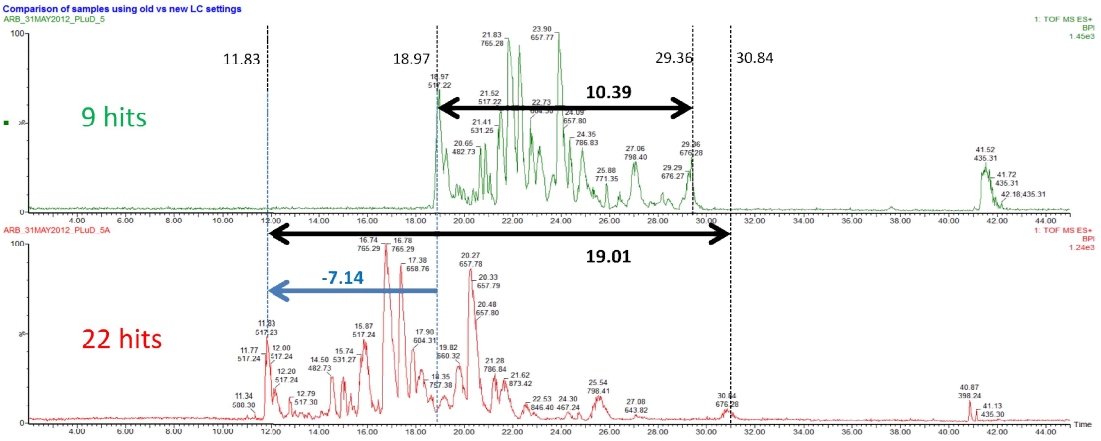

Figure 3.12 Comparison of chromatography from a sample analysed using old (green) and optimised (red) liquid chromatography inlet methods. Number of protein identifications ('hits') is given to the left, and expansion of the chromatography is illustrated by the black arrows.

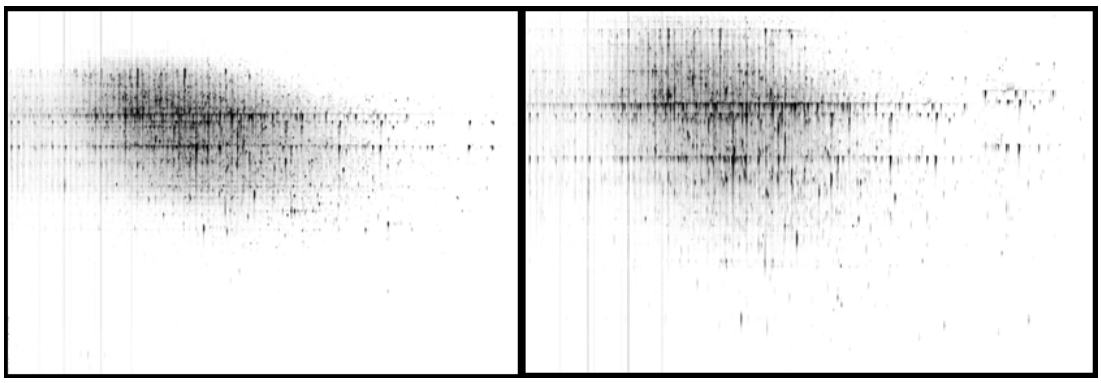

Figure 3.13 Computer reconstructed 'gel' of the two samples shown in Figure 3.12 demonstrating improved spread

3.2.8 Mass spectrometry

Following elution from the LC column, the peptides are ionised by spraying a continuous volume of peptide mixture through a cone through which an electric current is applied (electrospray). The ionised peptides are then guided through the vacuum of the mass spectrometer by means of an electromagnetic field generated by a quadrupole. In conjunction with a time of flight component, this allows highly detailed analysis of the mass/charge ratio of the peptides.

Using previously defined standard laboratory protocols [Bennett et al 2010], peptides were analysed in positive ion mode using an ESi Q-Tof Premier mass spectrometer (Waters Corp, Manchester; UK). Post calibration of data files were corrected using the doubly charged precursor ion of [glu1]-fibrinopeptide B (m/z 785.8426), with a sampling frequency of 30 seconds. Mass data were collected in a data independent and alternating low and high collision energy mode. Each low/high acquisition was 1.5 seconds with a 0.1 second interscan delay. A constant collision energy of 4 Volts was used for low collision energy data acquisitions. A 15-40 Volt ramp over a 1.5 second time period was used for high-energy acquisitions. A complete low/high collision energy acquisition was achieved every 3.2 seconds.

3.2.9 Data analysis

Complex bioinformatic software is used to match peptide fragments to protein sequences; resulting in a statistical likelihood of a protein being present in the original protein sample [Washburn, Wolters and Yates 2001]. In this instance, data were analysed using ProteinLynx Global Server (PLGS) version 2.5 (Waters Ltd, Elstree; UK). The amino acid sequence for yeast enolase *Saccharomyces cerevisiae* (P00924) was added manually to the human UniProt database. Protein identification from the low/high collision spectra for each sample was processed using a hierarchical approach requiring matching of at least three fragment ions per peptide, five fragment ions per protein, and one peptide per protein. Protein identification parameters used in the database searching included a <10ppm mass accuracy tolerance, fixed modifications of carbamidomethylation of cysteine, and dynamic modifications of deamidation of asparagine, deamidation of glutamine, and oxidation of methionine.

The algorithm utilised by the software during analysis usually relies on inclusion of the specific primary cleavage specificity of the enzyme used during the digestion process. For example, trypsin specificity is usually defined as cleavage C-terminal to arginine or lysine residues, except where a proline residue immediately follows. In reality, the situation is more complex, and the effect of trypsin on a peptide is also influenced by other surrounding peptides, particularly those which are negatively charged such as glutamate and aspartate (Figure 3.14). Such residues may result in a “missed cleavage” site in the peptide sequence, which can also be of use in analysing the data [Siepen et al 2007].

In this study, the use of such specific searching resulted in extremely low numbers of protein identifications (less than 10 per sample). It was hypothesised that the non-specific breakdown of proteins as part of the process of post mortem decomposition before tissue fixation and processing was responsible for this. The software programme was therefore modified to allow assessment of all peptide cleavage sites, rather than a specific search for cleavages and missed cleavages related to the enzymes used in digestion. This approach markedly increased the protein identifications achieved, though at the

cost of a significantly increased analysis time, and some decrease in confidence in the protein identifications.

Position relative to cleavage site						
P4	**P3**	**P2**	**P1**	**P1'**	**P2'**	**P3'**
		W Y or F	R or K	not R or K		
		not R or K	R or K	W Y or F		
		D or E	R or K	not R or K		
		not R or K	R or K	D or E		
			R or K	not R or K	D or E	D or E
D or E	D or E	not R or K	R or K			
	D or E	not R or K	R or K	not R or K	D or E	
		not R or K	R or K	R or K		
		not R or K	R or K	H		
			R or K	P		

Figure 3.14 Peptide sequence patterns imparting a negative effect on trypsin activity (as P4-P3-P2-P1-||-P1'-P2'-P3'-P4' where cleavage takes place between P1 and P1') After Siepen et al 2007

3.3 Targeted Proteomics

3.3.1 Introduction

Candidate biomarkers must be validated before translation into clinical use. The most efficient method for achieving this is by the development of multiple reaction monitoring based assays on a triple quadrupole tandem mass spectrometer. This allows targeted quantitation of proteins using specific peptides, with a number of major advantages over alternative methods including low cost, high specificity and high throughput. The technique involves the use of a triple quadrupole tandem mass spectrometer to filter and measure a target precursor mass ('parent') of the selected biomarker peptide (MS1), and collision-induced dissociation (CID) leading to the formation of specific mass product ('fragment') ions (MS2). The combination of a specific parent and fragment ion identification provides a characteristic 'mass signature' which, in combination with a unique retention time using reverse phase liquid chromatography, provides robust peptide identification. Relative quantitation is achieved by spiking samples with a known quantity of a non-human peptide

(e.g. yeast enolase *Saccharomyces cerevisiae* (ATCC 204508/S288c)). Absolute quantitation can be achieved by the use of an isotopically-labelled amino acid incorporated into a peptide with an identical sequence to the target peptide ("AQUA peptide"), used as an internal control.

MRM method development was undertaken using a modification of previously used in the laboratory in which this work was undertaken using a Waters Xevo TQ-S mass spectrometer coupled to a Waters standard Acquity liquid chromatography system.

3.3.2 Peptide selection

Candidate peptides from proteins of interest may be selected using several techniques [James and Jorgensen 2010]. First, candidate peptides may be selected from previous biomarker discovery work. Second, the whole protein may be purchased and digested before itself being submitted for proteomic analysis, and peptides selected from the resulting data. Third, for proteins of interest which have not been identified in previous work, peptides can be selected based on openly published online data from other groups (e.g. using the Global Proteome Machine) and theoretical protein cleavage data (e.g. using Skyline).

3.3.3 Preparation of peptides for tuning

Selected peptides were custom synthesised by Genscript USA Inc. or Generon UK Ltd. The synthesised peptides that did not contain a cysteine residue were reconstituted in a solution of 50% acetonitrile. Those containing a cysteine residue were carbamidomethylated using the following technique: Peptides were generally supplied as 1mg dry peptide. This was reconstituted in 1ml of 50% acetonitrile to give a working concentration of 1mg/ml of peptide. The reconstituted peptide solution was divided between four Eppendorf tubes, each containing 0.25mg of peptide in 250µl, and dried using a speed vac. One of the

samples was reconstituted in 20µl of distilled water for carbamidomethylation, and the others were stored at -20° C for later use.

The reconstituted peptide was reduced by addition of 3µl of 100mM Tris containing 0.195M 1,4-dithioerythritol, pH 7.8 and left shaking for 1 hour at room temperature. Carbamidomethylation was achieved by addition of 12µl of 100mM Tris containing 0.195M 2-iodoacetamide, pH 7.8, and left shaking for 45 minutes at room temperature.

A 1mg C18 spin column was equilibrated using 200µl of 50% acetonitrile containing 0.1% trifluoroacetic acid. This was centrifuged at 1,500 x g for one minute. The column was washed through with 200µl of 0.1% trifluoroacetic acid and centrifuged at 1,500 x g for one minute. The wash step was repeated a total of three times. The peptide sample was added to the column, centrifuged for thirty seconds at 1,500 x g, and the flow-through re-applied. The column was washed through with 200µl of 0.1% trifluoroacetic acid and centrifuged at 1,500 x g for one minute. The wash step was repeated a total of three times. Elution of the bound peptide was achieved using a solution of 70% acetonitrile. The eluted peptides were dried down using a speed vac and reconstituted as required in a solution of 50% acetonitrile.

3.3.4 Manual tuning of peptides

An accurate mass of a precursor ion was determined using an MS1 scan with source settings of 2.5kV capillary voltage, 35 V cone voltage, and a desolvation temperature of 500°C. Gas flow settings were set to desolvation 1000L/hr, cone 15 L/Hr, and nebuliser set at 7.0 bar. Low mass resolution was set to 2.8 and high mass resolution to 14.9 with a collision gas flow of 0.15 ml/min.

A daughter scan was then performed on the precursor mass, and daughter ions with the best response were optimised with repeated scanning at differing collision and cone energies. Parent-daughter transitions were confirmed to be from the peptide of interest using commercially-available software (Skyline). A retention time for the selected transitions was then determined by running the synthetic peptide through the liquid chromatography system using a method as

given in Figure 3.15 over a duration of ten minutes. Three transitions were selected for use for each protein of interest.

Step	Time	Flow	%A (Water)	%B (ACN)	Curve
1	I	0.8	97	3	0
2	0.20	0.8	97	3	6
3	7.00	0.8	60	40	6
4	7.01	0.8	0.1	99.9	6
5	8.00	0.8	0.1	99.9	6
6	8.01	0.8	97	3	1
7	10.00	0.8	97	3	1

Figure 3.15 Liquid chromatography inlet method used for retention time determination for Multiple Reaction Monitoring

3.3.5 Automated tuning of peptides

As an alternative to manual tuning, reconstituted peptides can be 'tuned' using automatic methods programmed into the mass spectrometer software. In the case of the mass spectrometer being used (Waters Xevo TQ-S coupled to a Waters standard Acquity liquid chromatography system), an automated system "Intellistart" is pre-installed with the software and this was used for some peptide tuning.

3.3.6 Experimental testing

Once optimised, peptide detection in samples can be performed. Samples were prepared using the same methods as described above for biomarker discovery work on a Q-TOF mass spectrometer. The optimal quantity of digest to inject was established using repeated testing. It was not necessary to use molecular weight filters for the sample types used. Tests for different peptides and proteins were multiplexed together to avoid sample loss and increase throughput. Dynamic multiple reaction monitoring was performed over a ten minute gradient with a minimum of 0.01s dwell time for quantitative transitions and a minimum of twelve data points per peak.

A standard curve was prepared in water and sample matrix and run with each batch of samples to establish linearity and define the limit of detection. Inter- and intra-batch variation was determined by repeated sample injections over a number of days. A quality control sample created from pooled tissue was run at the start of each batch and repeated between every twenty samples. A variation in the quality control run of less than 15% was considered acceptable.

3.3.7 Data analysis

Data was analysed using Waters QuantLynx version 4.1 (Waters Corp. Milford; USA). Peak integration including smoothing and baseline subtraction was performed and the integrated peak areas ratioed to the internal standard. Absolute quantitation was performed by comparison with the known quantity of internal standard (yeast enolase *Saccharomyces cerevisiae* (ATCC 204508/S288c)).

3.4 Summary

Given the necessity of using formalin-fixed paraffin embedded samples for this study, it has been necessary to refine the proteomic techniques being used in order to optimise the data obtained. A deparaffinisation method has been utilised and it has been shown that in such samples common protein assays are ineffective; necessitating alternative measures of protein content. Optimisation of the liquid chromatography inlet method has resulted in a significant increase in proteins identified from samples derived from formalin-fixed paraffin-embedded tissue. In addition to allowing the use of formalin-fixed paraffin-embedded tissue in this study, these advances in technique will be of use in other studies using this tissue type.

Part Three

Results

Chapter 4 - Bacteria and Toxin Identification

4.1 Introduction

Infection is strongly implicated as aetiology for at least a proportion of unexplained sudden infant deaths. While some deaths may be due to a classical 'sepsis' mechanism, it has been hypothesised that bacteria may also result in infant deaths by means of toxin-mediated effects.

A number of methods are available for identification of bacteria in clinical samples, several which may be applied to post mortem tissue. These include traditional culture with biochemical identification techniques, PCR analysis of 16S rRNA [Kolbert and Persing 1999, Clarridge 2004], and analysis of bacteria by means of MALDI mass spectrometry [Holland et al 1996, Rodriguez-Sanchez et al 2016]. As discussed previously, it is difficult to assess the significance of bacteria in post mortem samples, and Chapters 5 and 6 of this thesis are dedicated to the identification of biomarkers which may assist in this regard.

In contrast to the identification of bacteria in post mortem samples, with the exception of toxin testing for Clostridium difficile, there are currently no widely-available tests for bacterial toxins which can be applied in clinical practice. The only feasible technique for most pathologists is referral of an isolate to a National Reference Laboratory for toxin genotyping. This is expensive and time consuming, and out of reach of 'routine cases' encountered in paediatric pathology practice. Furthermore, the presence of the gene for a toxin is not evidence of toxin production or a pathological effect induced by that toxin. In addition to genotyping, we are aware of at least one laboratory which utilises gel-based techniques for toxin identification, and there are reports in the literature of toxin testing using ELISA [Zorgani et al 1999, Shokrollahi et al 2014], however these techniques are limited to research practice and are not widely available.

A historic limitation to direct identification of toxin in clinical samples is the very low concentration of toxin which is present in biological fluids compared with the plethora of native proteins present. Proteomic techniques provide an

opportunity to develop a specific and sensitive test to directly identify protein-based toxins in clinical samples, even when present at very low concentrations. There has been some limited application of proteomic techniques to bacterial toxin identification, including some which have utilised MRM-based methods [Andjelkovic et al 2016, Yang et al 2015, Silva et al 2015]. To date such approaches have been limited to small numbers of toxins and only a small number have been applied to human samples. Many relate to the presence of bacterial toxins in foodstuffs and there are no published examples of such techniques being applied in the context of infant death or post mortem testing.

The aim of this work was, as proof of principle, to develop a direct test for bacterial protein toxins using targeted proteomics and multiple reaction monitoring mass spectrometry (MRM-MS) which could be successfully applied to post mortem samples. As they are relatively well characterised and have been implicated as a cause of sudden infant deaths, toxins produced by *Staphylococcus aureus* were selected for this project. Furthermore, in addition to the application of MRM-MS to toxin identification, it is also possible to use this technique to identify and separate bacteria by the identification of specific proteins [Larson et al 2013, Kruh-Garcia et al 2014]. It was therefore decided to develop MRM-MS methods for identification of *Staphylococcus aureus* and some of its common toxins with a view to combining the MRM methods into a single multiplex test, and applying them to bacterial isolates.

4.2 Method development

4.2.1 Bacterial identification

Staphylococcus aureus were retrieved from beaded stored clinical isolates from six clinical cases at Great Ormond Street Hospital for Children between 2011 and 2012. These included a variety of sites of infection, and variable expression of the gene for Panton Valentine Leukocidin. The basic anonymised demographics of these cases are described in Figure 4.1.

Specimen ID	Age	Gender	Sample	Date sampled	Bacteria	PVL status
11B25865	7 weeks	Male	Pleural empyema	08/05/2011	*S. aureus*	gene detected
12B16783	3 years	Male	Pleural empyema	14/03/2012	*S. aureus*	gene detected
12B20982	2½ years	Male	Blood	03/04/2012	*S. aureus*	gene not detected
12B53221	9 months	Male	Blood	31/08/2012	*S. aureus*	gene not detected
12B57863	2 weeks	Female	Pus finger wound	22/09/2012	*S. aureus*	gene not detected
12B61275	14 years	Female	Pus right knee	08/10/2012	*S. aureus*	gene not detected

Figure 4.1 Anonymised characteristics of six isolates of Staphylococcus aureus utilised for shotgun proteomic analysis

To identify the most suitable and specific peptides for *Staphylococcus aureus* for development into an MRM-based targeted assay, a shotgun 'omic' analysis of *Staphylococcus aureus* was performed. The bacterial isolates were cultured on blood agar for 24 hours at 37ºC. For each sample, six colonies were removed from the agar plate using a wire loop and placed into an Eppendorf tube. The retrieved colonies were immersed in 50µl of 100mM Tris pH7.8 6M urea 2% ASB-14 to assist in the degradation of the bacterial cells. The samples were vortexed well, subjected to 10 freeze-thaw cycles in dry ice and then left to shake overnight.

The samples were then reduced, digested with trypsin, prepared for LCMS, submitted to LCMS, and analysed according to the protocol described in Chapter 3. Bioinformatic analysis using ProteinLynx Global Server v.2.5 (Waters Corp; Milford, Mass.) took account of the use of trypsin rather than utilising a non-specific approach.

Analysis of the data revealed between 136 and 200 proteins identified in each of the six samples. In contrast to the usual application of comparative proteomics, where differential protein expression between samples is used to identify biomarkers, for this work it was necessary to identify proteins which were conserved between groups; these proteins being potential common

biomarkers for *Staphylococcus aureus*. Thirty-five proteins were identified which were present in all six strains (see Figure 4.2). Each of these proteins was assessed according to the criteria given in the flow chart in Figure 4.3.

Protein name	Gene Name
Putative aldehyde dehydrogenase	aldA
Aspartyl glutamyl tRNA Asn Gln amidotransferase subunit B	gatB
50S ribosomal protein L11	rplK
Thioredoxin	trxA
Cysteine synthase	cysK
Succinate dehydrogenase subunit B	sdhB
Cell division protein FtsZ	ftsZ
ALF1 Fructose bisphosphonate aldolase class 1	fda
ALF2 Fructose bisphosphonate aldolase	fba
Purine nucleoside phosphorylase DeoD type	deoD
Pyruvate dehydrogenase E1 component subunit alpha	pdhA
ODPB Pyruvate dehydrogenase E1 component subunit beta	pdhB
Ribose phosphate pyrophosphokinase	prs
Aconitate hydratase 1	acnA
ATP synthase subunit alpha	atpA
Succinyl CoA ligase ATP forming subunit alpha	sucD
Formate acetyltransferase	pflB
Glutamine synthetase	glnA
Glyceraldehyde 3 phosphate dehydrogenase	gap
Dihydrolipoyl dehydrogenase	pdhD
Glycine cleavage system H protein	gcvH
Serine tRNA ligase	serS
FTHS Formate tetraydrofolate ligase	fhs
DNAK Chaperone protein DnaK	dnaK
PCKA Phosphoenolpyruvate carboxykinase	pckA
EFG Elongation factor G	fusA
GREA Transcription elongation factor GreA	greA
NDK Nucleoside diphosphate kinase	ndk
EFTS Elongation factor Ts	tsf
ENO Enolase	eno
KPYK Pyruvate kinase	pyk
PGK Phosphoglycerate kinase	pgk
Transketolase	tkt
Trigger factor	tig
Triosephosphate isomerase	tpiA

Figure 4.2 Common proteins identified in six strains of Staphylococcus aureus subjected to shotgun proteomic analysis

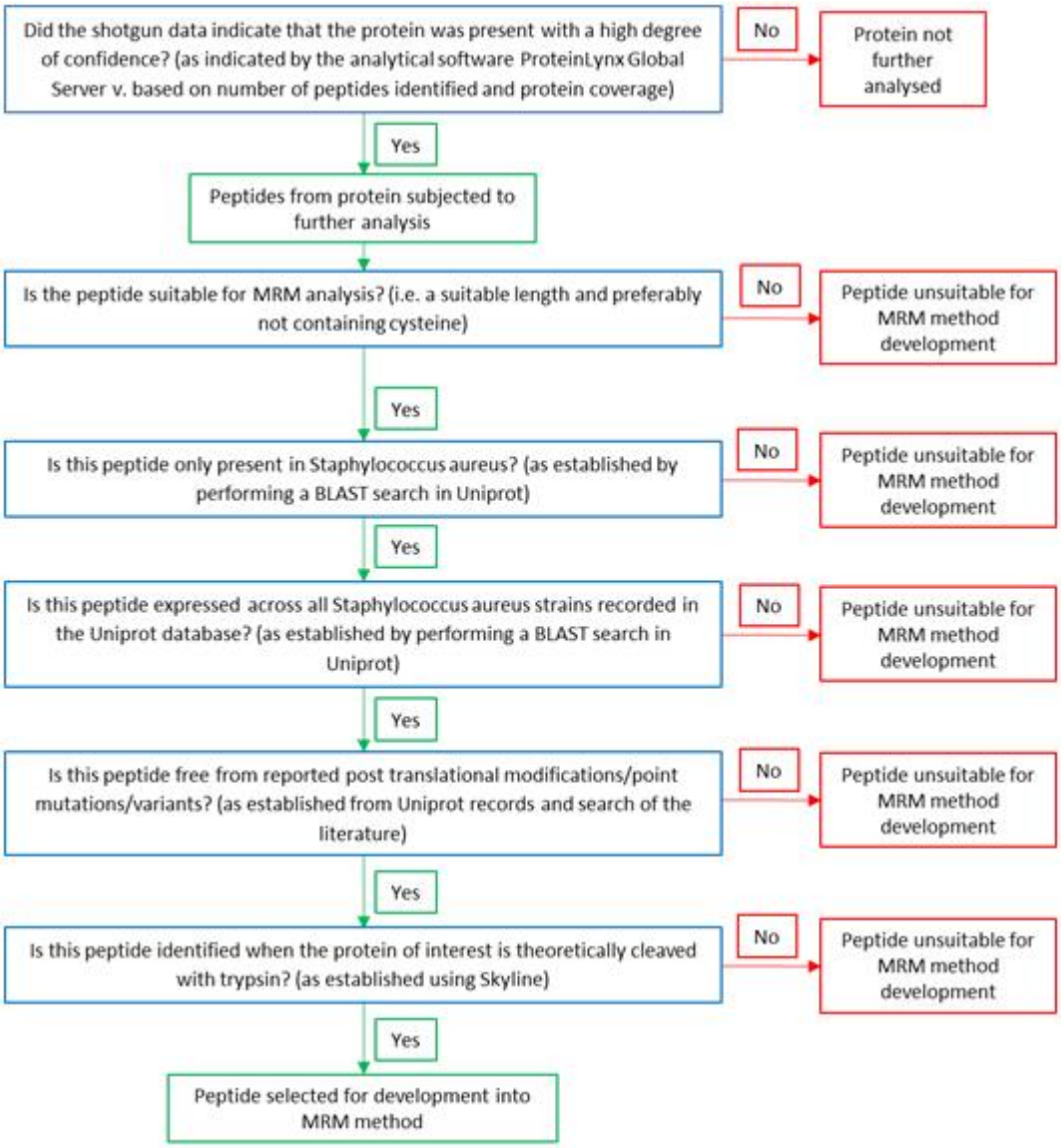

Figure 4.3 Flow chart detailing analysis of candidate peptides for Staphylococcal toxin identification

There were five peptides from five proteins identified which satisfied the given criteria and should therefore, if identified in a sample, indicate with a high degree of confidence whether or not *Staphylococcus aureus* is present. The characteristics of these proteins are given in figure 4.4.

