

Molecular Diagnostic Techniques in the Post-Mortem Investigation of Sudden Unexpected Infant Deaths: Current and Future Applications

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Abstract: Sudden unexpected death in infancy (SUDI) encompasses all infant deaths that occur relatively suddenly and unexpected by history; in around a third of cases, the post-mortem examination and/or review of the clinical history and death scene will reveal a cause of death (explained SUDI), whilst the remainder will remain unexplained using current investigative autopsy protocols (unexplained SUDI or sudden infant death syndrome, SIDS). This review highlights current and potential future molecular applications in the post-mortem investigation of SUDI/SIDS, specifically focusing on the potential role of infection, as well as cardiac, neuropathological and metabolic findings that may be associated with, or increase the infant's susceptibility to, sudden unexpected death.

Keywords: SUDI, SIDS, infant death, investigation, autopsy, molecular techniques.

INTRODUCTION

Sudden unexpected death in infancy (SUDI) encompasses all infant deaths (aged one week to one year of age) that occur relatively suddenly and unexpected by history; in around a third of cases, the post-mortem examination and/or review of the clinical history and death scene will reveal a cause of death (explained SUDI), whilst the remainder will remain unexplained using current investigative autopsy protocols [1]. The latter group is more or less equivalent to sudden infant death syndrome (SIDS), a diagnosis of exclusion which is broadly defined as the "the sudden unexpected death of an infant < 1 year of age, with onset of the fatal episode apparently occurring during sleep, that remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history" [2], and for the purposes of this article, the terms 'unexplained SUDI' and 'SIDS' will be used synonymously. However, exactly what constitutes a 'complete autopsy' in this setting remains poorly defined; for example, the above SIDS definition makes toxicological analysis mandatory in all cases, whilst the Kennedy autopsy protocol – the currently recommended post-mortem investigative protocol for all SUDI in England and Wales – suggests that toxicology be performed in selected cases only [3]. Furthermore, it is unclear what would be considered adequate microbiological or metabolic investigations, and none of the post-mortem guidelines provide guidance regarding the interpretation of positive laboratory results [3,4]. Equally, there appears to be much variation in pathologists' interpretation of the potential significance of pathological changes and whether these would represent an adequate explanation for the death (cause

of death) or whether the death should be classified as SIDS [5-8]. For these reasons, identifying novel investigative tools that may help – not only to determine cause of death – but also to provide additional, clinically useful data in equivocal cases in order to standardise the classification of death in SUDI is evidently needed.

In 2005, there were around 300 registered unexplained infant deaths in England and Wales, a rate of around 0.4 unexplained infant deaths per 1000 live births [9]. Many risk factors for unexplained SUDI/SIDS have been identified, including prone sleeping (and to a lesser extent side-sleeping) [10-14], co-sleeping or bed-sharing [5, 15-20] and maternal smoking [5, 21-23], as well as high ambient room temperatures, excessive clothing and/or bedding, head covering, preterm birth and/or intrauterine growth restriction, multiple pregnancy, high parity, young maternal age and low socioeconomic class [5, 24-28]. Previous studies have also consistently shown that use of pacifiers (soothers or dummies), room sharing without co-sleeping, and breastfeeding significantly reduce the risk of SIDS [27, 29].

OVERVIEW OF MECHANISMS OF UNEXPLAINED SUDI/SIDS

The triple risk hypothesis was first formulated by Wedgwood in 1972 and is based on the interaction of different risk factors, each with different probabilities of death [30]. The hypothesis was modified in 1994 by Filiano and Kinney [31], who proposed that SIDS occurs as a consequence of the concurrent interaction of three factors, namely 1) an intrinsically vulnerable infant, 2) a critical developmental period, and 3) exposure to exogenous stressors. It is suggested that individually these factors would not prove fatal: the infant would die only if all three factors intersected, and then only if the stressor(s) matched that infant's specific vulnerability [32]. The model proposes that SIDS infants are therefore not 'normal' but have an underlying biological or genetic predisposition that makes

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them vulnerable to specific environmental stressors (such as prone sleeping, co-sleeping, cigarette smoke exposure, or infections). The critical developmental period is said to be between one and six months of age, which is based on the typical age distribution of SIDS, rather than strong evidence of physiological (cardiovascular, respiratory, immunological) instability during this time of development [30]. Nevertheless, the model serves as a useful “conceptual framework” [32] to accommodate potential interactions between the various established epidemiological risk factors.

