An investigation into the use of a commonly available fabric dye as a routine stain for tissue samples to be used as a first line, low cost, diagnostic adjunct for the diagnosis of anaphylactic death at autopsy, in a resource-challenged environment.

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By

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ABSTRACT

Introduction

A retrospective study of deaths attributable to anaphylaxis at the Salt River Forensic Pathology Laboratory was undertaken, with a view to determine if eosinophilia was present in tissue samples of the spleen, in accordance with previously published research. Suitable cases of non-anaphylactic death were used as controls. Use was made of two commonly available fabric dyes as alternative stains to the traditional Haematoxylin –Eosin ["H&E"].

Methods

Use was made of two common fabric dyes, as stains to visualise eosinophils, one local, cheap and readily available, sold as Magenta™, the other sold as Pagoda Red ™ and manufactured by DYLON ™, also relatively cheap but locally unavailable. H&E stain was used as a control.

Simultaneously the study sought to determine if post-mortem mast cell tryptase levels were a reliable diagnostic indicator of anaphylactic death.

5µm sections of splenic tissue from samples and controls were stained using each of the three stains, and eosinophils were counted in 10 randomly selected 40x fields. Cell counts were performed by an experienced analyst and an inexperienced analyst. Cell counts were statistically analysed using the Wilcoxon rank-sum method to determine statistically significant differences between cell counts. Spearman correlation co-efficients were used to determine if a significant correlation existed between cell counts and mast cell tryptase levels.

Results

Statistical analysis of the results demonstrated the following:

- There was no statistically significant difference in eosinophil counts between anaphylactic death cases and control cases at the $p < 0.05$ level, thus negating previous research which postulated the spleen as a shock organ accumulating eosinophils in cases of anaphylaxis.

- Secondly, there was no statistically significant difference in eosinophil counts between the various stains used, but subjectively the use of Pagoda Red dye rendered eosinophils more easily visible, and resulted in a reduced amount of time being spent in counting them. This is of significance in a resource challenged environment with a heavy forensic autopsy load.

- Lastly, there was no significant difference [$p<0.05$] between the mast cell tryptase levels of test samples [anaphylactic deaths] and control samples.

Discussion

A retrospective study like this one has inherent limitations, such as dependency on previous data collection, laboratory test results being available and appropriate histology samples being obtainable. Our test and control sample cohorts were small given the relatively few cases which fitted inclusion criteria: i.e. full hard copy documentation, mast cell tryptase level and spleen section.

Nonetheless, using the limited population at our disposal we can show that the use

of an alternative stain to H&E reduces the time spent in cell counts, and allows rapid differentiation between eosinophils and mast cells, as a result of enhanced visualisation. Time saved is always welcome in busy work schedules. Money saved through the use of a cheaper stain is an obvious benefit

Further research is necessary into the use of other, possibly more appropriate, dyes as the dyes selected would appear to be composite in nature. The identification of the major component in each dye may thus identify a dye that will prove to be a cheap, locally available alternative.

The lack of correlation between mast cell tryptase levels and eosinophil counts warrants further extensive research, using a larger, multi-centre sample population.

Therefore, although this research project innocuously set out to verify the usefulness of fabric dyes to easily and readily visualize eosinophils in splenic tissue, the analysis of the results has shown it to be a veritable hornet's nest of controversy in the area of post-mortem diagnosis of anaphylaxis, and in the involvement of several different organs. Further extensive research is deemed necessary to address these several areas of confounding results.

Explanatory Notes.

In order not to burden this document with a papery sump of repetitive references, the references have been divided into two sections. Those utilized in the Research Proposal and Literature Survey is reflected in the Appendices.

In respect of the Journal article for publication, the references are found immediately after the conclusions, as for publication it is expected that the relevant references

appear here. In addition the convention specified by the journal is numeric in nature hence the UCT-Harvard method is not used here.

It being impossible to include the high resolution photomicrographs in the body of this document, a complete set of photomicrographs is available, on request, on CD-ROM.

Similarly, should copies of the actual spreadsheets be required these are also available on CD-ROM.