The five peptides from these proteins were taken forward for development into an MRM method. They are all involved in essential metabolic processes such as glucose metabolism and protein synthesis; meaning they are more likely to be conserved than proteins which form part of mechanisms of evasion of the host immune response or antibiotic resistance. All the peptide sequences

chosen for use in targeted analyses were shown to be species-specific for *Staphylococcus aureus* and thus suitable marker peptides for this species of bacteria.

Synthesised peptides were purchased (Generon Ltd; Maidenhead, UK) and used as the basis for development of multiple reaction monitoring tests, using both automated and manual tuning methods (as detailed in chapter methods). Following the development of the test, the three peptides with the strongest response were selected to form part of a multiplex test to accurately identify and quantify *Staphylococcus aureus* (See figure 4.5). This test was then used against the original six isolates to ensure its efficacy; yielding positive results.

Accession Number	UNIprotID	Gene	Protein	Function
PFLB_STAAC	Q5HJF4	pflB	Formate acetyltransferase	Regulates anaerobic glucose metabolism
E5QTM2_STAAH	E5QTM2	fusA	Elongation factor G	Catalyzes the GTP-dependent ribosomal translocation step during translation elongation
D0K5P7_STAAD	D0K5P7	tig	Trigger factor	Involved in protein export. Acts as a chaperone by maintaining the newly synthesized protein in an open conformation.
EFTS_STAA9	A5ISE1	tsf	Elongation factor Ts	Associates with the EF-Tu.GDP complex and induces the exchange of GDP to GTP
SYS_STAA9	A5INQ0	serS	Serine—tRNA ligase	Catalyzes the attachment of serine to tRNA(Ser)

Figure 4.4 Characteristics of proteins from which peptides were selected for multiple reaction monitoring method development

4.2.2 Toxin identification

The toxins selected for development into the test are listed in figure 4.6, along with their associated pathological effects. This group of Staphylococcal toxins was selected having been described in the literature as causing severe illness in living patients, or being associated with Sudden Infant Deaths. Protein sequences from each toxin were retrieved from Uniprot (http://ww.uniprot.org/)

and theoretically digested using Skyline (AB Sciex LLC; Framingham, Mass.) to identify candidate peptides for each toxin.

Purpose	Protein Name	Gene Name	Parent ion (m/z)	Daughter ion (m/z)	Cone voltage (v)	Collision energy (v)
Bacterial Identification	Trigger Factor	tig	544.7128	760.6167	56	16
			544.7128	873.7812	56	14
			1087.9200	501.0700	32	47
	Elongation Factor G	fusA	581.5851	575.9317	56	8
			581.5851	680.1261	56	14
			581.5851	787.2507	56	10
	Serine-tRNA Ligase	serS	729.2872	902.7066	6	22
			729.5400	547.1100	33	17
			729.5400	789.1800	33	17
Toxin Detection	Alpha-haemolysin	hly	432.4575	426.4793	8	6
			648.0319	639.0933	56	16
			648.0319	706.5872	56	20
	Toxic Shock Syndrome Toxin-1	tst	703.4575	564.4673	2	30
			703.4575	694.5812	2	16
			703.5500	980.4000	51	15
	Panton-Valentine Leukocidin chain F	lukF-PV	662.6490	329.3157	52	18
			662.6490	769.6239	52	20
			662.6490	882.7859	52	22
	Panton-Valentine Leukocidin chain S	lukS-PV	539.5425	426.3608	60	20
			539.6063	634.6361	14	12
			808.8404	643.7325	4	22
	Enterotoxin A	entA	578.0957	325.2861	14	30
			578.0957	528.5569	14	18
			1154.7340	618.4455	28	40
	Enterotoxin C1	entC1	516.6500	576.2200	38	9
			516.6500	739.2500	38	9
			775.0800	609.4100	41	26
	Enterotoxin G	entG	583.7400	421.1700	40	25
			583.7400	649.2700	40	25
			875.7400	439.1900	55	30
	Enterotoxin I	SEI	618.0900	560.2400	43	11
			926.7300	995.3700	85	25
			618.2872	447.3298	6	18

Figure 4.5 Final MRM Multiplex method including transitions for three S. aureus peptides and seven Staphylococcal toxin peptides

Suitable peptides were selected for each toxin and blast searched using Uniprot (http://ww.uniprot.org/) to ensure that there was no overlap with human or other proteomes which might compromise the utility of the test. Synthesised peptides were obtained (Generon Ltd; Maidenhead, UK) and an MRM method was developed for each using both manual and automated methods as described in Chapter 3.

Toxin	Gene	Mechanism of action	Role in Disease/Infant Death
Alpha-haemolysin	hly	Binds to membrane of eukaryotic (host) cells resulting in release of low molecular weight molecules leading to eventual osmotic lysis	Proven to play a role in damaging host organs/tissues e.g. in the formation of necrotising pneumonia due to S. aureus [Kebaier et al 2012, Bonesso et al 2016]; Antibodies/vaccine are protective against Staphylococcal pneumonia and soft tissue infections [Adhikari et al 2016, Dong et al 2012]
Toxic Shock Syndrome Toxin-1	tst	Superantigen-mediated release of pro-inflammatory cytokines; usually produced at local site of infection rather than in blood	Causes Staphylococcal toxic shock syndrome; present at increased levels in SIDS cases compared to controls [Zorgani et al 1999; Gordon et al 1999]; S aureus strains producing this toxin have been identified in SIDS cases [Weber et al 2011]
Panton-Valentine Leukocidin chain F	lukF-PV	Cytolytic effect	Causes purpura fulminans, skin sepsis (blistering skin disease), and necrotising bronchopneumonia [Morgan 2005]
Panton-Valentine Leukocidin chain S	lukS-PV		
Enterotoxin A	entA	Superantigen-mediated release of pro-inflammatory cytokines	Present at increased levels in SIDS cases [Zorgani et al 1999, Highet and Goldwater 2009]; S aureus strains producing this toxin have been identified in SIDS cases [Weber et al 2011]

Toxin	Gene	Mechanism of action	Role in Disease/Infant Death
Enterotoxin C1	entC1	Superantigen-mediated release of pro-inflammatory cytokines	Causes Staphylococcal food poisoning and toxic shock syndrome. Present at increased levels in SIDS cases [Zorgani et al 1999, Highet and Goldwater 2009]; S aureus strains producing this toxin have been identified in SIDS cases [Weber et al 2011]
Enterotoxin G	entG	Superantigen-mediated release of pro-inflammatory cytokines	Causes Staphylococcal food poisoning syndrome; Present at increased levels in SIDS cases [Highet and Goldwater 2009]; S aureus strains producing this toxin have been identified in SIDS cases [Weber et al 2011]
Enterotoxin I	SEI	Superantigen-mediated release of pro-inflammatory cytokines	Causes Staphylococcal food poisoning syndrome; S aureus strains producing this toxin have been identified in SIDS cases [Weber et al 2011]

Abbreviations: SIDS: Sudden infant Death Syndrome

Figure 4.6 Characteristics of Staphylococcal toxins chosen for method development

4.3 Multiplex test and sensitivity/specificity testing

As discussed above, the use of MRM allows the development of a rapid multiplex test which, using one sample, can both identify *Staphylococcus aureus* and its toxins. In order to achieve this, the transitions for *Staphylococcus aureus* identification and for each of the toxins were combined into a single MRM method. The details of this method are given in Figure 4.5.

In order to establish the sensitivity and specificity of the test for *Staphylococcus aureus*, this multiplex method was applied to 40 clinical isolates which included samples of *Staphylococcus aureus* and other structurally similar species such as coagulase negative Staphylococci. A total of 45 isolates was originally selected in the laboratory, but detailed analysis of organisms present was not available for 5 samples (W, AG, AI, AJ, AR), and these samples were therefore not analysed. While sequencing for strain typing was not performed due to

funding constraints, the antibiograms of the selected isolates have been examined for clinical purposes and their variability indicates that a number of different strains of *Staphylococcus aureus* and coagulase negative *Staphylococcus* are present, as would be expected in routine clinical practice (see Figure 4.7).

Positive control samples for toxins were derived from whole toxins for Alpha haemolysin (Sigma Aldrich Catalogue number H9395) and Enterotoxin A (Sigma Aldrich Catalogue number S9399). For other toxins, where whole toxin was not available, commercially available toxin-producing strains of *Staphylococcus aureus* were purchased from Public Health England (National Collection of Type Cultures; NCTC) (see figure 4.8) and incubated on the recommended media to produce colonies.

The clinical isolates were prepared according to the technique used to prepare the original isolates (see above) and subjected to MRM-MS analysis using the multiplex method developed (illustrated in Figure 4.5). The results were blindly reported without knowledge of the bacterial identifications provided by the clinical microbiology laboratory using conventional methods. The results of the testing are given in Figure 4.9 and indicate a specificity of 94.7% and a sensitivity of 100% for identification of *Staphylococcus aureus*. A single sample was identified as positive by the multiplex assay, but was in reality positive for coagulase-negative *Staphylococcus* and Coliforms, but not *Staphylococcus aureus*. MRM-MS analysis of this sample was repeated and yielded the same result. There are two main possibilities which might explain this positive result; first, the sample may have been contaminated with *Staphylococcus aureus* and the sensitivity of this test is such that even small amounts would lead to positive identification, or second there may be interference from other proteins present in the sample, particularly given the presence of a number of organisms. While the sensitivity and specificity levels for the test are excellent, it should be noted that the numbers here are relatively small, and the sensitivity and specificity established by analysis of isolates may not be reproduced to the same level in clinical isolates due to interference from native proteins.

Sample	Isolate	Sensitive	Resistant	Partial Resistance	MRSA/MSSA
A	CNS	A,T,V	E,F,G,P	-	n/a
	CNS	T,V	A,E,F,G,P	-	n/a
B	CNS	A,T,V	E,F,G,P	-	n/a
C	CNS	A,G,T,V	E,F,P	-	n/a
D	CNS	A,T,V	E,F,G,P	-	n/a
E	CNS	A,T,V	E,F,G,P	-	n/a
F	CNS	A,G,T,V	E,F,P	-	n/a
G	CNS	A,G,T,V	E,F,P	-	n/a
H	CNS	A,G,T,V	E,F,P	-	n/a
I	CNS	A,G,T,V	E,F,P	-	n/a
J	CNS	A,E,G,T,V	F,P	-	n/a
K	CNS	A,G,T,V	E,F,P	-	n/a
L	CNS	A,T,V	F	-	n/a
M	CNS	A,T,V	F	-	n/a
	CNS	T,V	A,F	-	n/a
N	SA	A,G,V	E,P	F	MSSA
O	SA	A,E,F,M,U	P	-	MSSA
P	SA	A,E,F,G,U	P	-	MSSA
Q	CNS	-	E,F,P	-	n/a
	Coliform	A,AMC,AMP,C,CIP,G,PTZ	-	-	n/a
	Coliform	A,AMC,C,CIP,G,PTZ	AMP	-	n/a
R	SA	A,E,F,M,U	P	-	MSSA
S	SA	A,E,F,M,U	P	-	MSSA
T	SA	U	F	-	MRSA
U	SA	E,F	P	-	MSSA
V	SA	E,M	F,P	-	MRSA
X	CNS	Not stated	-	-	n/a
Y	SA	A,E,F,G,U	P	-	MSSA
Z	SA	A,G,V	E,P	F	MSSA
AA	CNS	-	F	-	n/a
AB	SA	A,E,F,G,U	P	-	MSSA
AC	CNS	Not stated	-	-	n/a
AD	CNS	A,E,L,RIF,TRIM	F,P,U	-	n/a
AE	SA	A,F,G	E,P,U	-	MSSA
AF	SA	A,G,V	E,P	F	MSSA
AH	SA	E,F	P	-	MSSA
	Diptheroids	Not stated	-	-	n/a
AK	Mixed Coliforms	Not stated	-	-	n/a
AL	SA	A,E,F,U	P	-	MSSA
AM	SA	A,E,F,G,U	P	-	MSSA
	P aeruginosa	A,C,CIP,G	-	PTZ	n/a
AN	SA	A,G,V	E,P	F	MSSA
AO	SA	A,E,F,G,U	P	-	MSSA

Sample	Isolate	Sensitive	Resistant	Partial Resistance	MRSA/MSSA
AP	SA	A,F,G,M,P,U	E	-	MSSA
	CNS	Not stated	-	-	n/a
AQ	SA	A,E,F,G,U	P	-	MSSA
	Enterococcus sp.	Not stated	-	-	n/a
AS	SA	M,RIF,TRIM,U	E,F,P	-	MRSA

Abbreviations: A: Amikacin AMC: Amoxicillin/clavulanic acid AMP: Ampicillin C: Ceftazidime CIP: Ciprofloxacin E: Erythromycin F: Flucloxacillin G: Gentamycin L: Linezolid M: Mupirocin MSSA: Methicillin-sensitive Staphylococcus aureus MRSA: Methicillin-sensitive Staphylococcus aureus P: Penicillin PTZ: Piperacillin/tazobactam RIF: Rifampicin T: Teicoplanin TRIM: Trimethoprim U: Fucidin V: Vancomycin

Figure 4.7 Antibiogram of clinical isolates used for sensitivity and specificity testing

There were appropriate positive results in the positive control samples for Staphylococcal toxins. Of particular note is that while Staphylococcal enterotoxins are theoretically resistant to proteolytic enzymes such as trypsin, the positive results in these control samples indicate that the method developed allows trypsin-mediated cleavage. This is likely to be a consequence of disruption of the three dimensional structure of the protein by means of the incubation with urea and dithioerythritol, and the vast excess of trypsin which is used when compared with levels present in vivo.

NCTC Number	Feature of interest
06571	Antibiotic sensitive strain; produces PVL* ǂ
10655	Produces Enterotoxin C ǂ
11962	Produces Enterotoxin A and Toxic Shock Syndrome Toxin 1 ǂ
13300	Methicillin-resistant strain; produces Panton-Valentine Leukocidin ǂ

* While this strain of *S. aureus* was originally reported to produce no toxins, there is some evidence that it may harbour the genes for the production of Panton-Valentine Leukocidin [Kearns, Ganner and Holmes 2006]

ǂ Public Health England have no data regarding alpha-haemolysin production, but they state that the majority of strains are positive

Figure 4.8 Strains of Staphylococcus aureus from the National Tissue Culture Collection used for toxin positive controls

Sample	Clinical ID	Sample type	Routine methods	MRM Test
A	13B72528	Blood peripheral art	CNS x 2	NEGATIVE
B	13B72252	Blood Hickman line	CNS	NEGATIVE
C	13B72253	Blood PICC line	CNS	NEGATIVE
D	13B72254	Blood portacath	CNS	NEGATIVE
E	13B72254	Blood portacath	CNS	NEGATIVE
F	13B72253	Blood PICC line	CNS	NEGATIVE
G	13B72003	Blood peripheral vein	CNS	NEGATIVE
H	13B72003	Blood peripheral vein	CNS	NEGATIVE
I	13B72003	Blood peripheral vein	CNS	NEGATIVE
J	13B71890	Blood Hickman line	CNS	NEGATIVE
K	13B71890	Blood Hickman line	CNS	NEGATIVE
L	13B70821	Blood peripheral vein	CNS	NEGATIVE
M	13B70725	Blood Hickman line	CNS x 2	NEGATIVE
N	13B70827	Nasal swab	SA	POSITIVE
O	13B70952	Leg skin swab	SA	POSITIVE
P	13B71208	Neck abscess	SA	POSITIVE
Q	13B70890	Atrial Berlin cannula	CNS, Coliform x 2	POSITIVE
R	13B70889	Leg wound swab	SA	POSITIVE
S	13B70950	Foot skin swab	SA	POSITIVE
T	13B70940	Swab [site not spec.]	SA	POSITIVE
U	13B70506	Throat swab	SA	POSITIVE
V	13B71188	Nasal swab	SA	POSITIVE
X	13B71208	Neck abscess	CNS	NEGATIVE
Y	13B71208	Neck abscess	SA	POSITIVE
Z	13B70827	Nasal swab	SA	POSITIVE
AA	13B69331	CSF	CNS	NEGATIVE
AB	13B71209	Neck wound swab	SA	POSITIVE
AC	13B71209	Neck wound swab	CNS	NEGATIVE
AD	13B71245	Wound swab NOS	CNS	NEGATIVE
AE	13B71219	Ankle skin swab	SA	POSITIVE
AF	13B70827	Nasal swab	SA	POSITIVE
AH	13B71975	Sternal wound swab	SA, Diptheroids	POSITIVE
AK	13B71094	Skin swab	Mixed Coliforms	NEGATIVE
AL	13B71853	Gastrostomy site swab	SA	POSITIVE
AM	13B71840	Right orbit	SA, P aeruginosa	POSITIVE
AN	13B70827	Nasal swab	SA	POSITIVE
AO	13B71854	Skin rash swab	SA	POSITIVE
AP	13B71676	Ear swab	SA, CNS	POSITIVE
AQ	13B71675	Foot skin swab	SA, Enterococcus sp.	POSITIVE
AS	13B71794	Nasal swab	SA	POSITIVE

Figure 4.9 Results of sensitivity/specificity testing on clinical isolates

4.4 Discussion

The use of proteomics to identify bacterial specific peptides and multiple reaction monitoring to develop this process into a rapid test, has allowed the development of a test facilitating confident identification of *Staphylococcus aureus* and many of its major pathological exotoxins from clinical isolates. Blind testing on a number of Staphylococcal and other isolates showed a high specificity and sensitivity.

While alternative methods have been used for the identification of bacteria and toxins, this test has a number of advantages. First, confident bacterial identification and testing for seven common pathological Staphylococcal toxins can be achieved with a single test, meaning that only small amounts of sample are required. Second, the multiplex method used for this test takes only 10 minutes and costs only a few pounds. Third, this method may be easily translated to function on a standard tandem mass spectrometer found in most general hospital biochemistry departments; making toxin testing feasible in routine practice. When compared to the current alternatives, this represents a significant time and cost saving (for example, bacterial identification by MALDI mass spectrometry requires the purchase of a MALDI mass spectrometer, a regular charge for an updated database of organisms, and does not provide quantitative results).

The method of developing the test may theoretically be applied to any organism or toxin allowing multiplex tests to be produced which cover a wide range of pathogenic organisms or toxins. While the test has been applied only to isolates taken from culture media, in theory this technique can be applied directly to biological fluids such as blood, CSF or tissue samples. In the context of this thesis, this has obvious potential for application to post mortem examinations, where it could form a part of a robust minimally invasive autopsy technique. Given appropriate samples, the test may be utilised to investigate further the role of toxins in SIDS.

Perhaps most significantly, application of this and similarly designed tests to biological fluids allows the potential for utilising the test as a rapid tool in the assessment of living patients with apparent sepsis. With appropriate refinement

and automation, it is estimated that the test could be adapted to give a result in well under an hour with a minimal increase in cost.

A further feature of interest is that with the inclusion of a known quantity of a standard peptide, such as yeast enolase or a stable isotopically-labelled peptide standard, MRM-based assays such as the Staphylococcal multiplex test allow absolute quantitation. The majority of the techniques currently available for bacterial and toxin identification are either binary tests (positive or negative), or semi-quantitative (present at a high, moderate or low level). With the development of appropriate reference ranges, absolute quantitation of bacteria and peptides may be of use in interpreting the significance of the presence of an organism in both the post mortem and live clinical contexts, and identifying likely contaminants.

While initial sensitivity and specificity testing results are promising, these results are based on a relatively small number of isolates and translation to clinical practice will require validation using larger numbers of samples, including those from clinical tissues.

4.5 Conclusion

In conclusion, this piece of work has enabled the creation of a single, cheap, rapid, multiplex test for the identification of *Staphylococcus aureus* and seven of its pathological exotoxins, which can be adapted to run on most standard tandem mass spectrometers. Sensitivity and specificity is extremely high in isolates of cultured bacteria, and the technique can be easily applied to other bacteria and toxins. This test brings toxin testing within the reach of routine practice. A graphical representation (chromatogram) of the multiplex test is given in figure 4.10.

In addition, the MRM method can be theoretically adapted to allow analysis of biological fluids; creating the possibility of rapid post mortem sampling on fluids taken during a minimally invasive autopsy examination. Perhaps more significantly, if successfully applied to biological fluids, it represents a rapid and cost-effective clinical test which could be used in the context of sepsis of unknown origin in living patients.