POSSIBLE AETIOLOGICAL AND/OR CONTRIBUTORY FACTORS

1. Bacterial Infections

Infection has been previously identified as a cause of death in explained SUDI cases, usually based on a combination of histopathological and bacteriological findings [1, 5, 33-36]. Whilst infection supported by histological findings of acute inflammation (e.g. pneumonia or meningitis) is a relatively straight-forward diagnosis, meaningful and appropriate interpretation of microbiological culture results in the absence of histological evidence of infection is more difficult, even when pathogens are isolated. Positive post-mortem isolates may represent a true bacteraemia, be due to contamination, or may represent a ‘post-mortem artefact’ caused either by ‘agonal spread’ or post-mortem transmigration/translocation [36]. The first two also occur in life, whilst the latter two occur exclusively in the post-mortem setting. A bacteraemia, where bacteria have invaded the blood during life, represents a true positive result, but even this may not necessarily have a bearing on the cause of death, as an incidental ‘symptomless bacteraemia’ has been shown to occur in life in healthy individuals [37]. Contamination during sampling at post-mortem may be minimised, as in life, with good technique, using appropriate antiseptic preparations and sterile instruments. ‘Agonal spread’ is essentially a hypothetical concept, which postulates that the mucosal integrity is compromised by perimortem hypoxia and/or ischaemia, thus allowing bacteria to enter the tissues and/or blood around the time of death, but there appears to be little evidence to support its existence [36]. Post-mortem transmigration or translocation occurs as part of the normal putrefaction process, in which bacteria that have colonised the mucosal surfaces in life start to invade the body after death, but this does not appear to be a significant problem if bodies are stored in appropriately refrigerated units [36]. Whilst there appears to be good correlation between positive post-mortem blood culture isolates of selected pathogens and macro- or microscopic evidence of infection, with positive predictive values of around 80% [36], the interpretation of positive microbiological culture results in the absence of histological evidence of infection is presently based on empirical guidelines, viz. isolates are more likely to be significant if 1) they are recognised pathogens, 2) the same pathogenic organism is isolated from multiple sample sites, 3) the organism is isolated as a pure growth (single isolate), and 4) there are features in the clinical history of a developing infection, or laboratory markers of possible sepsis, such as a raised white cell counts or CRP [38]. The current challenge, therefore, is to determine optimal methods for differentiating

deaths that are truly infection-related from those in which positive microbiological cultures represent contaminants, post-mortem artefact or an incidental bacteraemia. In this regard, additional methods of investigation must be developed, including those directed at detection of the systemic response to infection rather than simply identification of the organism.

Whilst there is presently no unifying theory for the pathogenesis of unexplained SUDI/SIDS, data derived from epidemiological, pathological, genetic and animal studies suggest that the aetiology may be infection-related in at least a subset of SIDS cases, although the absence of histological evidence of infection in unexplained SUDI/SIDS makes a diagnosis of ‘classical infection’ due to direct microbial invasion less likely. The so-called common bacterial toxin hypothesis was first proposed by James Morris in 1987 [37, 39, 40] and is supported by an increasing body of evidence. In essence, the hypothesis maintains that SIDS is caused by bacterial toxins which are produced by the infant’s own bacterial flora, most likely that of the upper respiratory tract, although the gastrointestinal tract is also known to harbour toxigenic bacteria. It postulates that an antecedent viral upper respiratory tract infection may disrupt the normal bacterial flora and result in overgrowth of toxigenic strains. Unlike older children and adults, young infants would be susceptible to these toxins in the period when maternal IgG concentrations are dwindling prior to maturity of the infants’ immune system. Many of these toxins can act as so-called superantigens and elicit a massive release of cytokines resulting in a powerful, and often exaggerated, inflammatory response leading to a toxic shock-like syndrome or septic shock, and death [40, 41]; alternatively, it is speculated that the toxins may act directly on neural or myocyte membranes to induce sudden death in infants [40].

The hypothesis uses a mathematical model in which the risk of death from common bacterial toxins increases as the levels of protective maternal IgG in the infant decreases during the first few months of life; making the assumption that these bacterial toxins are common (i.e. that 50% of the population would be exposed to the toxin in any 50-day period), the model correctly predicts the characteristic age distribution of SIDS, with death being uncommon in the first month of life when protective levels of maternal IgG are high, peaking at two to three months and falling thereafter, death being uncommon after six months of age as the infants are exposed to and acquire immunity to these (common) bacterial toxins [37, 39].