SECTION A: RESEARCH PROPOSAL

Title: An investigation into the use of a commonly available fabric dye as a routine stain for tissue samples to be used as a first line, low cost, diagnostic adjunct for the diagnosis of anaphylactic death at autopsy, in a resource-challenged environment.

Principal investigators: P.J. Burgers; Dr. L Liebenberg [supervisor]; Department of Clinical Laboratory Sciences; Division of Forensic Medicine , University of Cape Town Health Sciences Faculty

Co-investigators: Ms. M. Perrins; Chief Medical Technologist; Ms. Y. Davis; Senior Medical Technologist; Department of Clinical Laboratory Sciences; Division of Forensic Medicine, University of Cape Town Health Sciences Faculty

Introduction

Anaphylaxis is widely recognised as the most dangerous response the body offers to challenge by a variety of allergens, and in doses that often bear no relation to the severity and speed of onset of the reaction (Scarlet, 2006).

It is characterised as a systemic reaction with an acute response varying in time from seconds to minutes after challenge.

Generally it is a reaction that can involve a multiplicity of body systems, is very often unpredictable, characterised by rapid onset, and can have fatal, alternatively life–threatening consequences in serious cases.

If the reaction is as a result of an antigen/antibody response and commonly involving Immunoglobulin E [IgE] with a subsequent widespread activation of mast cells and

the release of various cytokines, the reaction is termed an anaphylactic reaction.

If, however, the reaction follows in consequence of a non-antibody trigger [and is thus non-IgE mediated], the reaction is termed anaphylactoid, and is generally and clinically indistinguishable from anaphylaxis.(Walther & Bottiger, 2004; Ring, Behrendt & de Weck, 2010)

Despite this difference the management and treatment of both reactions is the same.

Often the response is fatal and deaths due to anaphylaxis may be due to acute respiratory distress, consequent on glottal œdema, bronchial obstruction or bronchospasm; alternatively due to cardiovascular collapse.

Owing to the non-specificity of symptoms, the determination of anaphylaxis [alternatively an anaphylactoid reaction] as the cause of death at autopsy is one fraught with problems. Determination of the cause of death may well revolve on a differential diagnosis, or an exclusion of other causes.

As a result, in cases of suspected anaphylactic /anaphylactoid death scrupulous attention to detail at autopsy is necessary; a full history is virtually obligatory, coupled with a complete autopsy. Histological and immunohistochemical testing can significantly aid the diagnosis, as can biochemical testing for mast cell tryptase ["MCT"], total IgE level and sometimes specific antigen IgE level.

Routine histology may be unhelpful, and immunohistochemical and biochemical testing is expensive. Autopsy findings alone are thus often non-specific.

Laboratory testing in the form of mast cell specific immunohistochemical testing as well as the determination of mast cell tryptase levels, and routine biochemical testing are useful as diagnostic adjuncts, however conflicting results have been obtained [Schwarz 2006].

Furthermore, in the case of mast cell tryptase, serial testing is required, with a baseline level determined, during 1st hour of presentation and then 24 hours later. Such baseline or ante-mortem level is seldom available in autopsy cases.

Cost of testing is a significant factor. Written quotes obtained in 2015 for biochemical testing were determined as follows: Multiple specific Antigen IgE ZAR 279.92; Combined Antigen Specific IgE ZAR 183.76 [written quote, National Health Laboratory Services, price list 2015:],

What seems to be required is a simple, low-cost, first line diagnostic tool which will support or negate an initial diagnosis of anaphylactic/anaphylactoid death, which may then be followed up by more sophisticated tests [such as immunohistochemical tests] if deemed necessary.

Such a diagnostic aid would have to be simple in nature, easily performed and use readily available materials, particularly in an environment that is resource-challenged. A study of the literature has shown that in certain instances a commonly available fabric dye, manufactured by the company DYLON and sold as Pagoda Red ™ can be successfully used to demonstrate eosinophilia and mast cell degranulation in stained histological slides of splenic tissue. This published research supports a theory that mast cell degranulation and hypereosinophilia particularly in splenic tissue and possibly in lung and liver tissue is indicative, and possibly pathognomonic, of anaphylactic/anaphylactoid death. (Trani et al., 2008; Reggiani Bonetti et al., 2014)

Although the dye does not differentiate between mast cells and eosinophils, the presence of mast cells, increased numbers of eosinophils and mast cell granules in the spleen would thus appear to be a valuable aid in supporting a diagnosis of

anaphylactic death.