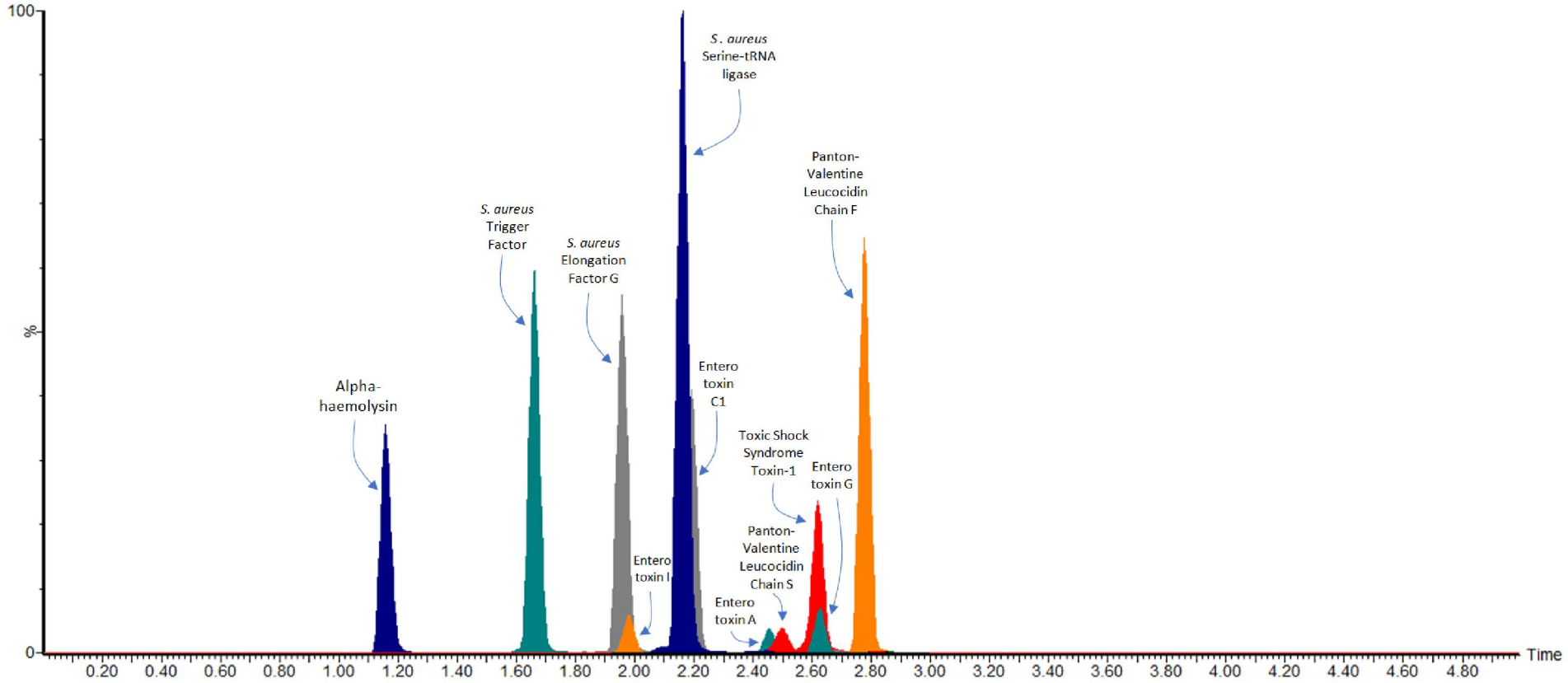

Figure 4.10 UPLC-MS/MS chromatogram of the final multiplexed test for the analysis of three biomarkers for Staphylococcus aureus (Trigger Factor, Elongation Factor G, and Serine-tRNA Ligase) and biomarkers for seven Staphylococcal exotoxins (Alpha-haemolysin, Toxic Shock Syndrome Toxin-1, Panton-Valentine Leucocidin Chains F and S, and Enterotoxins A, C1, G and I) in cultured bacterial samples

Appropriate samples for developing this utility of the test could be obtained prospectively from intensive care units, which care for large numbers of patients with sepsis. Alternatively, archived samples could be used if available.

The rapid and cost-effective nature of the test also means that prospective post mortem sampling from a large number of centres could be used as an alternative to archived samples to further investigate the role of toxins in SIDS.

Chapter 5 - Biomarker Discovery Using Proteomics

5.1 Introduction

As discussed in the introduction to this thesis, a major limitation to the investigation of infant deaths is the limited range of supplementary tests which are available to aid interpretation of post-mortem findings. In particular, while it is possible to identify bacterial organisms from samples taken during post-mortem examinations, interpreting the significance of such findings is difficult. In life, the significance of an organism identified in a clinical sample can be assessed using a combination of the patient's clinical history, current condition, examination findings, the results of white blood cell counts/differentials, and serum inflammatory marker levels. In a deceased patient undergoing post-mortem examination, possibilities are necessarily much more limited. A particular limitation is the lack of reliable inflammatory markers.

One of the aims of this piece of work is to use proteomics as a biomarker discovery tool to identify inflammatory markers which could be used in routine post-mortem practice. The first step in this process is to utilise comparative proteomics to compare the protein complement of tissues from individuals with and without infection. Differentially expressed proteins may then be further investigated for their potential role as biomarkers.

5.2 Method

5.2.1 Case selection

For the purposes of this work, cases were selected from the archive of post mortem tissue at Great Ormond Street Hospital for Children. In all cases, consent had been given by the parents for retention of tissue and use of tissue for research approved by the London (Bloomsbury) Research Ethics Committee. All cases selected were from post mortem examinations performed

prior to 2006, and therefore not subject to the requirements of the Human Tissue Act 2004.

As part of a previous study at this centre, a database was created containing detailed anonymised data from each autopsy performed at the centre over a period of 14 years [Weber et al 2008a]. The fields collected include anonymised demographic information, clinical history, post mortem findings, and results of microbiological testing. The database has previously been used with some success to identify trends in infection in SUDI [Weber et al 2008b] and the database therefore formed a good basis for the identification of appropriate cases for inclusion in this study.

Cases were selected for inclusion in one of four groups. A table containing details of each case in each group is given in figure 5.1. A description of the main features of each group and the rationale for their inclusion in the study is given below.

5.2.2 Trauma (negative control group)

Population-based studies of SUDI and SIDS usually rely on the inclusion of age-matched controls as the control group [Blair et al 2014]. It is never “normal” for a baby to die and as such a ‘control group’ in the context of post mortem investigations simply does not exist. It was therefore decided that for this study, where infection was the disease of interest, the most suitable negative control group would be infants who had died rapidly as a consequence of trauma. In no case selected was there a prolonged survival which might have given rise to infective/inflammatory complications such as ventilator-associated pneumonia or raised inflammatory markers secondary to head injury. None of the cases selected had any histological evidence of infection, and none had positive bacterial cultures.

Post mortem findings	Histological finding of infection	Bacteriology
Negative Control Group (Trauma)		
Head injury; NAI	No	Negative
Head injury; NAI	No	Negative
Rib fractures; NAI	No	Negative
Head injury; NAI	No	Negative
Head injury, multiple injuries; RTC	No	Negative
Head injury; NAI	No	Negative
Head injury; NAI	No	Negative
Head injury; NAI	No	Negative
Head injury; NAI	No	Negative
Sepsis Group		
Nil specific	No	*Staphylococcus aureus* in blood culture
Nil specific	No	GBS in blood culture, spleen, lung
Nil specific	No	GBS in blood culture, spleen, lung
Nil specific	No	GBS in blood culture, spleen, lung
Nil specific	No	GBS in blood culture, spleen, lung, trachea
Nil specific	No	*Staphylococcus aureus* in blood culture, spleen
Nil specific	No	*Streptococcus pneumoniae* in Blood, lung
Positive Control Group (Meningitis)		
Meningitis	Yes	*Streptococcus pneumoniae* in CSF
Meningitis	Yes	*Streptococcus pneumoniae* in CSF
Meningitis, HIE	Yes	*Enterococcus* in meningeal swab, lung
Meningitis, mild LRTI	Yes	GBS in spleen, CSF, lung
Meningitis	Yes	*Streptococcus pneumoniae* in CSF
Meningitis	Yes	*Staphylococcus epidermidis* and *Enterococcus* in lung, meningeal swab

Post mortem findings	Histological finding of infection	Bacteriology
Meningitis, pneumonia, empyema	Yes	Coliforms in CSF, urine, lung
Meningitis, mild LRTI	Yes	Mixed growth all sites
Meningitis, pericarditis	Yes	Mixed growth all sites
Meningitis, miliary tuberculosis lungs, kidneys	Yes	*Mycobacterium tuberculosis* in CSF, lung
Positive Control Group (Pneumonia)		
Pneumonia, tracheobronchitis	Yes	NG in lung, mixed growth other sites
Pneumonia, HIE	Yes	Coliform in lung, mixed growth other sites
Pneumonia, osteomyelitis	Yes	*Staphylococcus aureus* in blood culture, spleen, lung
Pneumonia, empyema	Yes	*Staphylococcus aureus* in blood culture, spleen, lung
Pneumonia	Yes	*Staphylococcus aureus* in blood culture, spleen, lung, HIV +, PJP
Pneumonia	Yes	GBS in lung
Pneumonia, HIE	Yes	Mixed growth all sites
Pneumonia, bronchiolitis	Yes	*Streptococcus pneumoniae* in lung
Pneumonia	Yes	*Haemophilus influenzae* type B and *Streptococcus pneumoniae* in lung, blood culture
Pneumonia	Yes	*Group A Streptococcus* in lung, spleen, blood culture

GBS: Group B *Streptococcus*, PJP: *Pneumocystis jiroveci* pneumonia

Figure 5.1 Detailed summary of the characteristics of cases selected for proteomic analysis

5.2.3 Infection – Meningitis (positive control group)

The first positive control group consisted of individuals who had died of meningitis and in whom there was an appropriate clinical history, histological findings, and bacteriological evidence of infection. These cases included

infection with a number of different organisms including *Neisseria meningitidis*, *Streptococcus pneumoniae*, and Group B *Streptococcus*; the variety of causes was chosen in order to minimize the possibility that biomarkers discovered would be specific to the inflammatory process triggered by a particular organism. In some cases there was also evidence of infection at other sites, such as the lungs or kidneys.

5.2.4 Infection – Pneumonia (positive control group)

It was decided that a second positive control group was necessary in order to allow for possible differences in inflammatory marker production in different types of infection. This group consisted of individuals who had died of bronchopneumonia/lower respiratory tract infection. Like the meningitis group, all of the cases included had an appropriate clinical history, post mortem findings, and microbiological results. Again, the cases covered a variety of causative organisms in order to ensure any potential biomarkers were as widely applicable as possible. These organisms included *Streptococcus pneumoniae*, Group A *Streptococcus*, *Pneumocystis jiroveci*, Group B *Streptococcus*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*.

5.2.5 Sepsis

As discussed in the introduction, deaths resulting from “Sepsis” prove particularly difficult at post mortem examination, as there may be no available clinical history or convincing histological findings. Histological evidence may also be missed as a consequence of inadequate sampling. For example subtle supporting evidence of sepsis such as reactive (secondary) haemophagocytic lymphohistiocytosis may be missed if lymph nodes, bone marrow or spleen are not examined histologically [Inai et al 2014]. Even where there is the suspicion of sepsis, interpretation of findings varies widely between consultants [Pryce et al 2011]. It was therefore decided to include a group of cases in which it was considered most pathologists would give the cause of death as sepsis, although

it is acknowledged that not all pathologists would agree with this interpretation in all cases. Cases included in this group have a positive culture result in blood and/or spleen and one other site, for an organism which, if it were discovered in the blood in life, would be considered pathological. In none of the cases was there histological evidence of infection, or a history or post mortem findings suggestive of an alternative cause of death. The cases predominantly involved Group B *Streptococcus*, but *Staphylococcus aureus* and *Streptococcus pneumoniae* were also represented in a small number of cases.

With respect to numbers, even amongst a cohort of the size available (over 1000 cases), it was difficult to find an appreciable number of cases which clearly satisfied the criteria for each group. Ten cases for the pneumonia and meningitis groups, seven cases for the sepsis group, and ten cases for the control group were therefore selected as a pragmatic compromise between achieving clean groups satisfying the inclusion criteria, and sufficient numbers for a representative selection. This was considered preferable to increasing case numbers by reducing the stringency of the selection criteria. Such numbers are generally acceptable in proteomic biomarker discovery studies of rare diseases. Given the small number of cases available, it was not possible to statistically balance the groups with respect to age, sex, or other potentially confounding factors. Nevertheless, a summary of these factors in each group is given in Figure 5.2. Statistical testing using an independent samples Kruskal-Wallace test demonstrated no significant difference between the groups with respect to age (p=0.216) or post mortem interval (p=0.979). There are a generally higher number of males than females; which reflects the demographic of infant deaths.

	Sex ratio of males: females	**Age median (range)*/days***	**Post mortem interval median (range)*/days***
Negative control (trauma) group	2	140 (10-299)	3 (1-7)
Positive control (pneumonia) group	1.5	108.5 (57-290)	3 (1-6)
Positive control (meningitis) group	1.5	144 (24-301)	3 (0-5)
Positive control (sepsis) group	1.3	41 (31-176)	2 (1-4)

Figure 5.2 Demographic details of experimental groups

Given that case selection was based on a database of cases, which was populated from post mortem reports, it was necessary to review the complete histology of all included cases in order to reliably exclude errors in the post mortem reports or database data entry. This review revealed evidence of significant pulmonary acute inflammation in one of the negative control cases which was not reported in the original post mortem report. This case was therefore excluded from the study and the negative control group therefore comprised nine cases.

Samples of heart, liver, kidney, spleen and lung were selected as being representative of the major organs sampled routinely at post mortem examination. The use of spleen and lung for the biomarker aspect of the study was discontinued at an early stage as extracts from these tissues yielded far smaller peptide numbers than the other tissues (typically up to 10-fold lower). The reason for this is uncertain, however it is well recognized that haemoglobin can interfere with proteomic assessment due to its presence at high concentration in blood-stained samples. Given the high erythrocyte content of the splenic red pulp, it seems likely that this is the cause of the practical difficulties using this tissue. There are techniques which can filter out haemoglobin which are successfully used on samples in our laboratory, but these techniques add a degree of complexity to the sample preparation, which might limit the more widespread use of the tests developed. In the case of lung the low yields may be a consequence of the relatively low cellularity of this tissue type.

5.2.6 Experimental process

A sample of each tissue of interest (Heart, liver, and kidney) was removed from the formalin fixed paraffin embedded blocks from each of the cases selected. The samples were prepared for proteomic analysis by means of the experimental technique described in Chapter 3. Once prepared, the appropriately pooled, fractionated and digested, protein samples were analysed using liquid chromatography-Mass spectrometry (see Chapter 3). The data from each fraction were analysed using specialist proteomic software (ProteinLynx

Global Server) and combined to create a full set of results for each tissue type in each experimental group (meningitis liver, meningitis heart, pneumonia kidney etc.). The results in each of these groups were then compared and the results are given below.

5.3 Results & Discussion

The number of confident protein identifications achieved for each study group in each tissue type is shown in Figure 5.3. The number of identifications is lower than seen in some published studies, but is easily sufficient for biomarker discovery analysis. The lower number of identifications observed in this work compared to other studies is likely due to tissue being obtained from archived formalin-fixed samples, which are well recognised to achieve lower numbers of protein identifications than fresh tissue.

	Heart	Liver	Kidney
Negative control group	44 (724)	115 (1368)	249 (4311)
Positive control group (meningitis)	103 (1570)	151 (1658)	260 (4144)
Positive control group (pneumonia)	204 (3731)	76 (800)	171 (2855)
Positive control group (sepsis)	182 (2806)	80 (846)	262 (3902)

Figure 5.3 Number of proteins identified per group (number of peptides)

Using the data, proteins were selected as candidate biomarkers if they fell into one of two groups (increased in infection or decreased in infection), and the criteria for inclusion in these groups are given in Figure 5.4. A total of 41 differentially expressed proteins were identified and these are listed in Figure 5.5 along with a summary of their function. A protein interaction map generated using STRING [Szklarczyk et al 2015] is given in Figure 5.6.

	Trauma	Meningitis	Pneumonia	Sepsis
Increased in infection	-	+	+	+/-
Decreased in infection	+	-	-	+/-

Figure 5.4 Criteria for selection of proteins for further analysis (+ Detected, - not detected, +/- detected or not detected)

Uniprot Entry Number	Uniprot Entry Name	Gene Name	Protein Name	Protein function
P01023	A2MG_HUMAN	A2M	Alpha-2-macroglobulin	Negative regulation of complement activation, lectin pathway
P02649	APOE_HUMAN	APOE	Apolipoprotein E	Negative regulation of inflammatory response
Q9UHQ4	BAP29_HUMAN	BCAP29	B-cell receptor-associated protein 29	Regulation of membrane transport and apoptosis
P01024	CO3_HUMAN	C3	Complement C3	Central component of classical and alternative complement pathways
Q8TD46	MO2R1_HUMAN	CD200R1	Cell surface glycoprotein CD200 receptor 1	Negative regulation of inflammatory response (inhibits expression of pro-inflammatory molecules)
P20273	CD22_HUMAN	CD22	B-cell receptor CD22	Mediates B-cell B-cell interaction
P12111	CO6A3_HUMAN	COL6A3	Collagen alpha-3(VI) chain	Serine type protease inhibitor
P31327	CPSM_HUMAN	CPS1	Carbamoyl-phosphate synthase [ammonia], mitochondrial	Involved in urea cycle (removal of excess ammonia from cell)
O75390	CISY_HUMAN	CS	Citrate synthase, mitochondrial	Involved in tricarboxylic acid cycle
P01034	CYTC_HUMAN	CST3	Cystatin-C	Cysteine protease inhibitor (involved in cellular response to insults)
Q93034	CUL5_HUMAN	CUL5	Cullin-5	Cell cycle protein (involved in protein ubiquitination)
Q7Z4W1	DCXR_HUMAN	DCXR	L-xylulose reductase	Participant in glucose metabolism
Q9NRZ9	HELLS_HUMAN	HELLS	Lymphoid-specific helicase	Plays role in expansion/survival of lymphoid cells
P02790	HEMO_HUMAN	HPX	Hemopexin	Involved in heme transport to the liver; also implicated in immunoglobulin production and positive regulation of IFNγ mediated signalling
P08238	HS90B_HUMAN	HSP90AB1	Heat shock protein HSP 90-beta	Involved in regulation of inflammatory signalling pathways

Uniprot Entry Number	Uniprot Entry Name	Gene Name	Protein Name	Protein function
P34931	HS71L_HUMAN	HSPA1L	Heat shock 70 kDa protein 1-like	Involved in regulation of cellular response to injury
P11142	HSP7C_HUMAN	HSPA8	Heat shock cognate 71 kDa protein	Binds bacterial lipopolysaccharide and mediates inflammatory response
P10809	CH60_HUMAN	HSPD1	60 kDa heat shock protein, mitochondrial	Involved in B-cell activation and positive regulation of inflammatory signalling pathways
P13232	IL7_HUMAN	IL7	Interleukin-7	Key factor in humoral immune response
Q12906	ILF3_HUMAN	ILF3	Interleukin enhancer-binding factor 3	Involved in defence response to viruses; negative regulation of viral genome regulation
Q9NWZ3	IRAK4_HUMAN	IRAK4	Interleukin-1 receptor-associated kinase 4	Involved in Toll-like receptor and IL-1R signalling pathways as part of innate immune response
Q8WWI1	LMO7_HUMAN	LMO7	LIM domain only protein 7	Involved in protein ubiquitination and cell signalling
P26038	MOES_HUMAN	MSN	Moesin	Involved in regulation of leukocyte migration and cell-cell adhesion
Q13469	NFAC2_HUMAN	NFATC2	Nuclear factor of activated T-cells, cytoplasmic 2	Involved in B cell receptor signalling pathway; positive regulation of B cell proliferation
Q9BYH8	IKBZ_HUMAN	NFKBIZ	NF-kappa-B inhibitor zeta	Involved in regulation of inflammatory response via toll-like receptor and IL-1R signalling pathways
P18669	PGAM1_HUMAN	PGAM1	Phosphoglycerate mutase 1	Key factor in gluconeogenesis
P00558	PGK1_HUMAN	PGK1	Phosphoglycerate kinase 1	Key factor in gluconeogenesis
P30048	PRDX3_HUMAN	PRDX3	Thioredoxin-dependent peroxide reductase, mitochondrial	Involved in cell response to injury, including that from lipopolysaccharide
P41222	PTGDS_HUMAN	PTGDS	Prostaglandin-H2 D-isomerase	Involved in maintenance of blood-brain barrier; inhibitor of platelet activation

Uniprot Entry Number	Uniprot Entry Name	Gene Name	Protein Name	Protein function
Q9H7Z7	PGES2_HUMAN	PTGES2	Prostaglandin E synthase 2	Involved in prostaglandin biosynthesis (part of lipid metabolism)
P01009	A1AT_HUMAN	SERPINA1	Alpha-1-antitrypsin	Acute phase protein
P01011	AACT_HUMAN	SERPINA3	Alpha-1-antichymotrypsin	Acute phase protein
P08697	A2AP_HUMAN	SERPINF2	Alpha-2-antiplasmin	Acute phase protein
P05155	IC1_HUMAN	SERPING1	Plasma protease C1 inhibitor	Involved in innate immune response and complement activation
Q9NTJ3	SMC4_HUMAN	SMC4	Structural maintenance of chromosomes protein 4	Cell division protein
P00441	SODC_HUMAN	SOD1	Superoxide dismutase [Cu-Zn]	Destruction of free radicals formed by insults to cell
P04179	SODM_HUMAN	SOD2	Superoxide dismutase [Mn], mitochondrial	Destruction of free radicals formed by insults to cell
Q9Y2C9	TLR6_HUMAN	TLR6	Toll-like receptor 6	Participates in innate immune response to Gram-positive bacteria and fungi
Q12931	TRAP1_HUMAN	TRAP1	Heat shock protein 75 kDa, mitochondrial	Involved in cellular response to stress
P10599	THIO_HUMAN	TXN	Thioredoxin	Involved in cellular response to stress
P0CG48	UBC_HUMAN	UBC	Polyubiquitin-C	Involved in innate immune response

Figure 5.5 List of differentially-expressed proteins with summary of protein function

It can be clearly established from the functions listed in Figure 5.5, that the differentially-expressed proteins identified cover a wide range of functions; many involving the immune response or the cellular response to stress (Figures 5.7-5.9). It is of particular note that there appears to be no particular inflammatory pathway which is favoured over others, and proteins are represented that form part of the innate immune response, adaptive immune response, response to viruses, response to cell damage by chemicals/radiation, response to lipopolysaccharide, complement pathway, Toll-like receptor pathway, and acute phase protein cascade. These are promising candidates for

biomarkers which could be used in the detection of infection in the post mortem setting. Furthermore, these results may provide insight into the disease mechanisms and pathways involved in fatal sepsis in infants.

There are also a small number of proteins which are not known to have a direct function in the immune or stress responses. These proteins are also suitable for further assessment, as they may prove to be useful markers even if not previously known to play a role in these processes.

While the proteins identified in this biomarker discovery phase are promising, these proteins will need to be validated using other techniques and in larger cohorts of samples. In addition the data obtained was reliant on pooled samples, meaning that there is no way of distinguishing from this data the difference between a protein present at a raised level in all cases, and a protein present at a raised number in a small proportion of cases, or even in a single case. In order to establish which of these markers are reproducibly raised in a significant number of individual cases, targeted techniques must be used to identify each protein in individual samples. Tandem mass spectrometry using multiple reaction monitoring was selected for this purpose and this is described in Chapter 6.