Much evidence has been forthcoming in recent years to support the common bacterial toxin hypothesis, with several studies demonstrating significantly higher carriage of pathogenic, potentially toxigenic organisms in the upper respiratory tract of SIDS infants compared to healthy controls, particularly in children sleeping prone, a well established risk factor for SIDS [42-44]. A Hungarian study of 13 SIDS infants and 100 healthy controls showed a significantly higher prevalence of toxigenic *Staphylococcus aureus* in SIDS infants [45]. In that study, post-mortem microbiological sampling included throat swabs collected shortly after death, and CSF, blood culture (heart blood) and lung tissue collected at post-mortem. The culture results of these were compared to the nasopharyngeal flora of the 100

control infants. Although the number of SIDS cases is small, the study nevertheless demonstrates interesting differences between the two groups: *Staphylococcus aureus* was more frequently isolated in SIDS (7 of 13, 54%) than in control infants (37 of 100, 37%; OR 2.0, 95% CI 0.6 to 7.3); overall, six (46%) SIDS infants carried toxigenic *Staphylococcus aureus* (screened for enterotoxins A to D (SEA to SED) and toxic shock syndrome toxin-1, TSST-1), while only 16% of control infants demonstrated a toxigenic strain (OR 4.5, 95% CI 1.2 to 17.7). Furthermore, of all children carrying *Staphylococcus aureus*, six of the seven SIDS infants (86%) carried a toxigenic strain, whilst only 16 of the 37 (43%) control infants demonstrated toxigenic *Staphylococcus aureus* (OR 7.9, 95% CI 0.8 to 191.9).

In a recent study of 130 SIDS cases, 32 SUDI due to infection and 33 SUDI due to non-infective causes, *Staphylococcus aureus* was isolated from post-mortem heart blood, spleen and/or cerebrospinal fluid (CSF) in almost 11% of SIDS and in almost 19% of infection-related deaths but not in any of the 33 non-infective SUDI deaths; although the observed differences did not reach statistical significance [46]. In the largest single-centre study to date, comprising more than 500 SUDI (including 379 unexplained SUDI, 72 non-infective explained SUDI and 56 explained SUDI due to bacterial infection), there were significantly more isolates of so-called 'group 2 pathogens' (including *Staphylococcus aureus* and *Escherichia coli*) in the bacterial infection group (24%) than in both unexplained SUDI (19%, $p=0.03$) and non-infective explained SUDI (11%, $p<0.0001$), and significantly more 'group 2 pathogens' in unexplained SUDI compared to the non-infective group ($p=0.001$), demonstrating that, on the basis of differences in detection rates between unexplained SUDI and non-infective explained SUDI, 10–35% of otherwise unexplained SUDI deaths in whom there is no histologically identifiable focus of infection at autopsy are associated with the presence of 'group 2 pathogens', further supporting the hypothesis that in a proportion of unexplained SUDI the pathogenesis of death may be related to the presence of *Staphylococcus aureus* and/or *Escherichia coli*, possibly via a toxigenic pathway [47].

To date, a large number of toxigenic bacteria have been proposed to contribute to the pathogenesis of unexplained SUDI/SIDS, including *Staphylococcus aureus* (enterotoxins, TSST-1), *Escherichia coli* (enterotoxins, verotoxins), *Bordetella pertussis* (pertussis toxin/endotoxin), *Haemophilus influenzae* (endotoxin), *Clostridium perfringens* (endotoxin), *Clostridium botulinum* (botulinum toxin), *Streptococcus pyogenes* (pyrogenic toxins) and even *Helicobacter pylori* (endotoxin, vacuolating toxin, urease) [48]. Only a few studies, however, have attempted to directly demonstrate the presence of toxin in the tissues at post-mortem, a more definitive way of showing a potential causative association in these deaths, as the presence of toxigenic bacteria merely confirms that bacteria were present than can produce toxins, whilst direct demonstration of the toxin in the tissues indicates that the bacteria have actually produced and released the toxins prior to death. The largest study to demonstrate this used enzyme-linked immunosorbent assay (ELISA) and flow cytometry techniques to test body fluids, and frozen or formalin-fixed tissues for pyrogenic toxins of *Staphylococcus aureus*, including TSST-1 and SEA, SEB

and SEC [49]. Samples were used from three different countries and included unfixed samples of serum, lung and kidney (Scotland), fixed samples of brain, kidney and spleen (France) and fixed brain tissue (Australia). Overall, toxins were identified in the tissues of 33 of the 62 (53%) SIDS – Scotland (10 of 19, 53%), France (7 of 13, 54%) and Australia (16 of 30, 53%); in the latter series, toxins were identified in only three of the 19 non-SIDS deaths (16%, $p < 0.02$). Subsequent studies of German and Hungarian SIDS infants showed similar results, with pyrogenic staphylococcal toxins in 13 of 20 (65%) and 13 of 23 (57%) SIDS infants [48]. In two earlier studies, immunohistochemical techniques, based on studies in rats, were used to identify toxins in SIDS in the proximal convoluted tubules of the kidneys: TSST-1 was present in nine of 50 (18%) SIDS compared to three of 50 (6%) cases who died of other causes (the comparison group included a wide range of autopsy cases, including adults), and SEC in 18 of 50 (36%) SIDS compared to 6 of 50 (12%) cases in the comparison group [50, 51]. However, immunohistochemical methods for detecting bacterial toxins are hampered by technical problems and background staining [40], making interpretation difficult, and are not currently available for routine diagnostic use.