Preliminary investigations have shown that the particular shade of dye, Pagoda Red used in the overseas studies mentioned below is not readily available in South Africa. Reference to previously published research indicated that perhaps other dyes would be equally successful (Kiyono et al., 1982; Yanagihara, Mehregan & Mehregan, 1984). Hence this study will attempt to determine if the use of a dye colour [Lady Dye "Magenta" ™] commonly available at retail pharmacies in the Cape Town area] will consistently reproduce the results shown by overseas researchers (Trani et al., 2008; Edston, 2013; Reggiani Bonetti et al., 2014).

In examining this problem the original dyestuff, Pagoda Red will be used to prepare samples for comparative study. As the study seeks to replicate results obtained and published (Trani et al., 2008; Edston, 2013), it was not deemed necessary to incur the expense of immunohistochemical staining as confirmation of the histological results. Nonetheless, attempts were made to determine if the appropriate and specific immunohistochemical staining could be performed locally. This proved not to be the case, as the closest appropriate stain was CD 117 at a cost of ZAR 900.00 per slide as against a cost of ZAR75.00 for the dye-stained slides [written quote, National Health Laboratory Services, 26 August 2014].

Methods

This will be a retrospective study of tissue samples retained at autopsy in confirmed anaphylactic deaths at Salt River Forensic Pathology Laboratory. A retrospective study has inherent limitations. The very fact that it is retrospective implies that fresh sampling cannot be performed, additional samples are unavailable, the autopsy

cannot be “redone”, and most significantly the methodology and reasoning of the pathologists involved may not be open to discussion they having left the employ of the Department.

Selection criteria for both test and control cases will be:

(a) MCT level as obtained from a spreadsheet record of tests performed for forensic pathology by the Lung Institute of UCT. Forensic Pathology Case numbers and mast cell tryptase level are recorded. This is the start off point of case selection.

(b) For cases where MCT results are available, full autopsy documentation including police information and medical records where available will be retrieved from the UCT Division of Forensic Medicine’s archive.

(c) From documentation it will be established whether tissue samples were retained for histological examination

(d) Full histological slide collections will be examined and it will be determined whether spleen section was done. A splenic sample for histological sectioning and staining, according to published protocol is necessary

(e) A diagnosis of anaphylactic versus non anaphylactic death cases will be identified by a senior pathologist by studying the full case records from the archive and special investigations including the full histological slide collection.

In amplification of the above, the diagnosis of anaphylaxis for the study samples should be in strict accordance with the protocols of Forensic Pathology Services. That is a full autopsy, tissue samples from all internal organs as per the standard operating protocol, full histological examination, coupled with a review of contemporaneous notes and the exclusion of other causes of death, followed by review by a senior pathologist. The presence of hospital records is regarded as a useful adjunct, but is often not available. Succinctly stated it should be a reasoned well researched differential diagnostic process to either include or exclude anaphylactic death.

In the case of the control samples, should sufficient samples be found that satisfy the initial criteria of Mast Cell Tryptase level, and spleen tissue samples, the records will then be examined in accordance with the protocol above to determine if anaphylaxis has been positively excluded.

The inclusion of an available MCT levels as a criterion is based on the literature which in earlier times supported a diagnosis of anaphylactic death, but not as a “gold standard. Randall Butts & Halsey (Randall, Butts & Halsey, 1995) showed that an appropriate clinical history together with an elevated MCT level and an allergen specific IgE level would be strong evidence of an anaphylactic death. It must however be noted that elevated tryptase levels may also occur in traumatic deaths, in particular those involving chest trauma, possibly as a result of mast cell lysis followed by degranulation

In the case of the control samples, the diagnosis of death must exclude anaphylaxis

and similarly, be confirmed by a senior forensic pathologist.