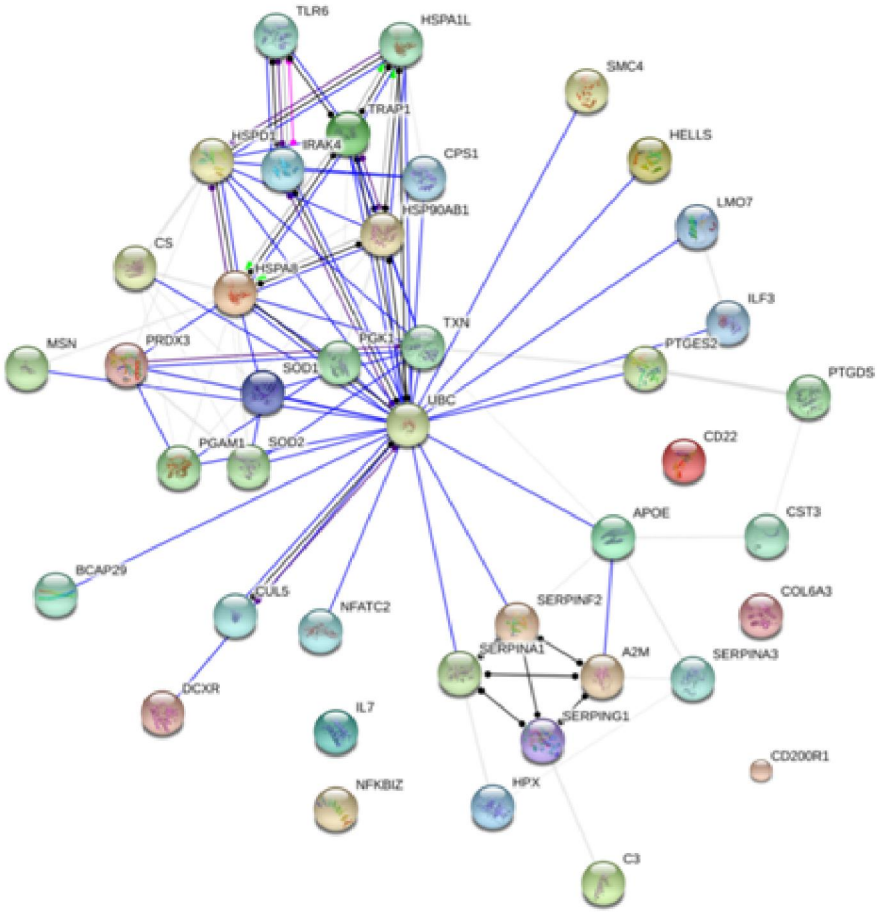

Figure 5.6 Protein interaction map demonstrating connections between differentially expressed proteins generated using STRING [Szklarczyk et al 2015]

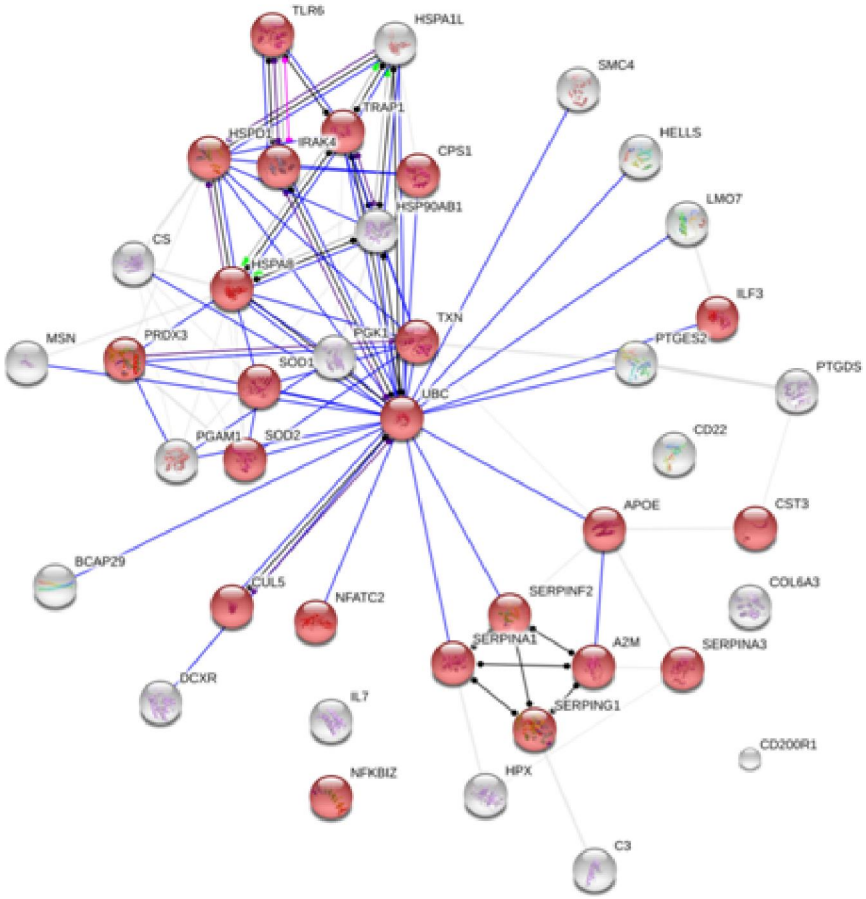

Figure 5.7 Protein interaction map of differentially expressed proteins, with proteins involved in response to stress highlighted, generated using STRING [Szklarczyk et al 2015]

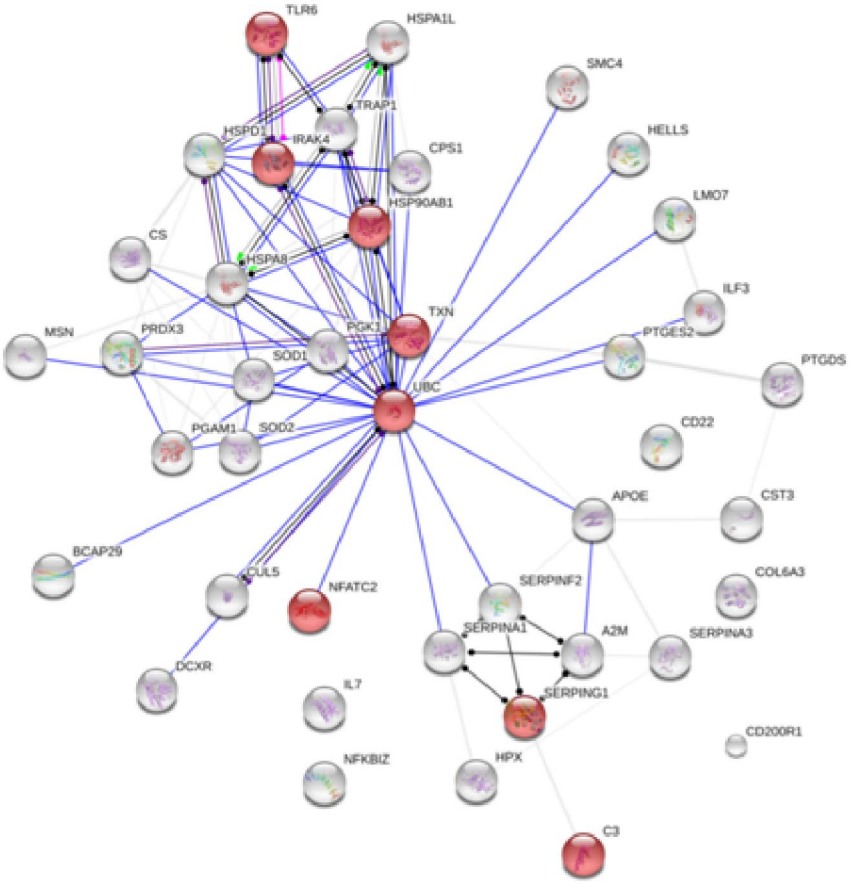

Figure 5.8 Protein interaction map of differentially expressed proteins, with proteins involved in the innate immune response highlighted, generated using STRING [Szklarczyk et al 2015]

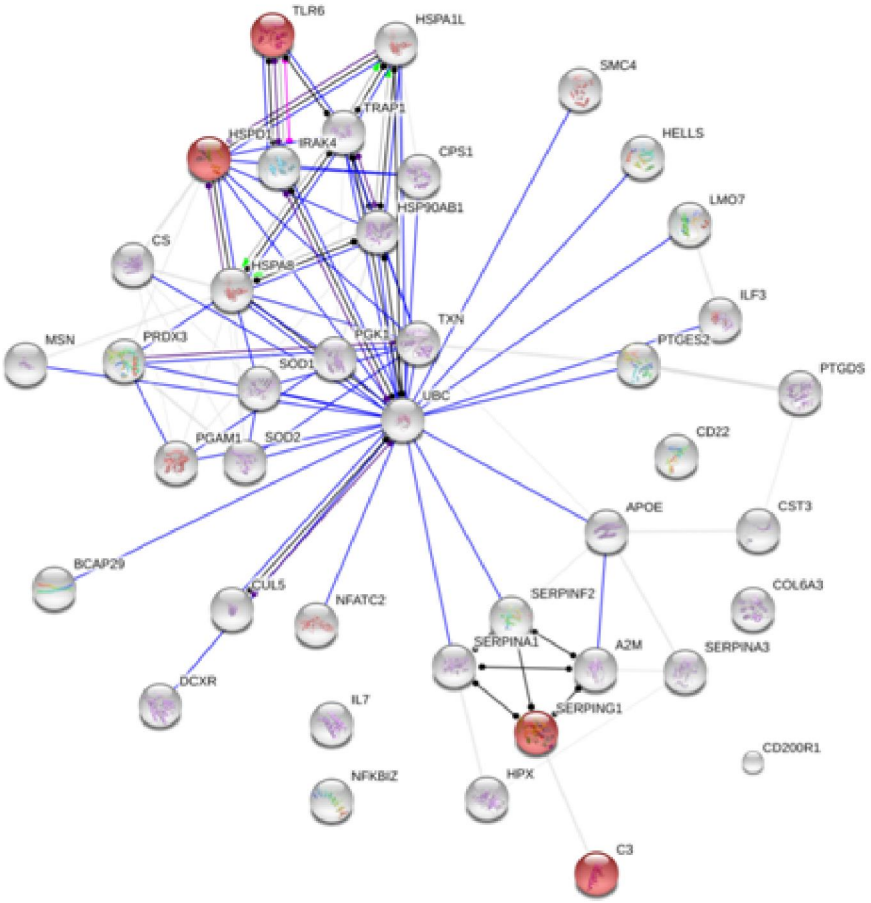

Figure 5.9 Protein interaction map of differentially expressed proteins, with proteins involved in the adaptive immune response highlighted, generated using STRING [Szklarczyk et al 2015]

Chapter 6 – The Development of a Targeted Proteomic Test for Quantitation of Inflammatory Markers

6.1 Introduction

In the last chapter, a number of candidate biomarkers were identified using shotgun proteomic analysis. As stated previously, such candidates may or may not be suitable for use as biomarkers as a consequence of pooling of samples. In order to confirm which, if any, of the candidates can be used reliably as a biomarker for the disease of interest it is necessary to develop targeted tests for the proteins of interest and apply them to individual cases rather than pooled groups. This can be achieved using a number of different methods, including targeted mass spectrometry and immunohistochemistry. While immunohistochemistry might appear to be the most suitable technique in a study aiming to develop post mortem tests, there are a number of disadvantages which make targeted mass spectrometry a more attractive option. First, the development of each immunohistochemical test is extremely time-consuming. Second, high quality positive controls are required to optimise the method and these are not available. Third Immunohistochemistry does not allow quantitation. Finally, the sensitivity of immunohistochemistry is considerably lower than that of targeted mass spectrometry. In light of these considerations, targeted mass spectrometry using multiple reaction monitoring was selected as the confirmatory method of choice. This decision does not preclude the development of immunohistochemical stains for any identified biomarkers at a later stage, once their utility is confirmed.

6.2 Method

Suitable peptides were selected or designed for each protein of interest identified in the biomarker discovery experiment (See Figure 5.5 in Chapter 5). These peptides were selected using a combination of methods as described in

Chapter 3. Where peptides were suboptimal, for example containing a cysteine residue, a second peptide was also selected for that protein.

In addition to those proteins identified as potential biomarkers in the biomarker discover experiment, it was decided to include a number of proteins which are known to play key roles in the inflammatory process. These proteins and their key functions (as described by UNIPROT; http://www.uniprot.org/) are given in Figure 6.1. Using freely-available functional annotation software (DAVID; https://david.ncifcrf.gov/) pathway diagrams with all the selected proteins highlighted have been generated and are given in Figures 6.2-6.4. These demonstrate the broad coverage of the innate and adaptive immune response which is covered by the selected proteins; maximising the chances of successful biomarker use.

Uniprot Entry Number	Uniprot Entry Name	Gene Name	Protein Name	Protein function [Uniprot]
P02741	CRP_HUMAN	CRP	C-reactive protein	Promotes agglutination, bacterial capsular swelling, phagocytosis and complement fixation
P05362	ICAM1_HUMAN	ICAM1	Intercellular adhesion molecule 1	Ligand for the leukocyte adhesion protein LFA-1 (integrin alpha-L/beta-2). Key role in leukocyte trans-endothelial migration
P01579	IFNG_HUMAN	IFNG	Interferon gamma	Produced by lymphocytes activated by specific antigens or mitogens. IFN-gamma, in addition to having antiviral activity, has important immunoregulatory functions. It is a potent activator of macrophages, and it can potentiate the antiviral effects of the type I interferons
P22301	IL10_HUMAN	IL10	Interleukin-10	Inhibits the synthesis of a number of cytokines, including IFN-gamma, IL-2, IL-3, TNF and GM-CSF produced by activated macrophages and by helper T-cells

Uniprot Entry Number	Uniprot Entry Name	Gene Name	Protein Name	Protein function [Uniprot]
P01583	IL1A_HUMAN	IL1A	Interleukin-1 alpha	Produced by activated macrophages, stimulates thymocyte proliferation by inducing IL-2 release, B-cell maturation and proliferation, and fibroblast growth factor activity. IL-1 proteins are involved in the inflammatory response, being identified as endogenous pyrogens.
P01584	IL1B_HUMAN	IL1B	Interleukin-1 beta	Potent proinflammatory cytokine. Initially discovered as the major endogenous pyrogen, induces prostaglandin synthesis, neutrophil influx and activation, T-cell activation and cytokine production, B-cell activation and antibody production, and fibroblast proliferation and collagen production. Promotes Th17 differentiation of T-cells
P60568	IL2_HUMAN	IL2	Interleukin-2	Produced by T-cells in response to antigenic or mitogenic stimulation, this protein is required for T-cell proliferation and other activities crucial to regulation of the immune response. Can stimulate B-cells, monocytes, lymphokine-activated killer cells, natural killer cells
P05231	IL6_HUMAN	IL6	Interleukin-6	Cytokine with a wide variety of biological functions. It is a potent inducer of the acute phase response. Plays an essential role in the final differentiation of B-cells into Ig-secreting cells Involved in lymphocyte and monocyte differentiation. Acts on B-cells, T-cells. Required for the generation of T(H)17 cells
P0DJI8	SAA1_HUMAN	SAA1	Serum amyloid A-1 protein	Major acute phase protein

Uniprot Entry Number	Uniprot Entry Name	Gene Name	Protein Name	Protein function [Uniprot]
P16581	LYAM2_HUMAN	SELE	E-selectin	Cell-surface glycoprotein having a role in immunoadhesion. Mediates in the adhesion of blood neutrophils in cytokine-activated endothelium through interaction with PSGL1/SELPLG
P01375	TNFA_HUMAN	TNF	Tumour necrosis factor alpha	Cytokine that binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. It is mainly secreted by macrophages. It is potent pyrogen causing fever by direct action or by stimulation of interleukin-1 secretion
P19320	VCAM1_HUMAN	VCAM1	Vascular cell adhesion protein 1	Important in cell-cell recognition. Appears to function in leukocyte-endothelial cell adhesion. Interacts with integrin alpha-4/beta-1 (ITGA4/ITGB1) on leukocytes, and mediates both adhesion and signal transduction. The VCAM1/ITGA4/ITGB1 interaction may play a pathophysiologic role both in immune responses and in leukocyte emigration to sites of inflammation

Figure 6.1 Characteristics of additional inflammatory proteins selected for targeted testing by multiple reaction monitoring mass spectrometry

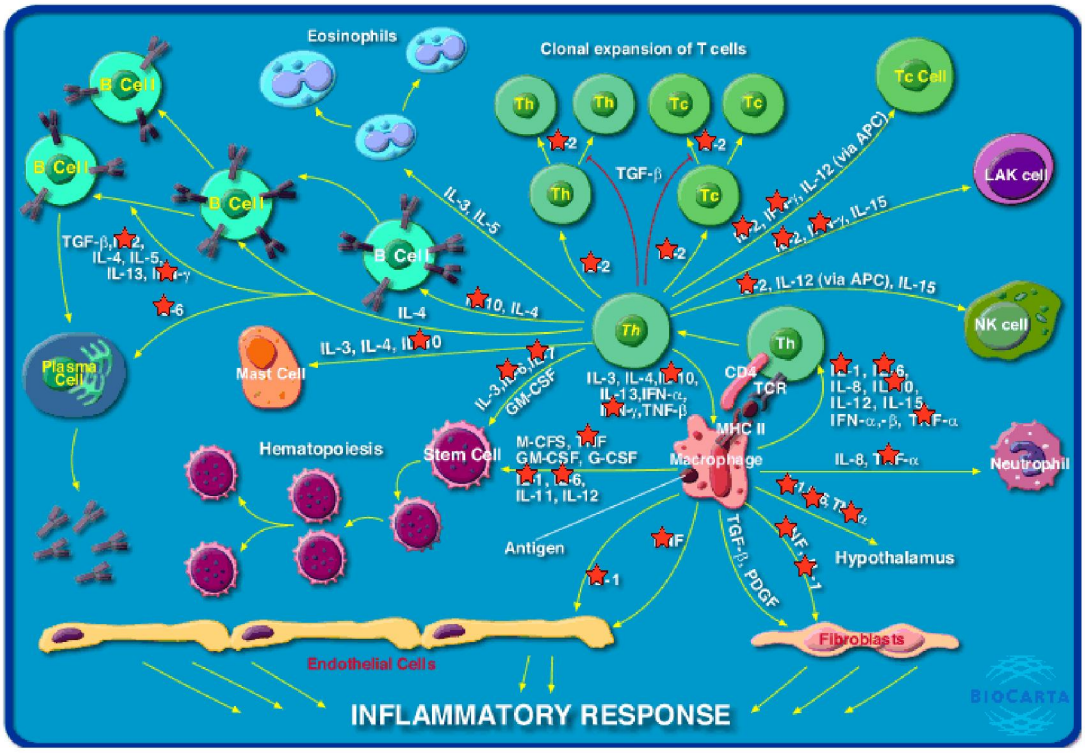

Figure 6.2 Pathway diagram of cytokines and the inflammatory response generated using DAVID Functional annotation software utilising pathway information generated by Biocarta [Huang, Sherman and Lempicki 2009]; proteins included for further analysis are highlighted.

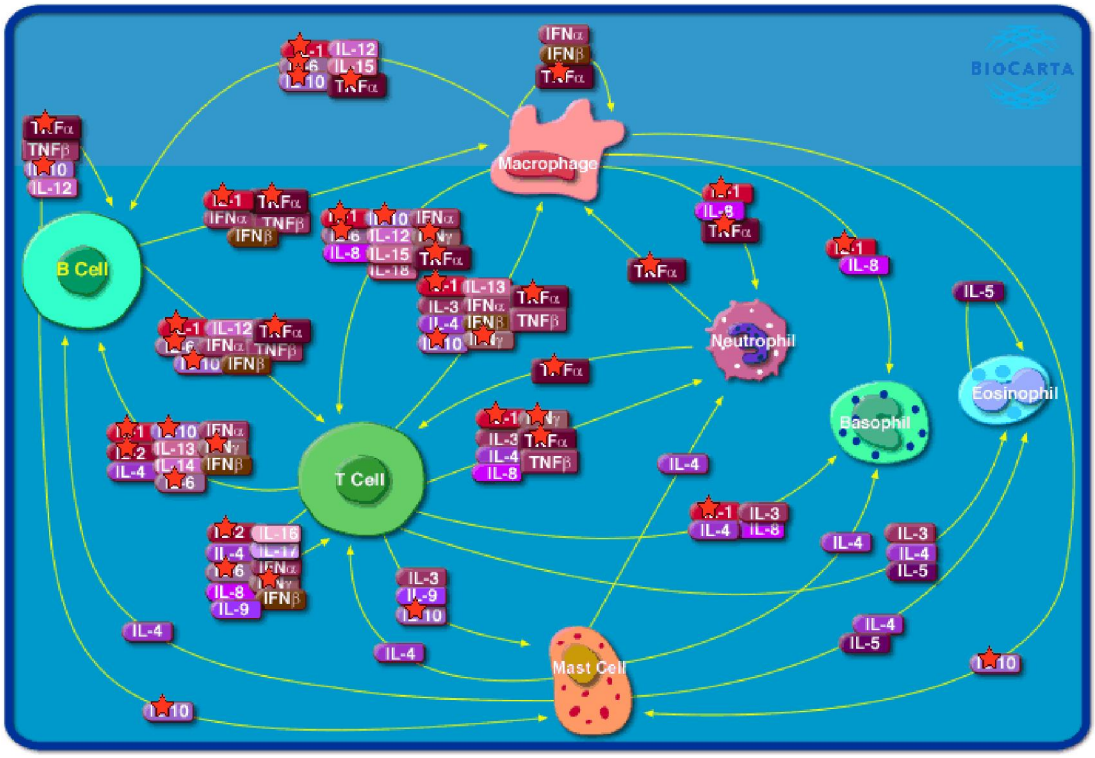

Figure 6.3 Pathway diagram of the cytokine network generated using DAVID Functional annotation software utilising pathway information generated by Biocarta [Huang, Sherman and Lempicki 2009]; proteins included for further analysis are highlighted.