Whilst it remains difficult at present to measure toxins in tissue samples, various molecular techniques such as FISH or broad range real-time PCR have been suggested to detect micro-organisms at post-mortem [52, 53]. However, although such techniques may prove more sensitive than conventional culture techniques, interpretation of positive results may be as difficult as the interpretation of positive culture results. In this regard, additional methods of investigation must be developed, particularly those directed at detecting the systemic response to infection rather than mere identification of the organism. Recent studies have suggested biochemical assays of pro-inflammatory cytokine levels or immunohistochemical stains to detect markers of endothelial activation in sepsis, including E-selectin (CD 62E), very late activation antigen-4 (VLA-4, CD49d/CD29) and intercellular adhesion molecule-1 (ICAM-1, CD54) [35,54], but further research is required to evaluate their sensitivity and specificity in the determination of sepsis in SUDI post-mortem examinations.

2. Viral Infections

Previous studies have implicated viral infections in the pathogenesis of unexplained SUDI/SIDS, usually by acting as 'environmental stressors' as part of the triple risk hypothesis for SIDS (*vide supra*); alternatively, sudden death may occasionally be caused by an overwhelming, disseminated viral infection (explained SUDI) [1]. Whilst there are conflicting epidemiological data on the role of viral (upper respiratory tract) infections in the pathogenesis of SIDS, the temporal association between SIDS and viral epidemics [55], and the winter peaks of SIDS in many countries, at least in earlier years [56–58], suggest an association between viral infection and SIDS. Furthermore, a higher proportion of SIDS infants display symptoms of infection prior to death compared to age-matched controls [59, 60] and several (though not all) studies have shown a higher prevalence of virus isolation in SIDS cases (on

average 20-25%) compared to controls (around 8%) [61, 62]. Moreover, the presence of mild inflammatory changes in the (particularly upper) respiratory tract is a common histopathological feature in SIDS and considered additional evidence of a preceding viral infection [63], although it has been shown that the extent of respiratory tract and pulmonary inflammation is similar in SIDS and control infants that died of non-infective causes, suggesting that the microscopic inflammatory changes in themselves are not lethal [64]. However, it has been shown that the presence of a viral infection may further increase the risk of SIDS if combined with other epidemiological risk factors associated with SIDS (*vide supra*), such as prone sleeping, head covering or smoking [61]. Potential mechanisms whereby viral infections may cause or predispose to sudden death are likely to include either direct induction of a 'cytokine storm', similar to bacterial toxins outlined above, or, more likely, by indirect, synergistic means [65], for example, by increasing nasopharyngeal colonisation of toxigenic bacteria and/or promoting the induction of pyrogenic toxins (*vide supra*).

The current SUDI autopsy protocol [3] recommends that routine virological investigations are carried out, either using a nasopharyngeal aspirate obtained in A&E, or by collecting a postnasal swab or nasopharyngeal aspirate, lung sample, CSF or faeces at post-mortem "if indicated" and not already taken in A&E. However, these recommendations give little guidance as to what viruses should be routinely tested for, or about preferred virus detection methods that should be used in the post-mortem setting (other than making reference to "viral cultures, immunofluorescence and DNA amplification techniques"). A recent study of more than 500 SUDI demonstrated that, when predominantly using immunofluorescence (IF) assays on post-mortem lung tissue, virus is identified in only a small proportion of deaths (4% of all infants; 2% of unexplained SUDI) [66], suggesting that IF may be of limited diagnostic use in the post-mortem setting. PCR is considered the more sensitive technique for virus detection [67], whilst other viral detection techniques include cell culture, rapid culture techniques such as the DEAFF (detection of early antigen fluorescent foci) test for cytomegalovirus (CMV) and electron microscopy (EM), as well as immunohistochemistry and in-situ hybridisation techniques using microscopic tissue sections [68]. However, the optimal sites for post-mortem virological sampling, as well as the effect of a prolonged post-mortem interval, with possible resultant degradation of viral particles, remain currently undetermined. Furthermore, unlike most bacteriological investigations, virus isolation/identification is usually determined by targeted virological analyses that can only detect those viruses against which the analysis has been specifically designed for, e.g. IF or PCR against influenza A virus will not detect respiratory syncytial virus (RSV) or vice versa. This does not apply to all virological techniques, and exceptions include the use of EM or cell cultures for virus isolation, but even the latter require a degree of anticipatory judgement as the different cell lines (such as VERO, HEL or MDCK) are sensitive to only a limited range of different viruses. Thus, whilst most centres will test for a panel of different viruses, and may occasionally employ EM or cell cultures, there remains a high likelihood that unless a particular virus is specifically tested for, it will remain undetected. Which viruses to test for, however, remains

controversial as - although many (predominantly respiratory) viruses have been implicated in SIDS, including, influenza virus, rhinovirus, RAV, adenovirus and CMV - none have been consistently associated with SIDS [61].