In order to validate the results negative controls comprising splenic tissue sections from confirmed non-anaphylactic deaths of the same age range and gender will be used. The tissue samples used in the study, as well as negative controls will be sourced from the registered Forensic Pathology Tissue data bank. The diagnosis for the control samples will be according to the protocol set out above; that is strict compliance with the standard protocols of Forensic Pathology Services, but with the difference that the diagnosis must positively exclude anaphylaxis as a cause of death

Samples of spleen tissue will be sectioned and stained according to the method laid out by Trani et al. [2008] using firstly, imported Pagoda Red dye as the stain, in accordance with the method set out below, thereafter using Magenta dye, and H&E as a control stain.

Briefly the paraffin tissue sections are floated in water, stained at 55 °Celsius for one hour in Pagoda Red stain comprising a 1 % aqueous solution of the dyestuff. Thereafter they are washed, counterstained with hæmatoxylin for 3 minutes, washed in tap water, dehydrated in alcohol, cleared in Xylene and then mounted for examination.

The same process will then be repeated using the Lady Dye “Magenta “ as the stain; both samples will then be examined microscopically to determine if eosinophils, mast cells and mast cell granules are readily visible as reported by Trani et al [2008].

It is theorised that the use of the locally available stain, Magenta while being capable of staining mast cells, eosinophils and granules, will demonstrate a significant difference in colour/hue of the stained eosinophils when compared to stained slides

using H&E and Pagoda Red, as the local dye Magenta exhibits a markedly different pH characteristic when compared to the imported dyestuff Pagoda Red, and possibly a different structure.

Study population and sampling

The study population comprises all traceable cases of death due to anaphylaxis at Salt River Mortuary over the period 2002 to date. At this point it is unknown exactly how many cases are on record. A search of the Department's records together with archived "hard copy" documents will yield the numbers and diagnoses, thereby providing both samples and negative controls.

Sampling.

Sampling will be of spleen tissue retained at autopsy, and preserved in formalin or alternatively in paraffin wax embedded cassettes for histological examination as an aid to determining the cause of death.

It is hoped that a sufficient number of cases will be available for a statistically valid sample to be obtained.

Measurements

The measurement criterion is that of Trani et al (2008): …"a diffuse perifollicular, cordonal and endosinusal splenic eosinophilia" determined by microscopy, coupled with Edston's [2013] numeric criterion of more than 10 eosinophils per 40x field. .

Eosinophils, mast cells and granules should, in accordance with Trani et al. and Edston's reports [*op.cit.*] be readily visible as bright red on a pale blue background. We suspect, as stated above that the use of the local dye will give a similar, but not exactly the same, result: i.e. the cells will still be stained but their relative contrast

against the blue background and the intensity of the colour produced may well be different.

Pilot studies

New sections of splenic tissue previously stained with H&E stain will be stained according to the protocol of Trani et al. [2008] with Lady Dye “Magenta”, and with Pagoda Red respectively. All three slides will [i.e. the H&E, Magenta & Pagoda Red stained slides] then be examined and compared to determine if the study is feasible or not.

Resources

Available resources

A sufficient quantity of “Magenta” dye has already been procured.

A sufficient quantity [30 g equating to 3 litres of made-up stain] of Pagoda Red dye has been purchased in Canada and imported.

In addition thereto, 30 g of recently manufactured Pagoda Red was donated by the manufacturer Spotless Punch plc., to be used in the study.

The Division of Forensic Medicine and Toxicology of UCT has the necessary infra-structure to process tissue sections and stain the selected sections.

Budget

This study is envisaged as costing, in total, no more than ZAR6000.00 made up as follows:

Lady Dye Magenta dye	30g	R 100.00
Dylon Pagoda Red dye	30g	R500.00
Cost of slide preparation 50 slides at R100.00 per slide		R5000.00
TOTAL		**R5600.00**

Ethical and legal considerations

The envisaged study is a retrospective study using archived autopsy documents, historic mast cell tryptase results and tissue samples retained at autopsy in terms of Section 3(2) read with Section 3(3) of the Inquests Act No. 58 of 1959 of South Africa as amended, for the determination of cause of death.