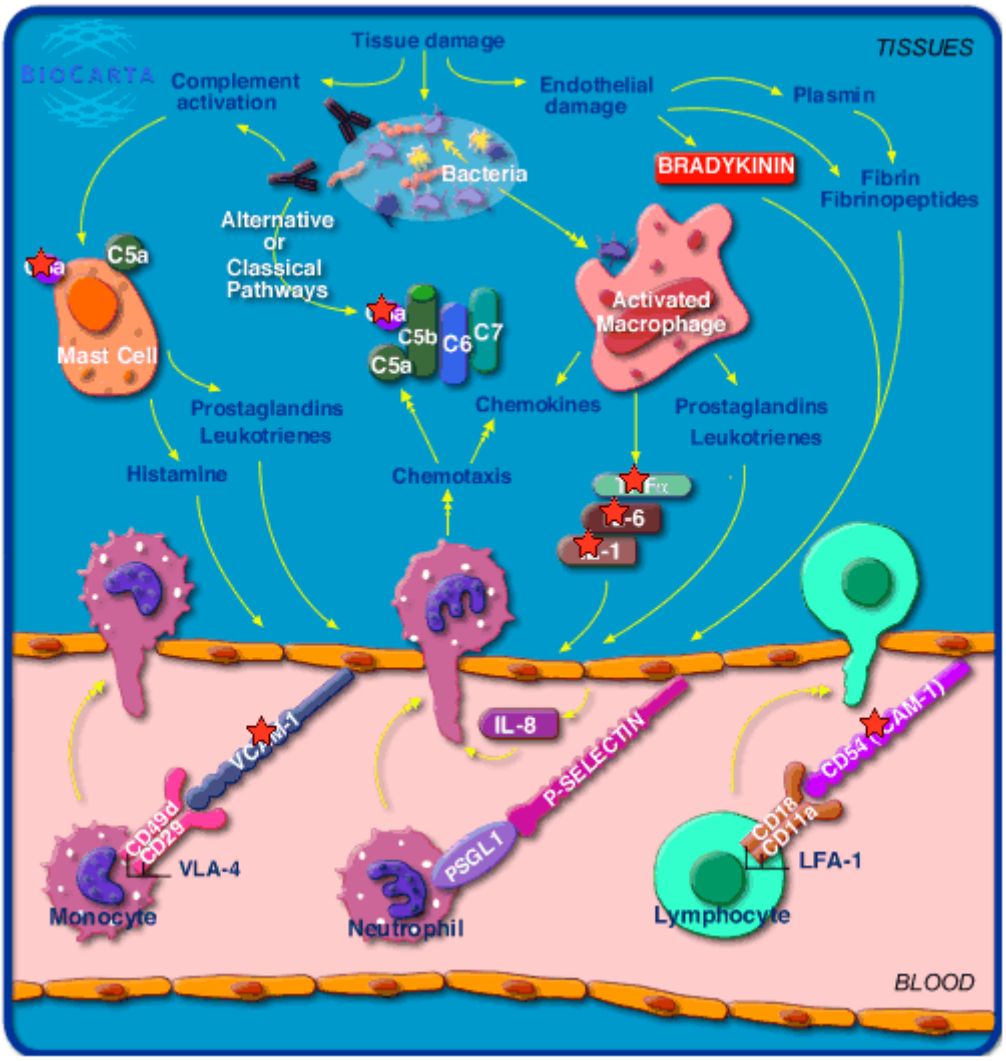

Figure 6.4 Pathway diagram of the local inflammatory response to an insult generated using DAVID Functional annotation software utilising pathway information generated by Biocarta [Huang, Sherman and Lempicki 2009]; proteins included for further analysis are highlighted.

The selected peptides were designed, synthesised commercially, prepared and tuned according to the technique given in Chapter 3. The selected proteins and the associated selected peptide are given in Figure 6.5. The individual methods were combined into five larger 10 minute multiplex methods in view of the limited amount of material available for analysis, and in order to increase the sensitivity and dwell time in the mass spectrometer (Figures 6.6-6.10). These tests were applied to the same groups of cases used for the biomarker discovery experiments, although the individual samples rather than pooled samples were used and the two infection groups were combined for analysis purposes. In addition to the groups used previously, a group of 19 unexplained

infant death cases were included in whom there were no significant clinical or post mortem findings, and in whom only commensal organisms or mixed bacterial growth were identified microbiologically. The general demographics of the groups are given in Figure 6.11. Analysis using an independent samples Kruskal-Wallis test showed no statistically significant difference between the groups with respect to post mortem interval (p=0.595) or age (p=0.058). There were generally more males than females; reflecting the demographics of infant deaths.

Gene name	Protein name	Peptide(s) selected
A2M	Alpha-2-macroglobulin	AFQPFFVELTMPYSVIR
APOE	Apolipoprotein E	LGPLVEQGR
BCAP29	B-cell receptor-associated protein 29	SSTSRPDAYEHTQMK
C3	Complement C3	Established test used*
CD200R1	Cell surface glycoprotein CD200 receptor 1	QITQNYSK
CD22	B-cell receptor CD22	EVQFFWEK
COL6A3	Collagen alpha-3(VI) chain	QLGTVQQVISER
CPS1	Carbamoyl-phosphate synthase [ammonia], mitochondrial	FVHDNYVIR
CRP	C reactive protein	APLTKPLK AFTVCLHFYTELSSTR ESDTSYVSLK
CS	Citrate synthase, mitochondrial	ALGVLAQLIWSR
CST3	Cystatin-C	ALDFAVGEYNK
CUL5	Cullin-5	YVEQLLTLFNR
DCXR	L-xylulose reductase	AVIQVSQIVAR TQADLDSLVR
HELLS	Lymphoid-specific helicase	LISQIQPEVDR
HPX	Hemopexin	YYCFQGNQFLR
HSP90AB1	Heat shock protein HSP 90-beta	SIYYITGESK
HSPA1L	Heat shock 70 kDa protein 1-like	VEIIANDQGNR
HSPA8	Heat shock cognate 71 kDa protein	DAGTIAGLNVLR
HSPD1	60 kDa heat shock protein, mitochondrial	VTDALNATR
ICAM1	Intercellular adhesion molecule 1	LLGIETPLPK ASVSVTAEDEGTQR
IFNG	Interferon gamma	IMQSQIVSFYFK AIHELIQVMAELSPAAK
IL10	Interleukin-10	AMSEFDIFINYIEAYMTMK DQLDNLLLK
IL1A	Interleukin-1 alpha	ESMVVVATNGK
IL1B	Interleukin-1 beta	SLVMSGPYELK
IL2	Interleukin-2	DLISNINVIVLELK

Gene name	Protein name	Peptide(s) selected
IL6	Interleukin-6	YILDGISALR FESSEEQAR
IL7	Interleukin-7	LNDLCFLK
ILF3	Interleukin enhancer-binding factor 3	FVMEVEVDGQK
IRAK4	Interleukin-1 receptor-associated kinase 4	SANILLDEAFTAK
LMO7	LIM domain only protein 7	KPQDQLVIER
MSN	Moesin	EDAVLEYLK
NFATC2	Nuclear factor of activated T-cells, cytoplasmic 2	YQQQNPAAVLYQR
NFKBIZ	NF-kappa-B inhibitor zeta	ASGQAVDDFK
PGAM1	Phosphoglycerate mutase 1	AMEAVAAQGK
PGK1	Phosphoglycerate kinase 1	VLPGVDALSNI
PRDX3	Thioredoxin-dependent peroxide reductase, mitochondrial	GLFIIDPNGVIK
PTGDS	Prostaglandin-H2 D-isomerase	TMLLQPAGSLGSYSYR AQGFTEDTIVFLPQTDK
PTGES2	Prostaglandin E synthase 2	QWADDWLVHLISPNVYR
SAA1	Serum amyloid A-1 protein	EANYIGSDK SFFSFLGEAFDGAR
SELE	E-selectin	YTHLVAIQNK
SERPINA1	Alpha-1-antitrypsin	LSSWVLLMK
SERPINA3	Alpha-1-antichymotrypsin	LYGSEAFATDFQDSAAAK
SERPINF2	Alpha-2-antiplasmin	LGNQEPGGQTALK HQMDLVATLSQLGLQELFQAPDLR
SERPING1	Plasma protease C1 inhibitor	LVLLNAIYLSAK
SMC4	Structural maintenance of chromosomes protein 4	SNNIINETTTR
SOD1	Superoxide dismutase [Cu-Zn]	Established test used*
SOD2	Superoxide dismutase [Mn], mitochondrial	LTAASVGVQGSGWGWLGFNK
TLR6	Toll-like receptor 6	DMPSLEILDVSWNSLESGR
TNF	Tumour necrosis factor alpha	ANALLANGVELR DNQLVVPSEGLYLIYSQVLFK
TRAP1	Heat shock protein 75 kDa, mitochondrial	ELGSSVALYSR
TXN	Thioredoxin	LEATINELV
UBC	Polyubiquitin-C	TITLEVEPSDTIENVK
VCAM1	Vascular cell adhesion protein 1	LHIDEMDSVPTVR NTVISVNPSTK

*In two cases, multiple reaction monitoring methods for the protein of interest had already been developed in our laboratory by other researchers, these methods were therefore used to avoid cost and effort of duplication.

Figure 6.5 Peptides selected for multiple reaction monitoring method development for each protein of interest

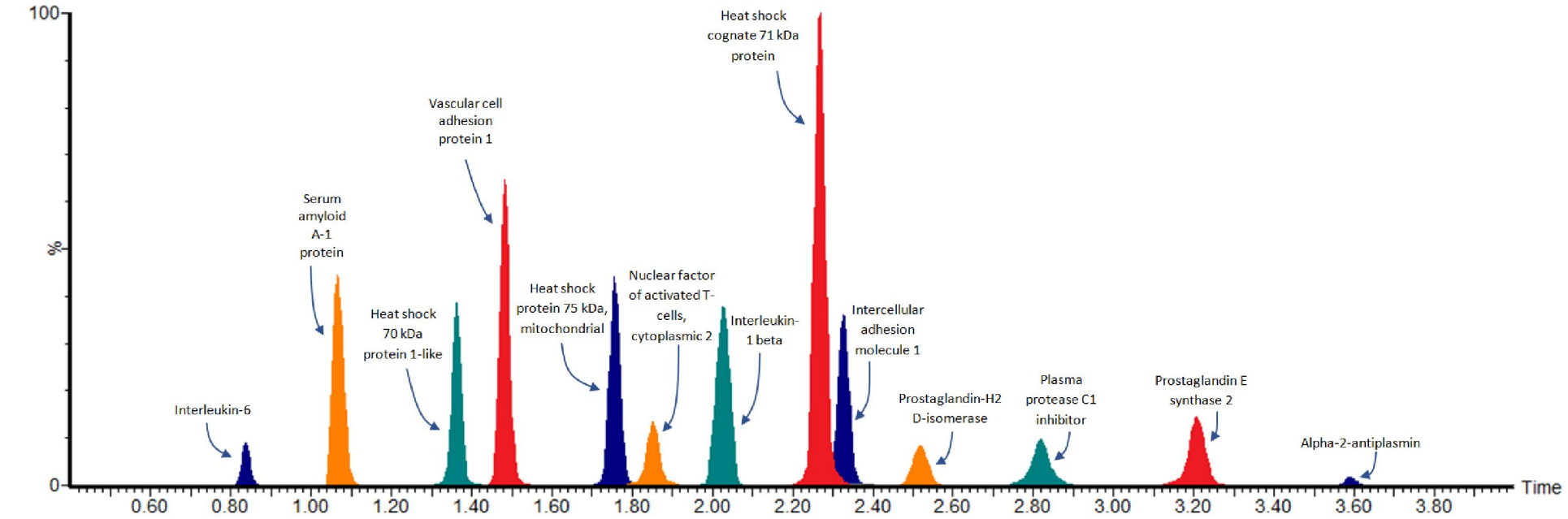

Figure 6.6 UPLC-MS/MS chromatogram of the first of five multiplex tests for the combined analysis of fifty-three candidate biomarkers for infection in human liver

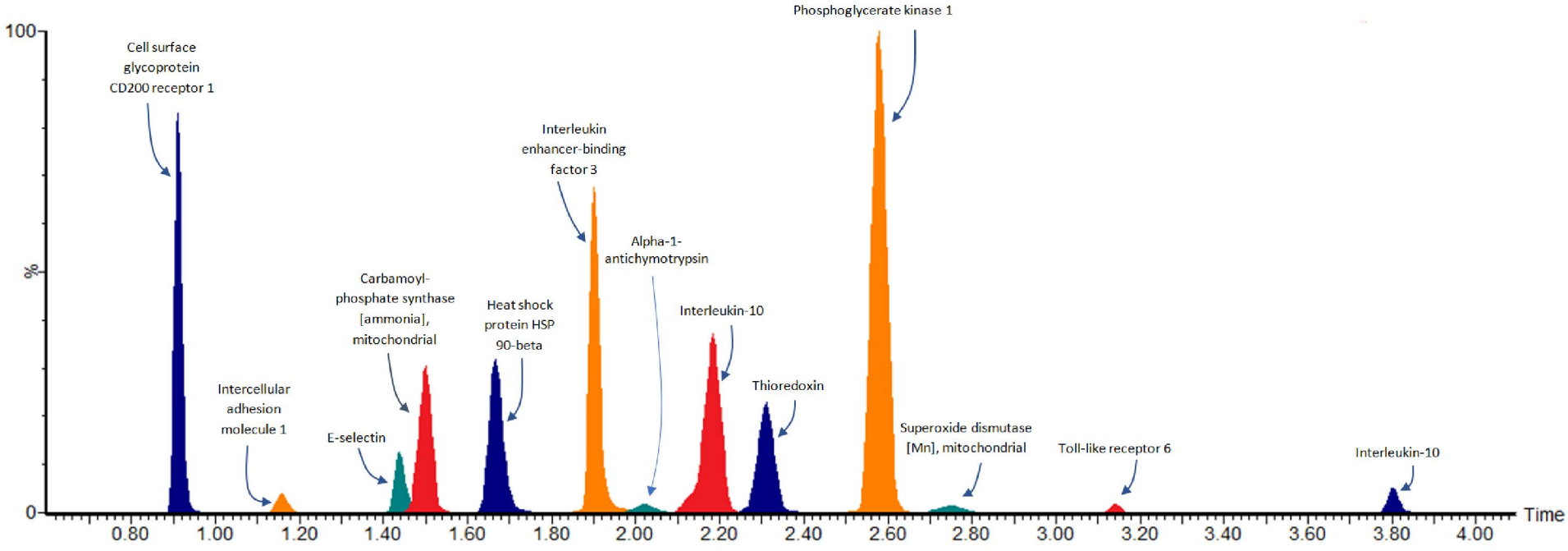

Figure 6.7 UPLC-MS/MS chromatogram of the second of five multiplex tests for the combined analysis of fifty-three candidate biomarkers for infection in human liver

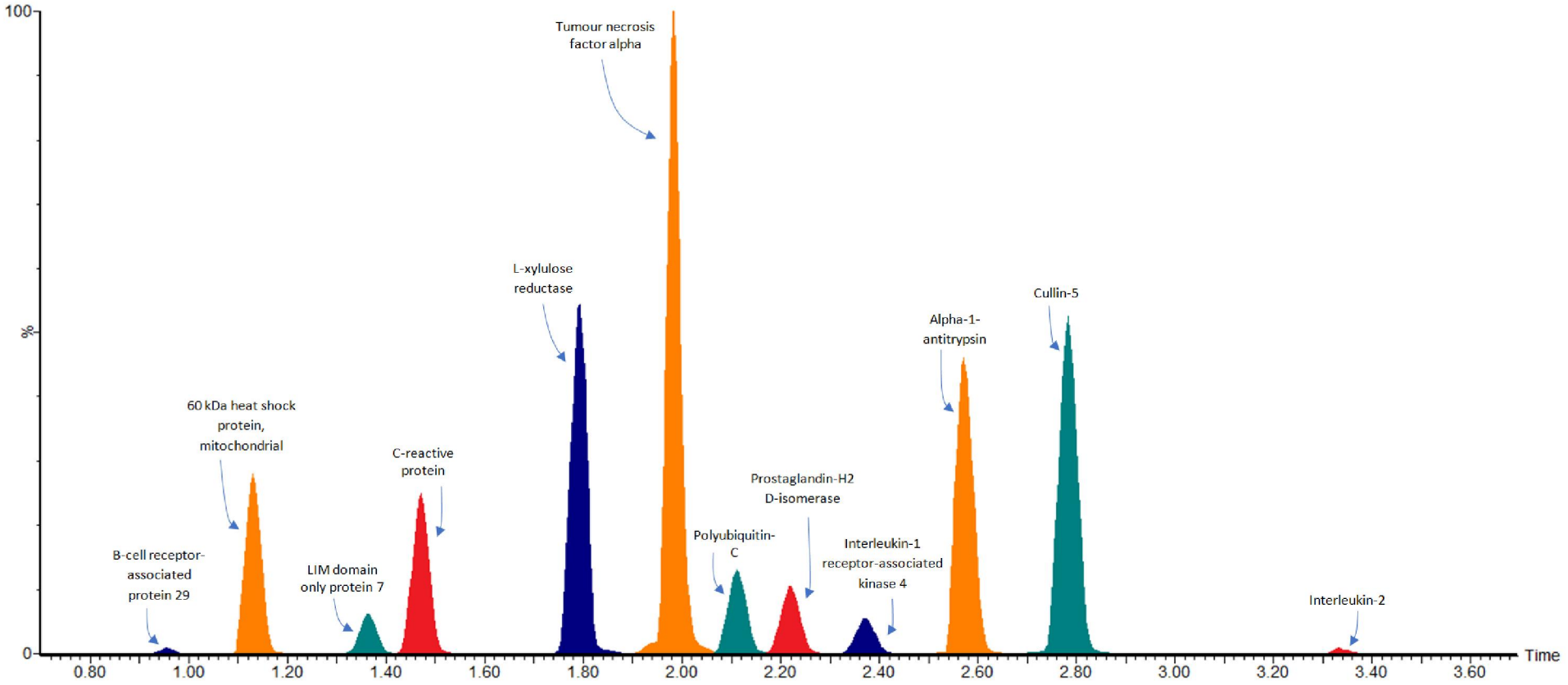

Figure 6.8 UPLC-MS/MS chromatogram of the third of five multiplex tests for the combined analysis of fifty-three candidate biomarkers for infection in human liver

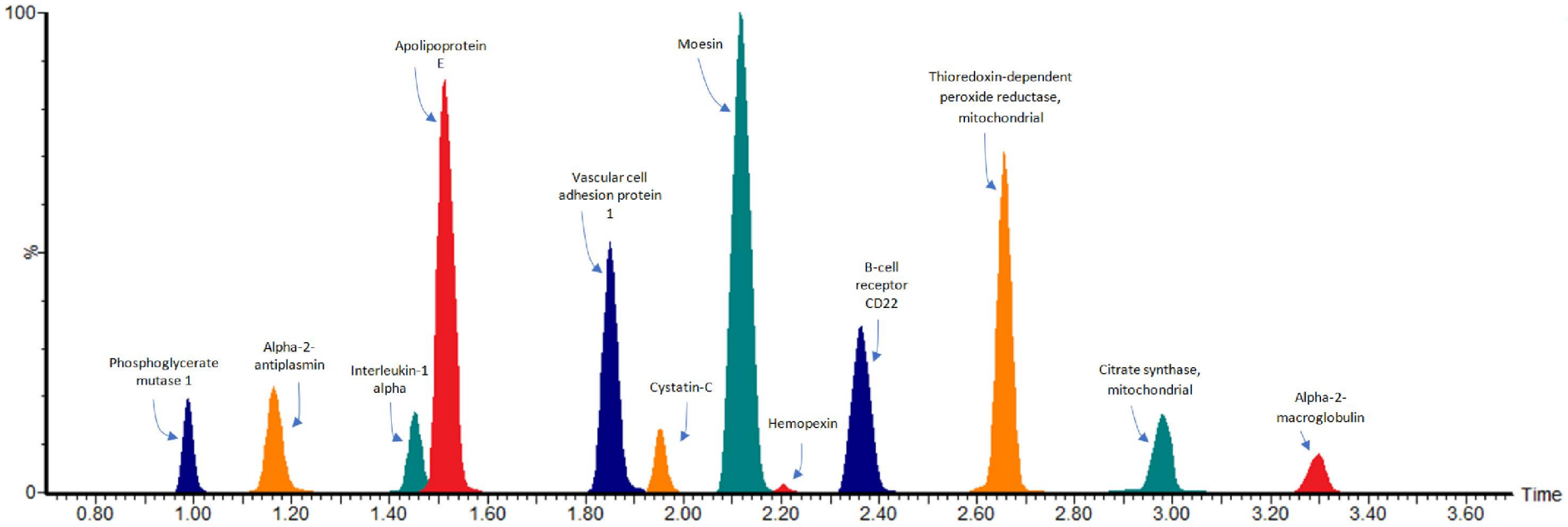

Figure 6.9 UPLC-MS/MS chromatogram of the fourth of five multiplex tests for the combined analysis of fifty-three candidate biomarkers for infection in human liver

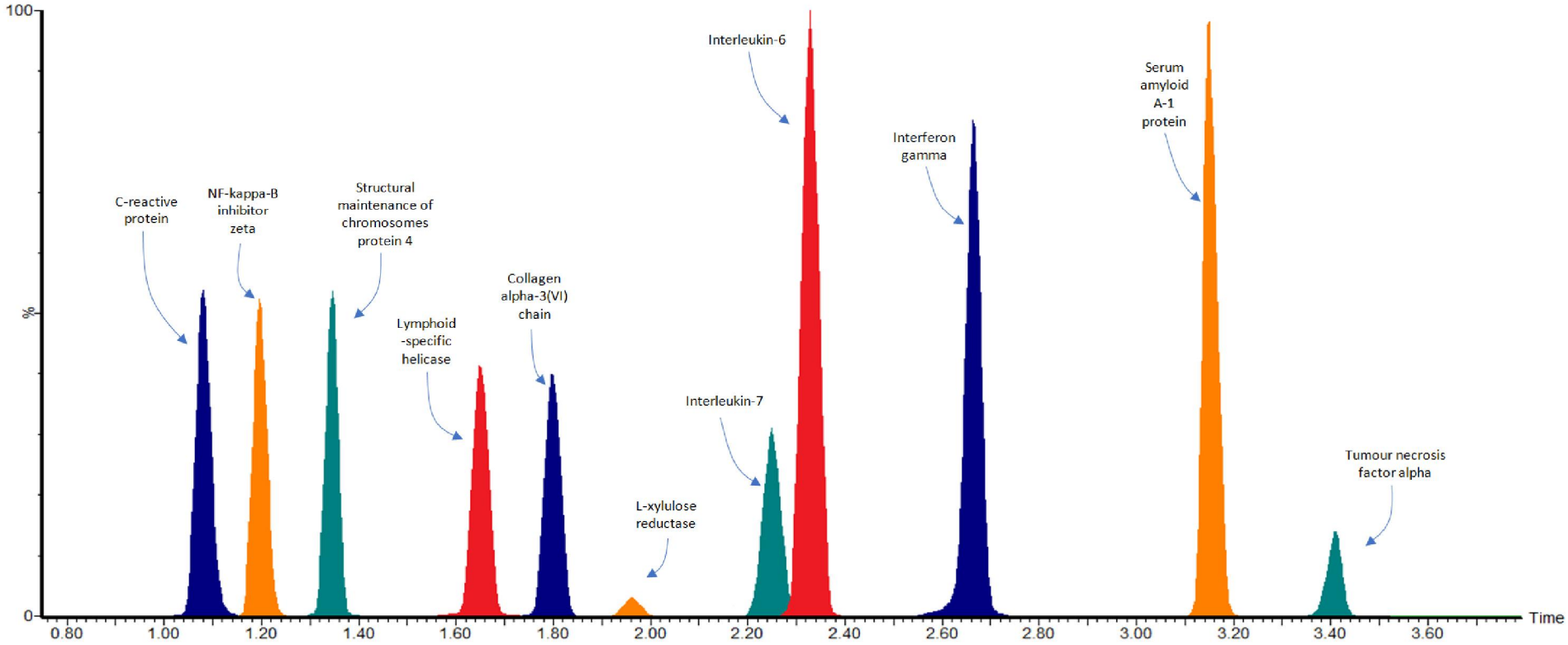

Figure 6.10 UPLC-MS/MS chromatogram of the fifth of five multiplex tests for the combined analysis of fifty-three candidate biomarkers for infection in human liver

	Sex ratio of males: females	Age (median (range))/days	Post mortem interval (median (range))/days
Negative control (trauma) group	2	140 (10-299)	2.5 (1-7)
Positive control (infection) group	1.2	112 (24-301)	3 (0-6)
Positive control (sepsis) group	1.3	41 (31-176)	2 (1-4)
Unexplained SUDI group	1.7	62 (9-308)	2 (0-7)

Figure 6.11 Summary of demographic details of the cases in each group of patient samples used for analysis

6.3 Results

6.3.1 General findings

Of the tests run on the different patient groups, 26 showed no measurable response in the individual samples, 15 showed a measurable response but with no significant difference between patient groups, and 12 showed a measurable response with a statistically significant difference between patient groups. A full breakdown of the proteins in each group is given in Figure 6.12.