Furthermore, the interpretation of a positive virological result in the absence of histological evidence of active infection remains difficult. Similar to the interpretation of a positive bacterial pathogenic isolate, the mere isolation of a virus does not necessarily indicate infection or even imply a contributory role leading to the infant's demise, as the viral isolate may 1) represent a contaminant (the contamination presumably having occurred during the sampling process); 2) represent 'normal/asymptomatic carriage' (e.g. herpes simplex virus (HSV), varicella-zoster virus and adenovirus); 3) represent an incidental infection with no bearing on the final cause of death; 4) indicate a contributory viral infection (e.g. a preceding viral infection that subsequently lead to an overwhelming secondary bacterial infection and death); or 5) indicate a fatal viral infection (e.g. disseminated HSV infection in a neonate). Most pathologists would agree that a significant viral isolate would be one that is associated with significant inflammatory changes, but even then the distinction between an 'incidental or contributory viral infection' and one that has directly led to the patient's demise can be a difficult one.

In summary, the current available data suggest that immunofluorescence using post-mortem lung samples rarely provides diagnostically useful information in order to establish a cause of death in SUDI. Further research is required to determine the most effective virus detection method(s) in the post-mortem setting, and to establish which virus(es) to routinely test for and which tissue or fluid sample(s) to take for virological analysis in SUDI.

3. Cytokine Responses and Genetic Polymorphisms

The inflammatory response forms an integral part of the host's immune defence to pathogens, the intensity of which is controlled by both pro-inflammatory cytokines and anti-inflammatory mediators [65]. As stated above, many of the toxins released by toxigenic bacteria can act as superantigens, inducing a 'cytokine storm' which, if not controlled, can cause severe tissue damage and death [69]. The balance between a normal and an exaggerated immune response may be further disrupted by polymorphisms involving various immunoregulatory genes that either up- or down-regulate circulating levels of cytokines, thereby increasing the infant's susceptibility to a 'cytokine storm' [65].

Interleukin-10 (IL-10) is an anti-inflammatory mediator, which acts by inhibiting the production of pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interferon- γ (INF- γ) and tumour necrosis factor- α (TNF- α); IL-10 also promotes antibody synthesis by B-lymphocytes [70]. Low levels of IL-10 may therefore predispose to SIDS by means of two possible mechanisms: one, by a reduced ability to inhibit the synthesis of pro-inflammatory cytokines; second, by a reduced ability to stimulate antibody production. Single nucleotide polymorphisms (SNPs) in base pairs -1082, -819 and -592 of the IL-10 gene - specifically the IL-10-1082*A -819*T -592*A haplotype (ATA haplotype for short) - have been shown to be associated with

low levels of IL-10 in *in-vitro* studies, although the opposite effect has been reported in *in-vivo* studies [71]. Nevertheless, these observations suggest that specific polymorphisms correlate with IL-10 cytokine production, and two studies have recently demonstrated that the ATA haplotype, specifically the -592*A allele, is significantly more prevalent in SIDS infants than in healthy control infants [70,72], suggesting that specific IL-10 gene polymorphisms increase the infant's susceptibility to SIDS. It is noteworthy that a Norwegian study did not demonstrate such an association, the ATA haplotype being significantly more prevalent in their cohort of explained SUDI due to infective causes but not in SIDS compared to healthy control infants [71], although it is possible that such differences are partly due to variations in the case definitions of SIDS vs explained SUDI used between the different studies, and/or due to regional ethnic differences, the patients from the Scandinavian study all being from the eastern part of Norway whilst the infants in the other two studies were British [72]. Even so, the Scandinavian study provides further evidence that an unfavourable IL-10 genotype may be associated with an imbalanced immune response, resulting in the infant's inability to deal with the infection. Moreover, a recent study showed that the IL-10 response to endotoxin was significantly lower from leucocytes of smokers who were homozygous and heterozygous for the IL-10-1082*A allele compared to non-smokers, suggesting a possible synergistic effect between specific IL-10 genotypes and other epidemiological risk factor for SIDS [73].

Various SNPs of pro-inflammatory cytokines have also been described in SIDS infants, which are associated with an exaggerated response to bacterial toxins [65]. These include a possible increased IL-1 β response for smokers who are homozygous for the IL-1 β -511*T SNP [74], as well as an association between the vascular endothelial growth factor VEGF-1154*G allele and SIDS, the latter a potent pro-inflammatory cytokine that acts as leucocyte chemoattractant, induces adhesion expression and regulates chemokine gene expression [75]. Cerebrospinal fluid IL-6 concentrations have been shown to be significantly higher in SIDS infants than in traumatic deaths [76] and two studies have demonstrated associations between the IL-6-174*G SNP (specifically the IL-6-174GG genotype) and SIDS [75,77], though in the latter study, this pertained only to the Australian SIDS group (n=19) and not the combined SIDS groups from Germany, Hungary and Australia, and in a recent Norwegian study of 175 SIDS and 71 living controls from eastern Norway, no such association was demonstrated [78].

In summary, there is increasing evidence that polymorphisms of immunoregulatory genes may increase an infant's susceptibility to SIDS, possibly *via* an infectious pathway as outlined above, but further research is required to ascertain the role of specific SNPs in the pathogenesis of SIDS. The differences in distribution of specific polymorphisms between different ethnic groups may also, at least in part, explain some of the observed ethnic differences in susceptibility to SIDS [69].

4. Impaired Autonomic Regulation and Arousal Responses

There is a large body of evidence suggesting that the final common pathway in SIDS involves both impaired

cardiorespiratory autonomic control and a failure of arousal from sleep [27]. Arousal from sleep is important in survival in life-threatening situations such as prolonged apnoea; arousal may occur spontaneously in response to internal stimuli or be initiated by external environmental triggers, and requires both behavioural and autonomic input, the latter involving the hypothalamus and brainstem (*vide infra*). A number of studies have identified impaired spontaneous arousal from sleep, as well as immature sleep patterns, in SIDS infants, and there is evidence that risk factors for SIDS such as prone sleeping and maternal cigarette smoking further reduce infants' arousability and alter autonomic control [27]. In contrast, it has been shown that apnoea, initially regarded as an antecedent to SIDS, neither precedes nor predicts SIDS, and that apnoea monitors do not reduce the incidence of SIDS [27].

Much SIDS research over the last decade has focused on pathological changes in the brain, particularly affecting the brainstem, which plays a critical role in cardiorespiratory autonomic regulation, sleep and arousal [79]. There is now consistent evidence of abnormalities involving serotonergic (serotonin, 5-hydroxytryptamine or 5-HT) pathways in the medulla oblongata, which are believed to facilitate and maintain homeostasis [79]. Abnormalities include reduced 5-HT receptor binding sites, increased 5-HT neuronal counts and density, and a reduced 5-HT transporter binding density [80,81]. Recent immunohistochemical studies have confirmed reduced 5-HT receptor expression in SIDS [82,83], which, in the latter study, correlates with smoking and prone sleeping in at least some brainstem nuclei. Furthermore, a recent study showed that IL-6 receptor expression is increased in 5-HT neurons in the arcuate nucleus, the latter being the presumed site for central carbon dioxide monitoring, suggesting that abnormal interactions between IL-6 and the arcuate nucleus may contribute to impaired arousal in the presence of infection [84].

Other reported neuropathological abnormalities in SIDS include evidence of subtle gliosis and other hypoxia-ischaemia related changes in the brainstem and other susceptible regions of the brain [79], although subtle differences in the distribution of affected areas were demonstrated between SIDS and those infants known to have been exposed to a hypoxic-ischaemic insult [85]. Also described are hypoplasia of the arcuate nucleus [79,86,87], persistence of the external granular layer of the cerebellar cortex [88], and excessive leptomenigeal neurons, predominantly in the brainstem [89]. Notably, hypoplasia of the arcuate nucleus was found to be more frequent in stillborn and SIDS infants in mother who smoked during pregnancy [90].

Whilst the data suggest that in a proportion of unexplained SUDI/SIDS these morphological and functional differences may contribute to the pathogenesis of sudden death, their demonstration at autopsy is technically difficult and often not possible given current time and financial constraints in the UK, and medicolegal limitations imposed on the pathologist following the implementation of the Human Tissue Act. Furthermore, some of the described changes (e.g. arcuate nucleus hypoplasia) have not yet been independently verified, and the diagnostic criteria for their detection remain controversial; moreover, their presence

does not offer an explanation of death; and thus, until further research becomes available, these changes should at best be regarded as risk factors for the development of SIDS and not as causes of death.

5. Cardiac Causes: Conduction System Abnormalities

Long QT syndrome (LQTS) refers to a family of cardiac ion channelopathies, heterogeneous heritable disorders characterised by a prolonged QT interval, ventricular arrhythmias and high risk of sudden death; approximately 75% of LQTS are caused by mutations involving three genes, viz. a loss-of-function mutation of the *KCNQ1* and *KCNH2* genes coding for potassium channel subunits, and a gain-of-function mutation of the *SCN5A* gene coding for a voltage-gated sodium channel [91,92]. In addition to the pathogenic mutations, a range of LQTS gene polymorphisms have also been shown to affect the QT interval [91]. Of patients with LQTS, 14% die during their first episode of arrhythmia and about a third of these deaths has been shown to occur in the first year of life [93].