Those proteins showing statistically significant differences between groups can be divided into inflammatory markers which are increased in sepsis/infection, proinflammatory markers which are decreased in infection/sepsis, and mitochondrial/metabolic proteins which are increased in SIDS. Further discussion of each of these groups follows.

6.3.2 Markers of infection/sepsis

Three of the tested markers showed a statistically significant difference between the SIDS or control groups and the sepsis or infection groups; C-reactive protein, Heat shock cognate 71 kDa protein, and Heat shock 70 kDa protein 1-like. The detailed results of the analysis of these markers are given below:

Measurable response with significant difference between groups	Measurable response with no significant difference between groups	No measurable response
C-reactive protein 60 kDa heat shock protein, mitochondrial Heat shock cognate 71 kDa protein Intercellular adhesion molecule 1 Heat shock 70 kDa protein 1-like Carbamoyl-phosphate synthase [ammonia], mitochondrial Phosphoglycerate kinase 1 Polyubiquitin-C L-xylulose reductase Interleukin 2 Interleukin 1-alpha Interleukin 6	Heat shock protein 75 kDa, mitochondrial Prostaglandin E synthase 2 Plasma protease C1 inhibitor Interleukin 10 Alpha-1-antichymotrypsin Heat shock protein HSP 90-beta Tumour necrosis factor alpha Thioredoxin-dependent peroxide reductase, mitochondrial Moesin Citrate synthase, mitochondrial Collagen alpha-3(VI) chain Serum amyloid A-1 protein Vascular cell adhesion molecule 1 Apolipoprotein E Thioredoxin	Nuclear factor of activated T-cells, cytoplasmic 2 Prostaglandin-H2 D-isomerase Alpha-2-antiplasmin E-selectin Cell surface glycoprotein CD200 receptor 1 Toll-like receptor 6 Superoxide dismutase [Mn], mitochondrial Interleukin enhancer-binding factor 3 LIM domain only protein 7 B-cell receptor-associated protein 29 Cullin-5 Interleukin-1 receptor-associated kinase 4 Alpha-1-antitrypsin Phosphoglycerate mutase 1 B-cell receptor CD22 Hemopexin Cystatin-C Alpha-2-macroglobulin Lymphoid-specific helicase Structural maintenance of chromosomes protein 4 NF-kappa-B inhibitor zeta Interleukin 7 Interferon gamma Interleukin 1 beta Complement 3 Superoxide dismutase [Cu-Zn]

Figure 6.12 Results of individual protein tests

6.3.2a C-reactive protein (CRP)

A Kruskal-Wallis test was conducted to determine if there were differences in detection of CRP between the control, sepsis, infection, and SIDS groups. Distributions of CRP detected were not similar for all groups, as assessed by visual inspection of a boxplot. CRP levels were statistically significantly different between the different groups of cases, $Chi^2(3) = 15.286$, $p = 0.002$. Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed statistically significant differences in CRP levels between the Trauma (control) (21.61) and Infection groups (38.42) ($p = 0.031$), and the SIDS (22.05) and infection groups ($p = 0.004$), but not between any other group combination (figure 6.13).

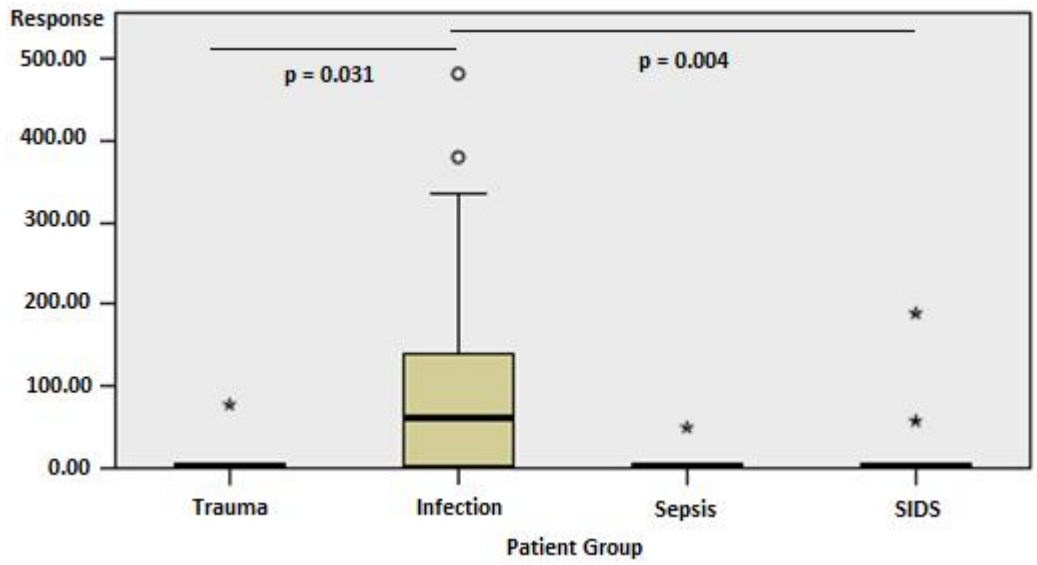

Figure 6.13 Graphical representation of C reactive protein measured in post mortem liver from four experimental groups

6.3.2b Heat shock cognate 71 kDa protein (HSP7C)

A Kruskal-Wallis test was conducted to determine if there were differences in detection of HSP7C between the control, sepsis, infection, and SIDS groups. Distributions of HSP7C detected were not similar for all groups, as assessed by visual inspection of a boxplot. HSP7C levels were statistically significantly different between the different groups of cases, $Chi^2(3) = 11.848$, $p = 0.008$. Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed statistically significant differences in HSP7C levels between the SIDS (23.95) and Infection groups (36.65) ($p = 0.031$), but not between any other group combination (see figure 6.14).

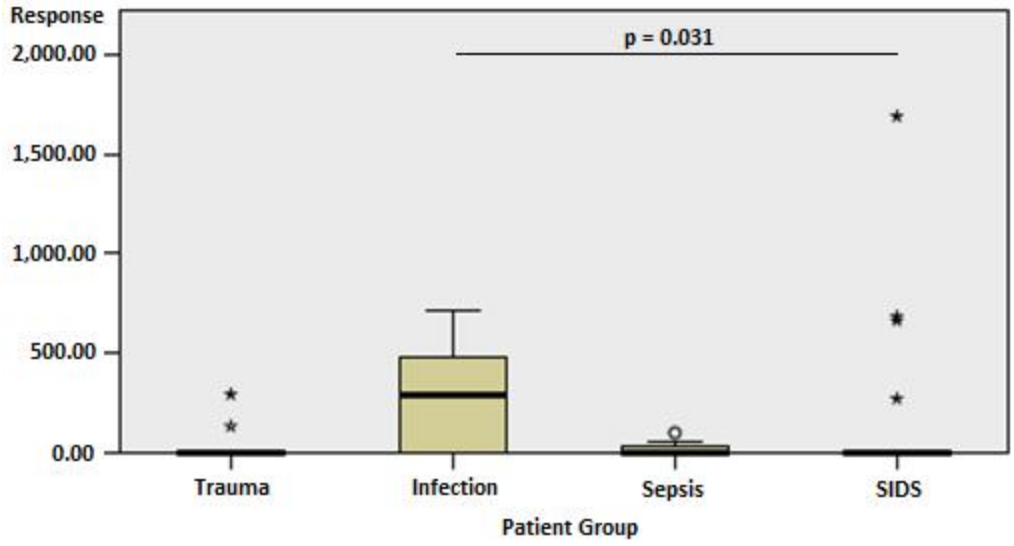

Figure 6.14 Graphical representation of Heat shock cognate 71 kDa protein measured in post mortem liver from four experimental groups

6.3.2c Heat shock 70 kDa protein 1-like (HS71L)

A Kruskal-Wallis test was conducted to determine if there were differences in detection of HS71L between the control, sepsis, infection, and SIDS groups. Distributions of HS71L detected were not similar for all groups, as assessed by visual inspection of a boxplot. HS71L levels were statistically significantly different between the different groups of cases, $Chi^2(3) = 26.790$, $p < 0.001$. Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed statistically significant differences in HS71L levels between the SIDS (17.05) and Infection groups (36.65) ($p = 0.002$), the SIDS and sepsis groups (47.57) ($p < 0.001$), and between the Trauma (control; 18.89) and sepsis groups ($p = 0.002$), but not between any other group combination (see figure 6.15).

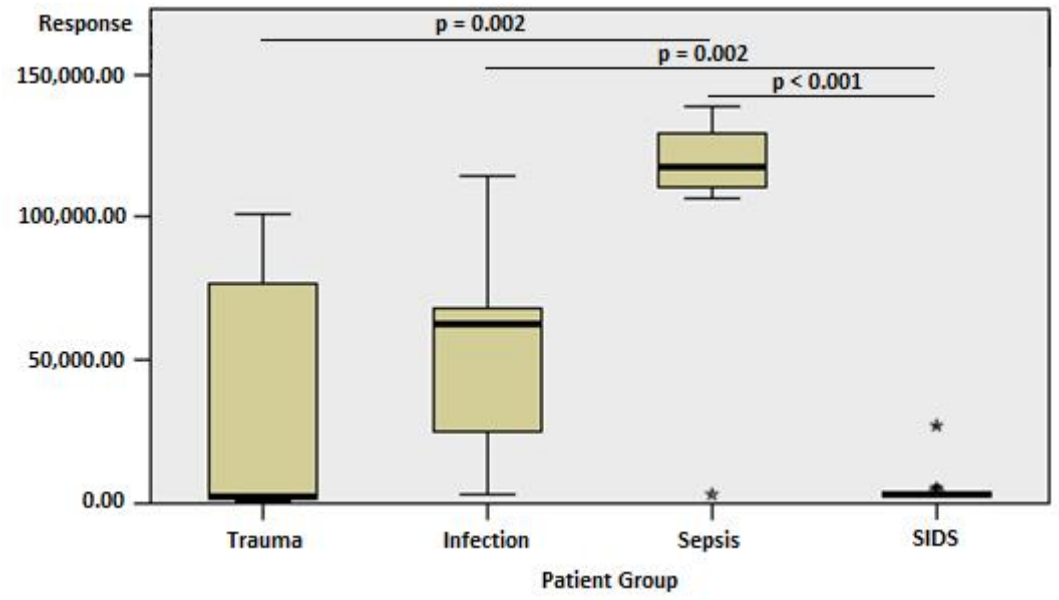

Figure 6.15 Graphical representation of Heat shock 70 kDa protein 1-like measured in post mortem liver from four experimental groups

6.3.3 Pro-inflammatory cytokines decreased in the infection/sepsis groups

Unexpectedly, four pro-inflammatory cytokines which would be expected to be increased in the case of infection/sepsis were in fact decreased when compared with controls/SIDS cases; Interleukin 1a, Interleukin 6, Interleukin 2 and ICAM1. The detailed analysis of these proteins is described below:

6.3.3a Interleukin 1a (IL1a)

A Kruskal-Wallis test was conducted to determine if there were differences in detection of IL1a between the control, sepsis, infection, and SIDS groups. Distributions of IL1a detected were not similar for all groups, as assessed by visual inspection of a boxplot. IL1a levels were statistically significantly different between the different groups of cases, $Chi^2(3) = 18.597$, $p < 0.001$. Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed statistically significant differences in IL1A levels between the Infection (17.20) and Trauma (control) groups (39.89) ($p = 0.002$), and between the Infection and SIDS groups (35.45) ($p = 0.002$), but not between any other group combination (see figure 6.16).

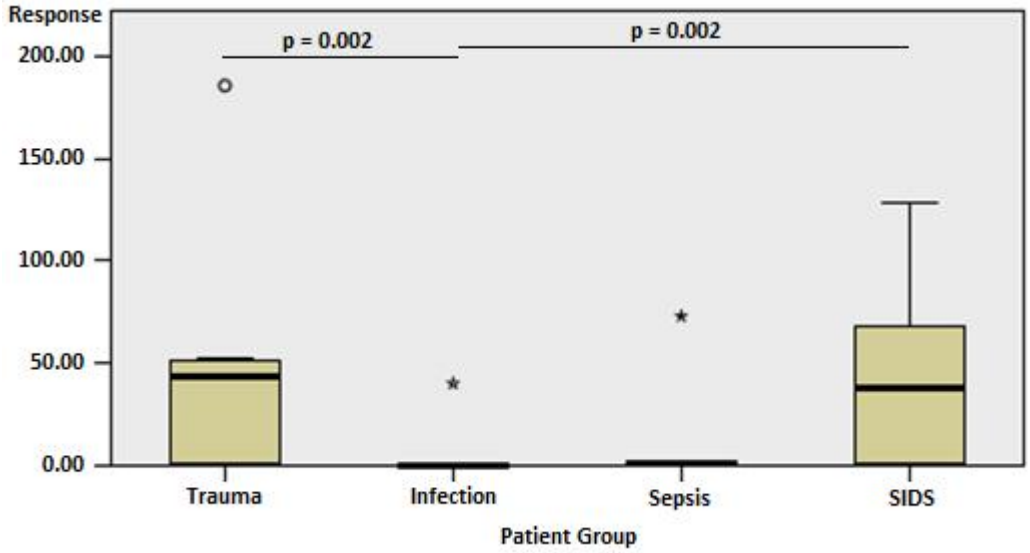

Figure 6.16 Graphical representation of Interleukin 1a measured in post mortem liver from four experimental groups

6.3.3b Interleukin 2 (IL2)

A Kruskal-Wallis test was conducted to determine if there were differences in detection of IL2 between the control, sepsis, infection, and SIDS groups. Distributions of IL2 detected were not similar for all groups, as assessed by visual inspection of a boxplot. IL2 levels were statistically significantly different between the different groups of cases, $Chi^2(3) = 28.438$, $p < 0.001$. Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed statistically significant differences in IL2 levels between the Infection (17.45) and Trauma (control) groups (42.67) ($p = 0.001$), the Infection and SIDS groups (38.20) ($p < 0.001$), the Sepsis (14.56) and Trauma (control) groups ($p = 0.002$), and the Sepsis and SIDS groups ($p = 0.003$), but not between any other group combination (see figure 6.17).

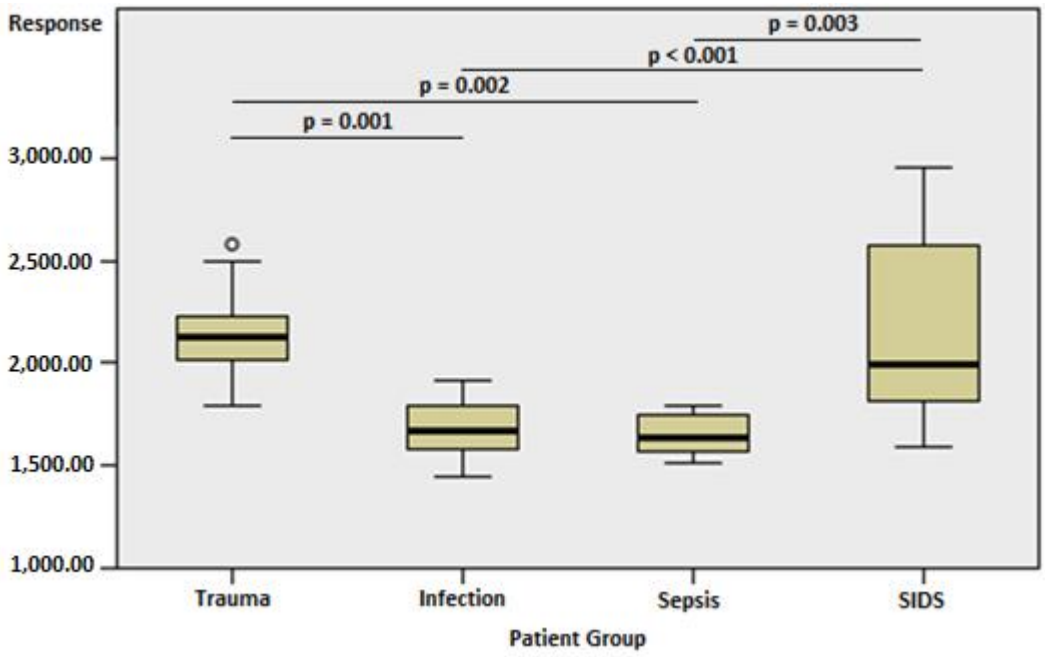

Figure 6.17 Graphical representation of Interleukin 2 measured in post mortem liver from four experimental groups

6.3.3c Interleukin 6 (IL6)

A Kruskal-Wallis test was conducted to determine if there were differences in detection of IL6 between the control, sepsis, infection, and SIDS groups. Distributions of IL6 detected were not similar for all groups, as assessed by visual inspection of a boxplot. IL6 levels were statistically significantly different between the different groups of cases, $Chi^2(3) = 15.607$, $p = 0.001$. Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed statistically significant differences in IL6 levels between the Infection (21.13) and Trauma (control) groups (39.89) ($p = 0.008$), and between the Infection and Sepsis groups (39.44) ($p = 0.016$), but not between any other group combination (see figure 6.18).

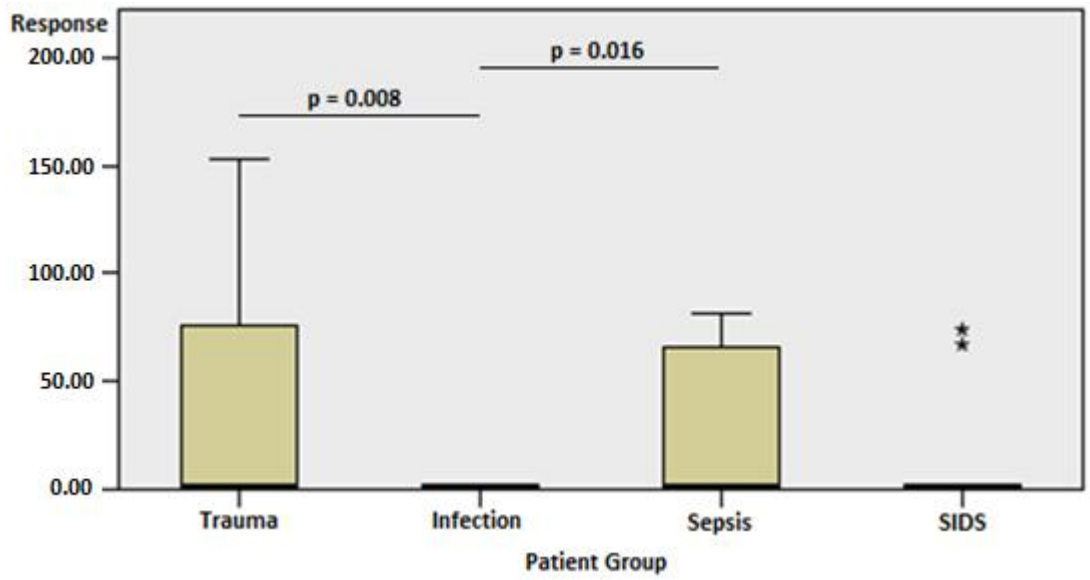

Figure 6.18 Graphical representation of Interleukin 6 measured in post mortem liver from four experimental groups

6.3.3d Intercellular adhesion molecule 1 (ICAM1)

A Kruskal-Wallis test was conducted to determine if there were differences in detection of ICAM1 between the control, sepsis, infection, and SIDS groups. Distributions of ICAM1 detected were not similar for all groups, as assessed by visual inspection of a boxplot. ICAM1 levels were statistically significantly different between the different groups of cases, $Chi^2(3) = 10.421$, $p = 0.015$. Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed statistically significant differences in ICAM1 levels between the Infection (18.84) and SIDS groups (34.65) ($p = 0.015$), but not between any other group combination (see figure 6.19).

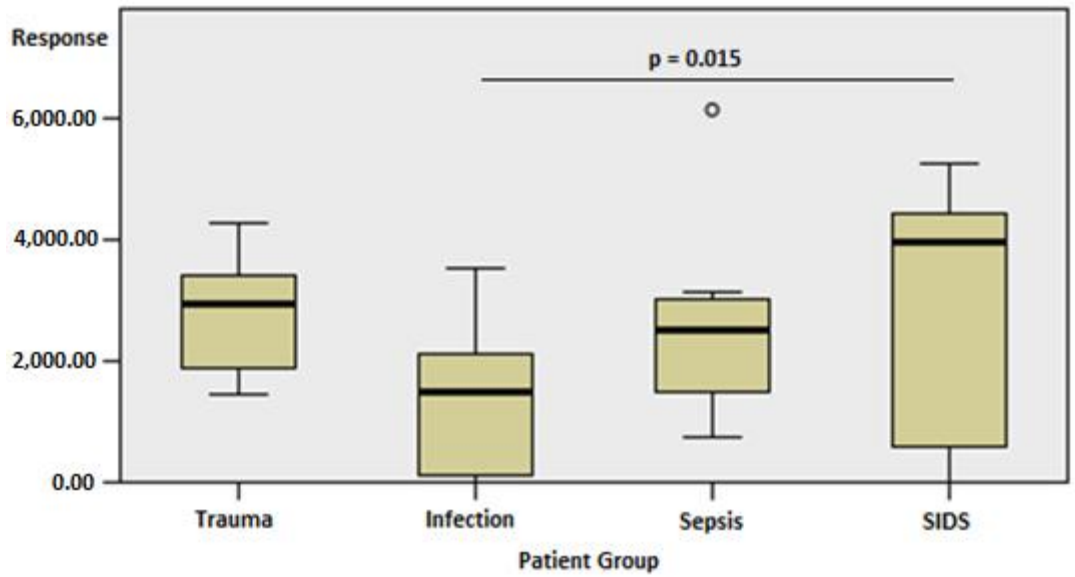

Figure 6.19 Graphical representation of Intercellular adhesion molecule 1 measured in post mortem liver from four experimental groups

6.3.4 Markers increased in SIDS when compared with all other groups

A group of five mitochondrial/metabolic proteins were statistically significantly increased in the SIDS group when compared with controls and the infection/sepsis groups: 60 kDa heat shock protein, mitochondrial, Carbamoyl-phosphate synthase [ammonia], mitochondrial, Phosphoglycerate kinase 1, Polyubiquitin C, and L-xylulose reductase. The detailed analysis of these proteins is given below:

6.3.4a 60 kDa heat shock protein, mitochondrial (CH60)

A Kruskal-Wallis test was conducted to determine if there were differences in detection of CH60 between the control, sepsis, infection, and SIDS groups. Distributions of CH60 detected were not similar for all groups, as assessed by visual inspection of a boxplot. CH60 levels were statistically significantly different between the different groups of cases, $Chi^2(3) = 10.483$, $p = 0.015$. Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed statistically significant differences in CH60 levels between the Trauma (control; 17.83) and SIDS groups (35.11) ($p = 0.046$), but not between any other group combination (see figure 6.20).