A large prospective study of >30,000 infants showed a positive association between prolonged QT interval, demonstrated on ECG during the first few days of life, and subsequent development of SIDS: infants who died of SIDS had a longer corrected QT interval than survivors or infants who died from other causes (non-SIDS deaths) [94]. In the study, 12 of the 24 (50%) SIDS infants – but none of the other infants – had a prolonged corrected QT interval, defined as >440 milliseconds. A subsequent study of ECG recordings of infants during sleep also showed longer QT intervals in SIDS compared to age-matched controls [95], and in a recent French cohort of 52 SUDI (comprising 32 SIDS, 18 explained SUDI and two ‘suspected SIDS’ but in whom an autopsy was not performed), LQTS mutations were found in three (9%) of the SIDS infants but in none of the 18 explained SUDI [91]. A Japanese study found LQTS mutations in five (12%) of 42 SIDS [96].

Other channelopathies implicated in SIDS include shortening of the QT interval (short QT syndrome, SQTS) and Brugada syndrome, both of which are also associated with a risk of cardiac arrhythmias and sudden death [92,97]. For SQTS, gain-of-function mutations have been identified in the potassium channel-encoding genes *KCNQ1*, *KCNH2* and *KCNJ2*, whilst around 20% of cases with the Brugada syndrome are associated with a loss-of-function mutation involving the *SCN5A* gene, although mutations involving glycerol-3-phosphate dehydrogenase 1-like protein (encoded by *GPD1L*), and mutations involving the L-type calcium channel alpha and beta subunits (encoded by *CACNA1C* and *CACNB2b* genes, respectively) have also been described [92]. Importantly, although subtle structural abnormalities of the cardiac conduction system have been described at post-mortem of SIDS infants [98], the functional significance of these, if any, is uncertain, and in the majority of deaths secondary to cardiac channelopathies the heart will not demonstrate any significant morphological changes discernable on light microscopy.

In summary, the currently available evidence suggests that, in a proportion of SIDS infants, cardiac ion channelopathies may cause or contribute to death; obviously, the mere presence of an ion channelopathy-associated gene

mutation does not in itself prove that death was due to a fatal cardiac arrhythmia, but in the absence of any other pathological findings it seems a justified conclusion, although other potential mechanisms may include synergistic effects between QT interval-associated gene polymorphisms and other factors related to autonomic dysfunction reportedly present in many SIDS infants (*vide supra*). Importantly, the demonstration of LQTS or other cardiac channelopathy has implications for the surviving family members who would require ECG and/or genetic screening [99]; unfortunately, routine post-mortem diagnostic testing for the presence of such mutations is not currently available in the UK, and further research is required.

6. Metabolic Disorders

Mitochondrial fatty acid oxidation disorders (FAODs) represent a group of rare inherited metabolic disorders which affect fatty acid transport and beta-oxidation; more than 20 defects have been described to date, and the clinical presentation varies from neonatal onset with metabolic acidosis, hypoketotic hypoglycaemia, cardiomyopathy, liver failure and sudden unexpected death, to late onset with myopathy, neuropathy and retinopathy [100]. FAODs have previously been implicated as a cause of death in around 5–8% of sudden infant deaths, based on metabolic studies of surviving siblings, so-called ‘near-miss’ SIDS cases and autopsy studies [100]. However, there were no reported FAOD-related deaths in a recent series of more than 500 SUDI infants aged one week to one year; combining the data from that with previously published studies, there were a total of nine deaths due to a metabolic disorder from a total of 2,054 infants presenting as SUDI, comprising 0.3% (95% CI 0.1% to 0.6%) of all SUDI deaths [1]. In two earlier studies of 67 SIDS (mean age 3.2 months) and 1224 SIDS (aged one week to one year), respectively, none of the infants were found to be homozygous for the A985G mutation in the medium-chain acyl-CoA dehydrogenase (MCAD) gene, which accounts for around 90% of all MCAD deficiencies, the commonest of the reported FAODs, with the G-985 heterozygote prevalence being similar to that of the general population, further suggesting that FAODs are an uncommon cause of death in SUDI [101,102].

In contrast, in a recent post-mortem study of 55 sudden unexpected early neonatal deaths (SUEND), representing as sudden and clinically unexpected deaths analogous to SUDI of infants during the first week of life (i.e. aged 0 - 7 days), there were three (6%) deaths from unsuspected metabolic disease, including two MCAD deficiencies and one case of carnitine-acylcarnitine translocase deficiency, suggesting that FAODs are more likely to present with sudden death in the first week of life [103]. These data are consistent with those from a previous series of more than 100 patients with metabolic disorders, including 97 paediatric deaths, in which one third died during the first week of life, including some that presented as SUEND [104]. Furthermore, in another series of 25 patients with FAODs, 19 infants presented as SUEND, these deaths being associated with exclusive breast feeding, which appears to be a risk factor for early death in these patients [105].

In deaths due to FAODs, it is expected that there would be abnormal fat accumulation in hepatocytes, renal tubules,

and cardiac and skeletal myocytes, and hence the Kennedy autopsy protocol [3] recommends oil-red-O-staining of frozen sections of liver, kidney, heart and skeletal muscle in all sudden unexpected deaths, even though the sensitivity and specificity of frozen section as a screening test for FAODs currently remains undetermined. Other recommended investigations include tandem mass spectrometric analysis of acylcarnitine profiles using post-mortem blood and bile spots collected on Guthrie cards if a metabolic disease is suspected clinically or if fat stains on frozen section are positive, as well as confirmatory enzyme assays using cultured fibroblasts derived from skin. DNA analysis for common disease-causing mutations is also possible.

In summary, FAODs appear to be more prevalent in SUEND deaths than in SUDI deaths, accounting for around 5-10% of the former. To date, a range of different FAODs have been reported in SUEND/SUDI, including MCAD deficiency, very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, glutaric acidaemia type 2, carnitine-acylcarnitine translocase deficiency, mitochondrial trifunctional protein deficiency, and carnitine uptake defect [103,106-108]. However, with the sensitivity and specificity of both frozen section, and post-mortem blood and/or bile acylcarnitine profiling, being undetermined for the detection of FAODs at autopsy, and novel FAOD-related mutations being identified, it is possible that some deaths due to FAODs are presently missed by following current investigative autopsy protocols, and further research is required.

CURRENT AND FUTURE APPROACHES TO THE POST-MORTEM INVESTIGATION OF SUDI

The currently recommended investigative protocol for SUDI in the UK was drawn up in 2004 by a joint working party of the Royal College of Pathologists and Royal College of Paediatrics and Child Health; the group was chaired by Baroness Helena Kennedy and hence the report is usually referred to as the 'Kennedy guidelines' [3]. In essence, the Kennedy guidelines recommend a multidisciplinary approach to the investigation of SUDI, which includes active participation by paediatricians, paediatric (and forensic) pathologists, the coroner, police and social services, and other health care providers. The report recommends that the medical investigation is lead by a designated 'SUDI paediatrician' who would also participate in home visits with the police, and that a local case discussion meeting be carried out within two-to-three months after the death; since 1 April 2008, the latter has been superseded by the local Child Death Overview Panel of the Local Safeguarding Children Boards, in accordance with the Children Act 2004 in England and Wales.

Guidelines specific to the post-mortem examination include recommendations that SUDI autopsies are carried out by specialist paediatric pathologists in tertiary centres with expertise in this area. If there is a criminal investigation (e.g. a suspected homicide), the autopsy is to be carried out by a forensic pathologist with appropriate expertise in paediatric pathology, or jointly by both forensic and paediatric pathologists. The post-mortem examination itself comprises a detailed macroscopic examination and minimum

set of tissue samples for histological examination, in addition to a range of ancillary investigations, including post-mortem radiological skeletal survey and post-mortem sampling for bacteriological, virological, metabolic and toxicological investigations. Nevertheless, it should be recognised that this protocol remains largely non-evidence based, being predominantly informed by expert opinion and perceived best practice. For example, the number and sites of bacteriological and virological samples, the sensitivity of frozen section and post-mortem acylcarnitine profiling as a screening test for metabolic disorders, the usefulness of routine toxicological investigations, and importantly, the interpretation of positive or 'borderline' findings, remain principally undetermined. Furthermore, the potential role of future applications of molecular investigations in a post-mortem setting, such as routine screening for specific mutations (e.g. cardiac channelopathies, FAODs), genome-wide screening for SIDS-predisposing SNPs, as well as immunohistochemical assessment and tissue-specific gene expression studies for cytokine activation and other markers of the inflammatory response to infection, or the detection of bacterial superantigens in tissues, have yet to be addressed.

In conclusion, at present, despite using the recommended UK autopsy guidelines [3], which are similar to those advocated in the internationally recommended post-mortem protocol for the investigation of SIDS [4], almost two thirds of SUDI deaths remain unexplained, clearly highlighting the need for identifying alternative and/or additional diagnostic techniques to further improve the detection rate of identifiable causes of death at autopsy [1]. With a paucity of published evidence on the utility of such molecular investigations at post-mortem, there is clearly an urgent need for further focused research to assess the application of such novel post-mortem techniques in this context.

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