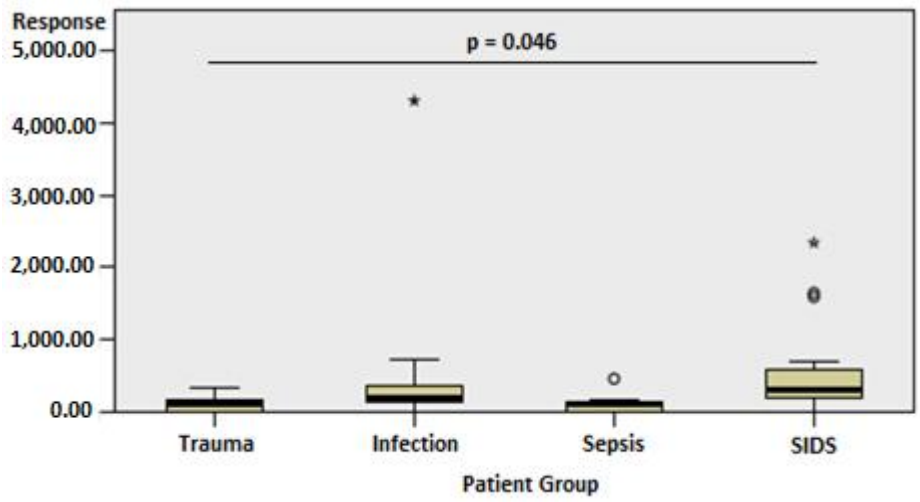

Figure 6.20 Graphical representation of 60 kDa heat shock protein, mitochondrial measured in post mortem liver from four experimental groups

6.3.4b Carbamoyl-phosphate synthase [ammonia], mitochondrial (CPSM)

A Kruskal-Wallis test was conducted to determine if there were differences in detection of CPSM between the control, sepsis, infection, and SIDS groups. Distributions of CPSM detected were not similar for all groups, as assessed by visual inspection of a boxplot. CPSM levels were statistically significantly different between the different groups of cases, $Chi^2(3) = 10.277$, $p = 0.016$. Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed statistically significant differences in CPSM levels between the SIDS (37.42) and Infection groups (23.75) ($p = 0.045$), but not between any other group combination (see figure 6.21).

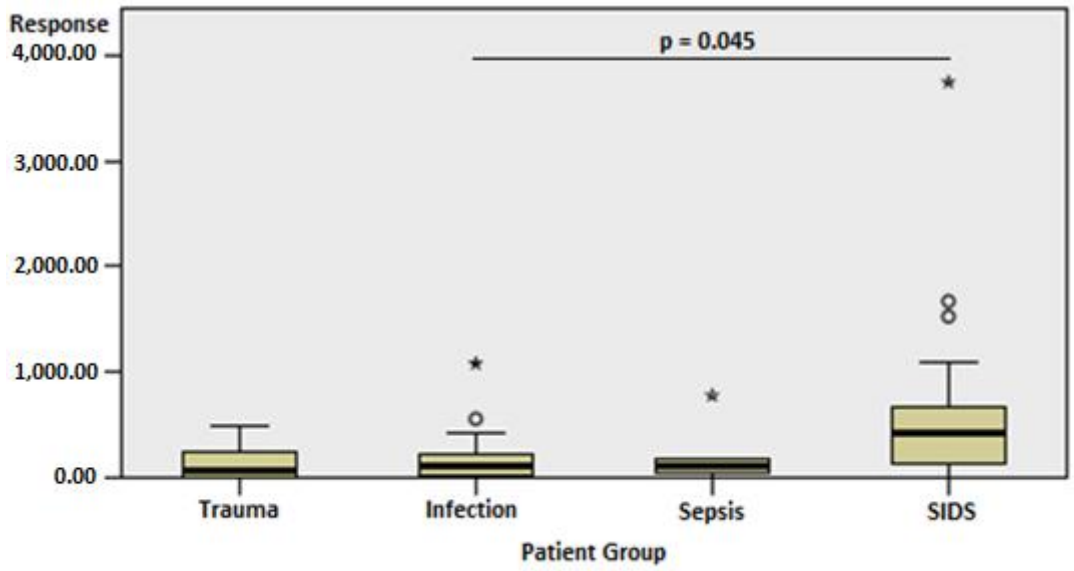

Figure 6.21 Graphical representation of Carbamoyl-phosphate synthase [ammonia], mitochondrial measured in post mortem liver from four experimental groups

6.3.4c Phosphoglycerate kinase 1 (PGK1)

A Kruskal-Wallis test was conducted to determine if there were differences in detection of PGK1 between the control, sepsis, infection, and SIDS groups. Distributions of PGK1 detected were not similar for all groups, as assessed by visual inspection of a boxplot. PGK1 levels were statistically significantly different between the different groups of cases, $Chi^2(3) = 9.695$, $p = 0.021$. Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed statistically significant differences in PGK1 levels between the SIDS (33.74) and Sepsis groups (14.07) ($p = 0.014$), but not between any other group combination (see figure 6.22).

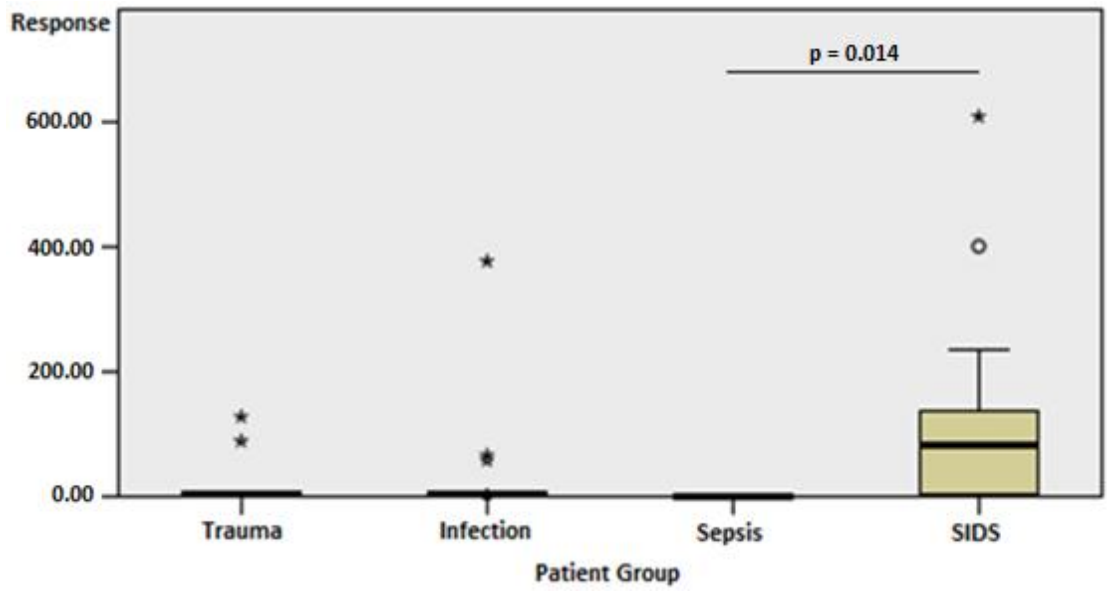

Figure 6.22 Graphical representation of Phosphoglycerate kinase 1 measured in post mortem liver from four experimental groups

6.3.4d Polyubiquitin C (UBC)

Kruskal-Wallis test was conducted to determine if there were differences in detection of UBC between the control, sepsis, infection, and SIDS groups. Distributions of UBC detected were not similar for all groups, as assessed by visual inspection of a boxplot. UBC levels were statistically significantly different between the different groups of cases, $Chi^2(3) = 13.482$, $p = 0.004$. Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed statistically significant differences in UBC levels between the Trauma (control; 17.44) and SIDS groups (37.58) ($p = 0.010$), and the Sepsis (18.57) and SIDS groups ($p = 0.040$), but not between any other group combination (see figure 6.23).

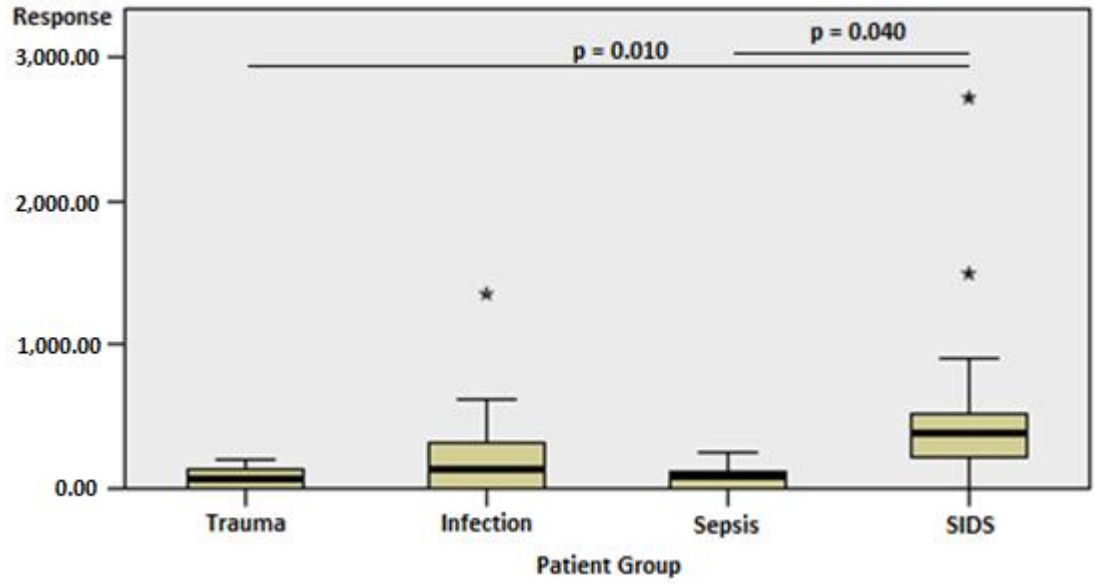

Figure 6.23 Graphical representation of Polyubiquitin C measured in post mortem liver from four experimental groups

6.3.4e L-xylulose reductase (DCXR)

A Kruskal-Wallis test was conducted to determine if there were differences in detection of DCXR between the control, sepsis, infection, and SIDS groups. Distributions of DCXR detected were not similar for all groups, as assessed by visual inspection of a boxplot. DCXR levels were statistically significantly different between the different groups of cases, $Chi^2(3) = 24.181$, $p < 0.001$. Subsequently, pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. This post hoc analysis revealed statistically significant differences in DCXR levels between the Trauma (control; 10.11) and SIDS groups (40.53) ($p < 0.001$), the Sepsis (21.57) and SIDS groups ($p = 0.045$), and the Infection (26.40) and SIDS groups ($p = 0.035$), but not between any other group combination (see figure 6.24).

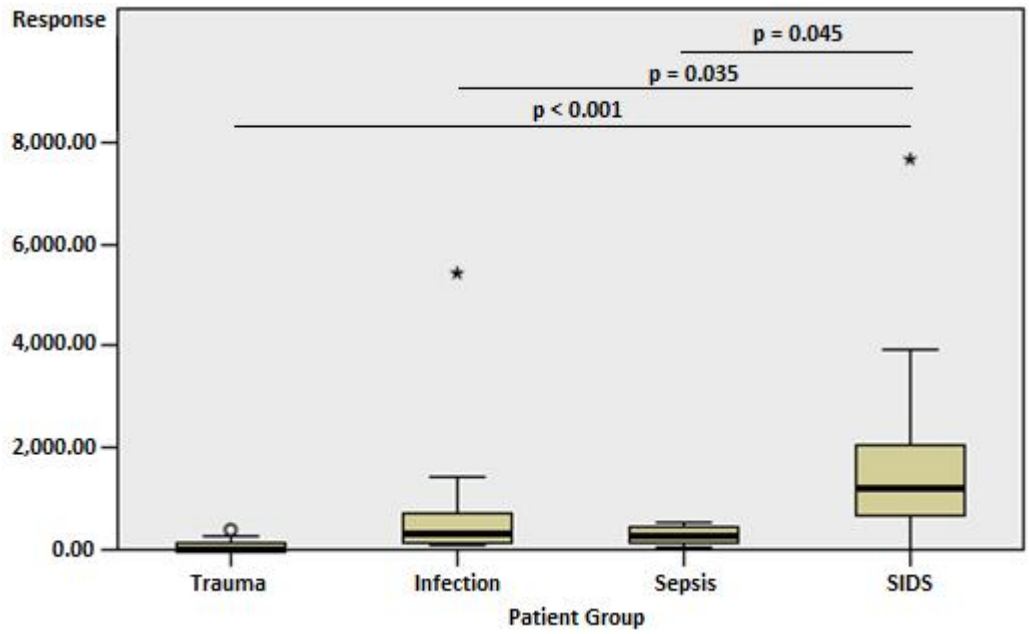

Figure 6.24 Graphical representation of L-xylulose reductase measured in post mortem liver from four experimental groups

6.4 Discussion

6.4.1 A post mortem test for infection

Statistically significant differences between groups were identified in 12/52 (23%) of the tested markers, and three (3/52, 6%) were statistically increased in infection cases when compared with controls or SIDS cases and are therefore suitable biomarkers for use in identifying infection in SUDI cases. While it may appear disappointing that so many markers remained undetected in individual samples, in general terms it is expected that around 5% of markers identified in biomarker discovery experiments will be proved useful markers and the number identified in this study are clearly around this level. That so many markers remained completely undetected is likely to be a reflection of the very low relative concentrations at which inflammatory markers are present in vivo.

The three markers identified with most potential for use as a test are C-reactive protein, and two heat shock proteins. C-reactive protein is member of the pentraxin family and acts as an acute phase protein which is generated in the liver in response to an inflammatory stimulus [Thompson, Pepys and Wood 1999]. It acts by binding to lysophosphatadyl choline on the surfaces of some bacterial organisms and dying native cells, and activates the complement system via the classical pathway [Pepys and Hirschfield 2003]. It is in wide use in clinical practice as an inflammatory marker in hospital inpatients. It is not specific to infection and may be markedly raised in many inflammatory conditions and in response to trauma. Furthermore, small increases in CRP have been noted in a wide range of physical conditions even in the absence of infection or evidence of tissue injury [Antonelli and Kuschner 2017].

CRP measurement in post mortem samples has been investigated by a number of groups, primarily in serum and pericardial fluid [Palmiere and Augsberger 2014], although one group also measured CRP in fresh liver samples after storage and found the levels to correlate well with serum levels [Astrup and Thomsen 2007]. A previous study in this group by Pryce and colleagues also examined CRP expression in liver in the same cohort of patients as used in this study be means of immunohistochemistry and found strong hepatocyte

expression in infection [Pryce et al 2014]. The current study is the first to quantitatively examine CRP levels in liver tissue which has been formalin-fixed and paraffin embedded; proving that the protein remains relatively stable for many years when processed and stored in this way.

An interesting finding in the measurement of CRP in these cases is that the levels are significantly only in the infection group when compared with the control and SIDS groups. With regard to the control group this is an interesting finding because the majority of those cases involved trauma which is also known to result in a CRP rise in the living. It is likely that these deaths have occurred before that rise in CRP has occurred and this validates the used of this group of cases as a negative control group for infection. The significant difference when compared with the SIDS group suggests that a relatively small number of cases, or possibly no cases, of infection are being incorrectly included in the SIDS group (i.e. the number of missed cases of infection is likely to be low).

The other two markers identified were Heat shock cognate 71 kDa protein and Heat shock 70 kDa protein 1-like. Both of these proteins are members of the heat shock protein 70 family and are encoded by the gene HSPA8 on chromosome 11, and HSPA1L on chromosome 6 respectively. They function as chaperone proteins and play important roles in protein folding and apoptosis [Mayer and Bukau 2005].

Published research on Heat shock cognate 71 kDa protein focuses on its role in viral infection, particularly with respect to virally-induced malignancy [Takashima et al 2003, Moerdyk-Schauwecker, Hwang and Grdzelishvili 2009, Liu et al 2013]. There are also studies which have identified a role for this protein in cases of bacterial infection; it was identified as a part of the pathway of lipopolysaccharide signal transduction [Triantafilou, Triantafilou, and Dedrick 2001]. Detection of this protein in the clinical samples in this study showed a similar pattern of response as CRP, although the only statistically significant difference identified was between the infection and SIDS groups.

Heat shock 70 kDa protein 1-like has been less extensively studied, but has been studied in the context of inflammation. Mutations in the gene HSPA1L have been reported to be increased in patients with inflammatory bowel disease when compared with controls [Takahashi et al 2017], and increased levels of

mRNA have been identified in patients with graft-versus-host disease [Atarod et al 2015]. It has also been suggested that the protein may play a role in the inflammatory response in trauma patients, but studies have been contradictory and whether this is a genuine association is unclear [Schroder et al 2003, Bowers et al 2006, Bronkhorst, Patka, and Van Lieshout 2015].

The pattern of expression in this study varied from that of the other two proteins, with extremely high levels seen in the sepsis cases when compared with the controls and SIDS cases, and increased levels in infection cases when compared with SIDS cases. The extremely high levels in the sepsis cases (around 30 times the levels seen in controls and 10 times the levels in infection cases) are particularly interesting as they imply that those cases which pathologists conclude are likely to be the consequence of "sepsis" are different from both the cases of histologically-proven infection and the cases categorised as SIDS. This is reassuring in that it implies that pathologists may be right to highlight sepsis as a possible cause of death in these cases, although given the relatively small number of sepsis cases in this study, further work would be required to conclude this with certainty.

With respect to the aims of this thesis (i.e. the creation of a multiplex test to accurately identify infection in post mortem cases), these three markers have significant potential. The positive and negative rates for each marker in the control and Infection groups are given in Figure 6.25. Included in this figure are also figures for positive and negative rates in the control and infection groups if the three markers were to be used as a multiplex, with positive results for any two being considered a positive result.

	C-reactive protein	HSP7C	HS71L	Any two of the three markers positive
Infection	12/20 (60%)	14/20 (70%)	17/20 (85%)	18/20 (90%)
Controls	1/9 (11%)	2/9 (22%)	3/9 (33%)	1/9 (11%)

Figure 6.25 Positive and negative rates for potential markers of infection in the infection group and control group

Using this data, specificity, sensitivity and other relevant parameters were calculated for a combined test of the three markers, with detection of any two of the three being considered a positive result. The results of this analysis are

given in Figure 6.26. These findings clearly indicate that the test using these parameters is appropriate for use in the post mortem setting, where for a Coroner's purposes a sensitivity and specificity of only 51% would be required to satisfy the "balance of probabilities" test for cause of death.

Sensitivity	90.0%
Specificity	88.9%
Positive Predictive Value	94.7%
Negative Predictive Value	80.0%
False Positive rate	11.1%
False Negative Rate	10.0%

Figure 6.26 Sensitivity, specificity and other parameters of a test for infection in liver from infant deaths undergoing autopsy using three markers (CRP, HSP7C and HS71L), with positivity in any two of the three markers being interpreted as a positive result

It should be noted that the combined test using the positive parameters discussed above would identify only two of the sepsis cases (sensitivity 28.6%, specificity 88.9%). It is notable that while the sepsis cases clearly behave differently than the controls and SIDS cases, they also behave differently than the infection cases; raising the possibility of an alternative inflammatory pathway. As an alternative to the test defined above, given the extremely high level of HS71L in the sepsis cases, if a level of HS71L more than 30 fold the level in the control group was considered a positive test result, all but one of the sepsis cases would have been correctly identified (sensitivity 85.7%, specificity 88.9%). Using this definition of a positive test only one of the infection group would have been labelled as sepsis, and since levels of the other two markers were also raised in this case it would also have been labelled as infection.

This study has therefore shown the potential utility of a multiplex test comprising tests for CRP, HSP7C and HS71L (Figure 6.27), with a positive result for any two of the three markers having a high sensitivity for tissue infection, and if the test was negative using these parameters, a positive result for HS71L at a level greater than 30 times the baseline level in the control group, has a high sensitivity for sepsis without evidence of tissue infection. Given the relatively small numbers used in this method development, the test would benefit from further sensitivity and specificity testing in a larger group of unselected cases (see Chapter 8).

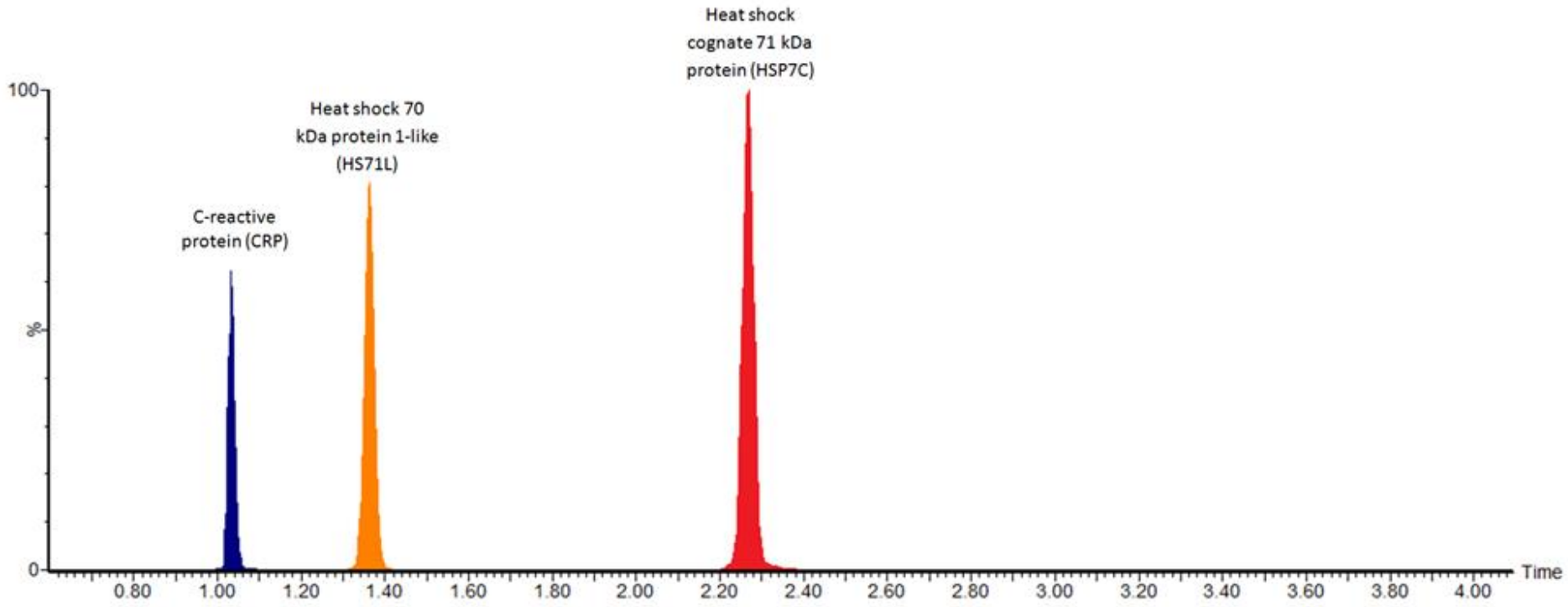

Figure 6.27 UPLC-MS/MS chromatogram of a multiplex test for the analysis of C-reactive protein, Heat shock 70 kDa protein 1-like, and Heat shock cognate 71 kDa protein in human liver

6.4.2 Decreased detection of pro-inflammatory cytokines in infection cases

Statistically significant differences between the infection and sepsis groups and the SIDS and control groups were observed in four pro-inflammatory cytokines: Interleukin 1a, Interleukin 2, Interleukin 6, and Intercellular adhesion molecule 1. What is interesting about these results is that they are essentially in the opposite direction than one would expect (i.e. the levels of these markers are lower in infection than in controls). The pattern of response in the CRP and heat shock proteins described above, which is exactly what would be expected in infection, suggests that this is not error related to the tests performed or the sampling undertaken.

There is an increasing recognition that 'inflammation' is in fact a complex interaction of pro and anti-inflammatory pathways and that immunological dysfunction is an important factor in the response to sepsis [Bermejo-Martin et al 2016]. Studies have shown alteration in the function of both monocytes and dendritic cells in severe sepsis, with decreased production of pro-inflammatory cytokines [Poehlmann et al 2009, Bhardwaj et al 2015, Fan et al 2015, Shalova et al 2015]. One possibility is that such acquired immunological dysfunction is a significant factor in infants who die suddenly and unexpectedly as a consequence of infection. This seems a more likely explanation than the obvious alternative, which is that all of these children have some form of immunodeficiency which is yet to be described.

Although they represent the largest group of explained deaths in infants, fatal infection in the context of SUDI is a relatively under-investigated area from a pathological perspective as the cause of death is usually readily apparent. In light of these striking results, further investigation of these patients is warranted with a view to determining if there is any element of immune paresis which might be predicted, and therefore targeted so as to avoid a proportion of these deaths. This would be an interesting area of collaboration with intensive care medicine and this is discussed further in Chapter 8.

6.4.3 Upregulation of metabolic pathways in SIDS

The final group of statistically significant differences involved five proteins which form part of the normal metabolic machinery of the cell, in particular the mitochondria. These proteins were statistically significantly increased in SIDS, but not in the other groups of cases. These proteins are: 60 kDa heat shock protein, mitochondrial, Carbamoyl-phosphate synthase [ammonia], mitochondrial, Phosphoglycerate kinase 1, Polyubiquitin C, and L-xylulose reductase.

While all of these proteins are involved in the normal metabolic functioning of the cell, three of the proteins have been suggested to have some role in inflammatory pathways. Carbamoyl-phosphate synthase [ammonia], mitochondrial is postulated to play a role in lipopolysaccharide binding, and 60 kDa heat shock protein, mitochondrial, and Polyubiquitin-C are thought to have roles in the innate and adaptive immune response [Uniprot; http://www.uniprot.org/].

While there is a possibility that the upregulation of these proteins in SIDS represents evidence that there is an underlying inflammatory component to SIDS deaths, it is also possible that this represents a non-specific increased metabolic response to another stimulus, such a prolonged agonal period caused by deaths due to respiratory causes. It is not possible to determine the nature of these changes in more detail based on the data collected in this study.

6.5 Conclusions

Targeted testing of a group of inflammatory markers discovered through biomarker discovery techniques and selection based on data from the literature has identified a total of 15 proteins whose concentrations in post mortem formalin-fixed paraffin-embedded liver differ significantly between disease groups. It has been shown that three proteins used in combination can be used to identify infection and sepsis in these cases with a high degree of sensitivity

and specificity. This can be rapidly translated into a test for routine clinical use, for example in minimally-invasive post mortems.

Statistically significant decreases in common pro-inflammatory cytokines were identified in the infection group when compared with other groups and this supports the role for immune dysregulation as a significant aspect of the physiological response to infection in infants dying suddenly and unexpectedly. Finally, a statistically significant increase in a group of metabolic proteins in SIDS when compared with the other disease groups raises the possibility of a prolonged agonal period or other cause for increased metabolic demand in these cases. These two findings require further investigation in order to determine their significance.

Part Four

Discussion, Conclusions and Future Work

Chapter 7 – Discussion

7.1 Introduction

The principle aim of this thesis was to utilise proteomic techniques to further investigate infection as a potential cause of SIDS. A particular aim of the work was to detect new biomarkers and develop them into a robust test for infection in post mortem tissue which could be used to identify cases of infection which may be being missed and incorrectly being defined as SIDS cases. This has the dual benefit of giving accurate information to the family of the deceased infant, and any healthcare professionals involved in their care before their death, and of removing infective deaths from the SIDS umbrella; allowing more accurate research studies. The techniques applied during the work have allowed the identification of markers for infection, sepsis, specific bacteria, and bacterial toxins, and have provided valuable insight into the mechanisms which may underlie both infant deaths due to infection, and infant deaths arising as a consequence of other causes. These outcomes are discussed further below in the context of current understanding of this area of investigation.

7.2 Markers of infection and sepsis in autopsies

As was discussed in the introduction to this thesis, not only is infection the most common cause of explained SUDI, but there are numerous factors which imply that infection or inflammation may play a significant role in the pathogenesis of SIDS. While it is not difficult to identify bacteria in the post mortem period using conventional microbiological techniques such as culture, interpreting the significance of any bacteria identified is difficult and interpretation in clinical cases varies widely between pathologists [Pryce et al 2011]. A major hindrance to the interpretation of these findings is the lack of robust, widely-available inflammatory markers which may indicate that any identified bacteria was having a physiological effect in the infant. While a number of inflammatory markers have been recommended for use in post mortem samples [Palmiere

and Augsburger 2014, Palmiere and Egger 2014], there are significant limitations to these techniques which limit their use in practice.

First, analysis of such markers often relies on specialist tests or equipment which may not be available at a local laboratory and may be extremely costly. Second, studies of candidate markers have generally been performed on fluids such as pleural fluid, CSF, or serum, which may be difficult to collect in sufficient quantity to allow the routine testing which forms part of infant death investigation and the novel investigations recommended. Finally, the reliability of such markers for identification of infection, rather than inflammation from other sources, is not proven in large scale cohorts of patients.

Using proteomic techniques to identify candidate biomarkers and then develop targeted tests to these biomarkers and to others identified in the literature, it has been possible to identify a group of three proteins which, when used in combination allow the identification of infection and sepsis in post mortem formalin-fixed paraffin-embedded liver samples with a high degree of sensitivity and specificity. This test uses multiple reaction monitoring on a tandem mass spectrometer; a technique which may be easily translated for use on the mass spectrometers available in local centres with minimal cost in terms of money and time.

The real benefit of these markers over previously published tests is the genuine potential for use in clinical practice which they present, in addition to the use of a widely available tissue which, as this study has proved, remains suitable for analysis for many years after the post mortem examination has taken place. This allows both prospective and retrospective use of the test.

With the advent of the Medical Examiners system, which was initially planned as part of the Coroners and Justice Act 2009, and in the context of an increasingly multicultural population, there is great demand from the public and Coroners for tests which might allow full invasive post mortem examinations to be avoided in favour of more limited examinations. Much of the research in this area has so far focussed on the application of radiological techniques to the deceased in an effort to establish the cause of death [Addison, Arthurs, and Thayyil 2014]. The development of this test allows the rapid diagnosis of infection or sepsis on the basis of a post mortem liver biopsy; a technique which is likely to be much more acceptable to the public than a full invasive autopsy,

and provides diagnosis of infection with a sensitivity and specificity far above that required by the Coroner. There is also good evidence that minimally invasive post mortem examinations are more acceptable to families [Lewis et al 2017]

The finding that sepsis may be distinguished from infection is useful clinically, but is also reassuring in that it the diagnosis of sepsis in post mortem cases at present is based on rather non-evidence based interpretation of the species of bacteria present and their location in the body. The fact that the sepsis cases show a response to the inflammatory marker panel which differs from that of controls indicates that there is something different about these cases from an inflammatory perspective which would be consistent with sepsis. However, it must be noted that the pattern of marker response also differs from that identified in infection; suggesting that the 'sepsis' deaths may be arising as a consequence of a different inflammatory mechanism.

7.3 Infection and sepsis as causes of SIDS

In addition to the development of a reliable test for infection and sepsis, the application of the developed tests to a variety of infection cases, sepsis cases, SIDS cases, and controls, has allowed a greater insight into infection both as a cause of SUDI and of SIDS. While many authors hypothesise that at least a proportion of SIDS cases arise as a consequence of undiagnosed infection [Goldwater 2017], the fact that so few of the SIDS cases in this study tend to cluster with the infection or sepsis cases when the tests are applied suggests that a very small number are due to undiagnosed infection or sepsis. This does not necessarily mean that other SIDS cases aren't the consequence of a disturbance in the inflammatory network; rather it may indicate that these deaths are not occurring by the same mechanism as 'classical' infection or sepsis deaths.

Over the last decade there has been a shift in the infant death literature to a focus on a more complex inflammatory theory of SIDS causation involving the integration of key bacterial species, toxin production, genetic predisposition, and other risk factors (particularly smoking) [Moscovis et al 2004, Goldwater 2017].

The data arising from this study goes some way to supporting the supposition that SIDS deaths do not arise as a consequence of a more straightforward 'classical' infective mechanism.

Another interesting finding in this study was the increased concentration of a number of metabolic proteins in the SIDS cases when compared with the other disease groups. This also provides some support to the suggested inflammatory cause of some SIDS cases discussed above, in that a number of these proteins are known to play a role in inflammatory pathways but, as the data from this study shows, they are not significantly upregulated in infection or sepsis. Neither are they involved in the same inflammatory pathway; although to some extent the network of interactions in inflammation is sufficiently complex that all pathways of inflammation interact at some stage. This supports the theory of an alternative and more complex inflammatory mechanism than classical sepsis.

However, this does not fully explain the findings of this study, as two of the metabolic markers increased in the SIDS group are not known to play any direct role in infection. This raises the possibility that the increase of these five proteins in SIDS cases is in fact more representative of a generally increased metabolic demand in SIDS than in other cases. Clearly infection causes an increased metabolic demand, but the SIDS cases have a much higher increase in the proteins than the infection group.

Increased metabolic demand may be seen in a number of physiological and disease states, including a prolonged agonal period. There are a number of SIDS theories which relate to mechanisms which would give rise to a prolonged agonal period, particularly theories which involve central respiratory depression [Machaalani and Waters 2014, Salomonis 2014, Bergman 2015]. It is possible that the increase in these metabolic proteins identified in the SIDS cases is a consequence of such a mechanism.

7.4 Immune dysregulation in SUDI arising from infection

Another key finding of this study is the decreased concentration of four key pro-inflammatory cytokines in the proven cases of infection. Other studies have shown an increase in such markers in cases of infection [Pereira et al 2014], but the area has not been extensively researched from a pathological perspective as the cause of death is usually clear.

There is a growing recognition that the physiological response to infection is far more complex than had previously been suspected, with the inflammatory response representing a complex interplay of pro- and anti-inflammatory pathways [Bermejo-Martin et al 2016]. It is well established that some pro-inflammatory pathways are downregulated in cases of severe sepsis; particularly those relating to monocyte and dendritic cell activation and antigen presentation [Poehlmann et al 2009, Bhardwaj et al 2015, Fan et al 2015, Shalova et al 2015]. The observed low levels of the pro-inflammatory cytokines in the infection cases in this study raises the possibility that such immune dysregulation is a key mechanism in infants dying suddenly and unexpectedly of infection. This may go some way to explaining why some infants appear to die of infective causes rapidly and with minimal symptoms in the community, where most infants have sufficient time from onset of symptoms to collapse to allow presentation to medical care, treatment and recovery. As this is a relatively newly developing area, it has not been extensively investigated, particularly in the context of infant deaths, although other abnormalities of the monocyte activation pathways have been shown in neonates and infants [Heinemann et al 2016, Juskewitch et al 2015]. Further investigation of these mechanisms in the context of explained infant deaths has the potential to enrich the understanding of infection as a cause of death, and perhaps more importantly in the living as a potential target for preventative strategies or therapy.

7.5 Bacterial and toxin identification and quantitation using tandem mass spectrometry

One of the established theories in the study of SIDS is the possibility of deaths arising as a consequence of toxin production by bacteria [Morris et al 1987]. Like the inflammatory processes in infection discussed above, the recent literature has seen a move to theories which integrate bacterial toxin production with other factors such as nicotine exposure, genetic polymorphism, and the inflammatory response [Moscovis et al 2004, Goldwater 2017].

A limitation in the investigation of theories of infant death relating to toxins is the lack of suitable and widely applicable tests for common bacterial toxins. Specific qualitative and quantitative methods of identification are available, but not outside funded research studies. Even in clinical practice focussed on living patients, direct toxin measurement is rarely used and reliance is placed on genotyping of suspected toxin-producing strains from cultured organisms.

The use of the proteomic techniques in this study has allowed, as proof of principle, the development of a direct assay allowing quantitation of *Staphylococcus aureus* and its major toxins. This test may be translated to run on most mass spectrometers available in local hospital biochemistry departments, and is relatively cheap. It is therefore of considerable potential in the further investigation of toxin-based theories of SIDS by the routine application of direct toxin identification in prospective post mortem cases. It also has potential for use as a rapid test for toxins in living children to allow appropriate management to be conducted in a timely fashion.

7.6 Conclusions

In conclusion, this study has allowed a greater insight into the mechanisms of infection in explained infant deaths, and the potential inflammatory and metabolic mechanisms underlying unexplained infant deaths. A rapid, low cost, easily translatable assay has been developed which can detect and distinguish infection and sepsis in post mortem material; allowing use in the context of

routine practice and, in the future, limited autopsies. As proof of principle, a test has also been developed to allow the identification and quantitation of specific bacteria and bacterial toxins, which has the potential to greatly aid the knowledge and understanding of the role of toxins in SIDS.

The combined output of this work is substantial and has the potential for numerous further avenues of investigation, and these are explored in Chapter 8.

Chapter 8 - Limitations and Future Work

8.1 Limitations

There are a number of limitations to this work which were the consequence of the necessary use of post mortem and archived tissue. The principle limitations lay in the availability of material for research use. The source material was drawn from cases undertaken at Great Ormond Street Hospital, London over a period of many years; a centre which undertakes a relatively large number of paediatric post mortem examinations. Nevertheless it was only possible to identify a small number of suitable cases for inclusion in the positive and negative control groups for the study.

Furthermore, as was discussed at length in the method and results chapters, even though numerous methods are now described for the extraction of samples from formalin-fixed paraffin-embedded tissue, such extracts continue to perform poorly in proteomic studies when compared with fresh tissue, and the presence of post mortem autolysis requires significant adjustments to the analysis of the data obtained. As described in the method development chapter, protein assay is also compromised in formalin-fixed paraffin-embedded tissue; making comparison of samples challenging.

While this study has demonstrated that useful results can be achieved in such circumstances, the combined effect of the relatively small case number and suboptimal sample type is that further biomarker discovery/mechanism identification work in this area will continue be difficult; taking more time and money than similar studies on fresh tissue, with an increased possibility of poor quality data limiting results and interpretation.

Nevertheless, there are numerous avenues of interest in SIDS which could be very effectively explored by means of comparative proteomics and associated techniques such as transcriptomics, metabolomics, and lipidomics. If new guidelines were introduced that include the routine storage of fresh tissue in SUDI cases (with consent), this would open up immense potential for the use of these techniques in investigating SIDS. This is an established research tool used in numerous areas including tumour biology and degenerative brain

disease, and systems used for collection of samples in these areas could be utilised as a template for the creation of a SUDI tissue bank.

An added complication in the context of SUDI are the limitations imposed by the Human Tissue Act, and the perceived negative public perception of paediatric post mortem research following the organ retention scandals of the last century. Nevertheless there is clearly a fundamental difference between systematic sampling of small fresh tissue samples for research in an open, transparent way, and retention of whole organs with no parental knowledge or consent. Properly managed, and with the support of parental groups, such a tissue bank could be effectively advocated to parents, the Chief Medical Officers, the Chief Coroner, and the Human Tissue Authority. Even were specific consent required in every case, there are few enough SIDS cases in each year that one dedicated research nurse at the centre where the bank is held could contact every family suffering an unexpected infant death to discuss and obtain consent for sampling for research.

It is conceivable that there may be some resistance from pathologists to such an approach, particularly given that it will involve close collaboration between centres and some investment of time. However, the investigation of infant deaths needs to move forward from basic morphological assessment of the deceased to an integrated examination involving next generation tests and active participation in research. It may be that it is desirable in the long term to centralise the investigation of such cases to a small number of specialist centres who are able to provide an integrated examination which includes molecular and genetic testing and a research contribution. This would also have the advantage of increasing the experience and expertise of the pathologists performing the examinations. The potential disadvantages of centralisation, such as transport of bodies, are likely to be outweighed by the benefits.

8.2 Future work

A number of exciting potential areas for future research have arisen from the research forming the basis of this thesis, which have the potential to contribute greatly not only to the investigation of infant deaths, but also to the investigation of disease in the living.

8.2.1 Application of tests to routine infant death investigation

A small number of further research steps are required before the tests developed during this research can be routinely applied in post mortem practice. First, the refined inflammatory marker test must be applied to a large cohort of unselected historic cases in order to establish its specificity, sensitivity and robustness as a diagnostic test. There is also a role for prospectively applying the test in routine practice in a pilot centre or small number of centres. This will have the effect of proving the utility of the test in practice.

Once the utility of the test is proven, it can easily be applied in clinical practice, either by sending of samples to a reference laboratory for testing, or by applying the test locally. A great advantage of the MRM-based tandem mass spectrometry method is that it can be straightforwardly translated onto any tandem mass spectrometer, such as may be found in most district general hospital laboratories.

The test also has great utility for incorporation into less invasive autopsies by means of applying the test to biopsy samples taken following imaging studies.

8.2.2 Further investigation of the mechanisms of infant death

The development of a robust test for *Staphylococcus aureus* and many of its major toxins is a valuable tool which may now be applied to samples from post mortem examinations in infant deaths resulting from infection and SIDS cases.

This has the potential to add greatly to the understanding of the disease mechanisms which may play a role in the aetiology of SIDS and sepsis.

Using the techniques applied in this study, it is also possible to investigate other areas of mechanistic interest in SIDS, and indeed some groups have already used proteomics to approach the issue of central nervous system development in SIDS [Broadbelt et al 2012, Hunt et al 2016].

The increased integration of proteomics with other 'omic' techniques such as genomics, transcriptomics, metabolomics, and lipidomics also opens the door to using 'omic' technology to investigate abnormalities in SIDS identified using these other techniques. For example, inactivating protein mutations identified in whole exome studies in SIDS cases, which has been recommended as routine practice [Morris 2015], could be further investigated by proteomic analysis of the downstream pathways which such proteins regulate.

The identification of increases in a number of metabolic proteins also has potential for further investigation with regard to whether it represents part of a more complex inflammatory aetiology, or a less specific agonal response to other stimuli, for example central respiratory depression. These markers may be applied to larger number of cases of infants dying in more varied circumstances, including as a consequence of asphyxia and metabolic disease, to further characterise the meaning of these observed changes.

8.2.3 Investigation of disease in the living

While a number of inflammatory markers are in common use in clinical practice, such as C-reactive protein (CRP), there are still areas in which novel tests for established markers would be of use. A good example of this is in the context of intensive care medicine in head injury, where CRP is known to rise over the days following injury, giving rise to a clinical difficulty when there is suspected concurrent infection (such as ventilator-associated pneumonia). The application of this new panel of inflammatory markers to samples from intensive care patients presents an opportunity for development of a small panel of markers allowing a distinction to be drawn between infection and other processes.

As discussed at length in the bacterial and toxin identification chapter, while there are numerous methods available for bacterial identification in clinical and post mortem samples, options for identification and quantification of toxins are extremely limited. For practical purposes, toxin expression testing in clinical isolates is limited to genotyping at a reference laboratory. The *Staphylococcus aureus*/Staphylococcal toxin test can be applied to clinical isolates and allows positive identification of *Staphylococcus aureus* and a quantitative assessment of its toxins. This test requires significant further testing using large numbers of clinical isolates to ensure its efficacy before it can be reliably used clinically, but samples are so freely available that this should be achievable in a relatively short space of time at minimal cost. If it proves to be reliable in large numbers of cases, then a very useful application for the technology is in rapid positive identification of bacteria in sepsis in the Accident and Emergency Department setting. Adapting the laboratory technique to allow rapid processing would take a small amount of work and a relatively slight increase in cost.

Using the technique applied in this study, there is also potential for developing similar tests for other bacterial toxins of interest which can be tested for individually, or using a multiplex method. Furthermore, there is no reason why this technique could not be expanded to include other pathogens to rapidly identify the pathogen causing an infection; allowing early therapy with appropriate specific antibiotics. Such a precision medicine-based approach might prove invaluable in approaching the problem of broad spectrum antibiotic use and antibiotic resistance.

The identification of cytokine downregulation suggestive of immune dysregulation in infants dying suddenly and unexpectedly of infection raises the possibility of a more complex inflammatory mechanisms underlying fatal infection in this patient group. Application of these markers to a wider variety of fatal infection samples, and to samples of infants suffering infection severe enough to require intensive care therapy may provide valuable insights into the pathophysiology of infection and allow the development of preventative or therapeutic strategies which may reduce the incidence of these deaths.

8.2.4 Investigation of other inflammatory conditions

Another very useful outcome of the research is the development of a large panel of inflammatory markers. Although only a proportion of these have proven useful in identifying infection in SUDI, the larger panel of markers is still of use in other areas of research where inflammatory markers may be of interest, including inflammatory bowel disease and autoimmune disease. Furthermore, there is increasing recognition that inflammatory pathways may play a role in complex chronic disorders, particularly in neurodegenerative diseases [Hong, Kim and Im 2016]. Quantitative interrogation of these markers may now be incorporated into studies of a wide range of conditions using the methods developed as part of this study.

8.3 Conclusion

In summary, while there were unavoidable limitations surrounding the samples available for examination, this research has resulted in a number of outcomes which will be useful in clinical practice. First, it has allowed the development of a multiplex inflammatory marker test which has shown to be effective in the identification and distinction of infection and sepsis in post mortem formalin-fixed paraffin-embedded tissue. Second, it has allowed the development of a multiplex test allowing identification and quantitation of *Staphylococcus aureus* and its principle toxins. Both of these tests will be of great use in the development and refinement of the post mortem examination in Sudden Unexpected Deaths in Infancy, and in understanding the mechanisms underlying these deaths. Furthermore, they may also be of use in assessment of living patients in the Emergency Department and Intensive Care settings. The methods for individual inflammatory markers developed during the biomarker discovery phase of this research can be applied to clinical samples from a wide range of clinical conditions in which inflammatory markers may be of interest, such as inflammatory bowel disease, autoimmunity, and neurodegenerative conditions. Finally, the insights into the physiological mechanisms underlying

fatal infection in infants may be further investigated so as to advance understanding of infection, and develop strategies for prevention.

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