Anonymity of the persons from whom the tissue was retained is guaranteed as the researcher will use only the Forensic Pathology Service reference number allocated to the body at the time of autopsy [e.g. WC/11/1234/xxxx], where xxxx indicates the relevant year in which the autopsy was performed. In cases selected prior to September 2006 the reference number follows the format DR xxxx/ YYYY, where xxxx represents the sequential number allocated, and YYYY represents the year in which the autopsy was performed.

The outcome of the research is independent of, and will not in any way influence any past, current or future legal proceedings of any " open inquest", should the samples later prove to be from an "open inquest " case.

No other ethical or legal problems were identified.

Reporting of results

As the determination of success in using the alternative dye rests on visual comparisons with the Pagoda Red dye, it is envisaged that the results will be recorded and interpreted using photomicrographs of the tissue sections.

SECTION B: LITERATURE SURVEY

Title: An investigation into the use of a commonly available fabric dye as a routine stain for tissue samples to be used as a first line, low cost, diagnostic adjunct for the diagnosis of anaphylactic death at autopsy, in a resource-challenged environment.

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LITERATURE SURVEY

Introduction

Anaphylaxis has been defined as the most dramatic and potentially catastrophic manifestation of immediate hypersensitivity. The term "Anaphylaxis" is derived from the Greek [ανά - against; φύλαξις - protection] (Bochner & Lichtenstein, 1991). In addition thereto the diagnosis of this oft deadly disease state presents unique and

challenging problems, ascribable not only to the multiplicity of symptoms it can present with, but also to the paucity of diagnostic tools that are disease specific. To paraphrase Knight, the “feeble armamentarium” (Knight, 1996) of diagnostic aids.

It manifests as a plethora of symptoms and reactions that can range widely in their severity from mild to deadly.

The term anaphylaxis was coined in 1902 by Portier and Richet, as a descriptive term for a particular immunisation protocol they used which exhibited a paradoxical effect.

As a result of their work it was determined that anaphylaxis was an acquired sensitivity, manifesting after exposure and re-challenge with an intervening period of several weeks.

Later work, discussed in more detail hereunder, determined the role of antigen specific Immunoglobulin E [“IgE”] with the release of preformed mediators such as histamine from both mast cells and basophils.

A second type of reaction dubbed “anaphylactoid” has arisen and is characterised by a clinically indistinguishable symptomatology, but lacking the IgE antibody mediation, and not necessarily requiring previous exposure to the trigger agent.

In this regard published research seems to implicate IgG1, IgG2 antibodies and their recognition by FcɣRIII and FcɣRIV receptors on neutrophils (Lowell, 2011).

Common practice however labels both these reactions as “anaphylaxis” despite the marked dissimilarities in aetiology.

Generally, however, it would appear that mast cells and basophils are the primary functionaries in the development of an anaphylactic reaction.

The Role of Mast cells *per se*

The role of mast cells in inflammatory and allergic reactions is well documented.

Anaphylaxis has been reported as arising from “generalised mast cell degranulation caused by immunological reaction” (Osawa et al., 2008).

Mast cells are defined as heavily granulated wandering cells found in connective tissue and are abundant beneath epithelial surfaces (Payne & Kam, 2004). In similar fashion to eosinophils they stain an intense red with eosin, and are often seen to have degranulated in tissue sections.

Mast cell granules contain a number of proteases, including tryptase together with heparin and histamine. Mast cell tryptase [“MCT“] is categorised as a tetrameric neutral serine protease comprising four non-covalently bonded subunits. Two main types exist – α and β tryptases.

It has been reported that in severe systemic anaphylaxis serum β-tryptase levels are elevated, with levels peaking over a range of fifteen to twenty minutes with a half-life of some one and a half to two hours [Payne &Kam 2004], which permits testing of

samples obtained over a window period of about one to six hours after the onset of the reaction.

It has been proven that mast cell tryptase levels are relatively stable in the post-mortem setting, but can exhibit a post-mortem increase, (Horn, Halsey & Zumwalt, 2004); (Edston & van Hage-Hamsten, 1998); (Edston, Eriksson & van Hage, 2007)therefore, this permits post-mortem sampling of blood for the determination of post-mortem serum tryptase levels, as an indicator [albeit not a determinative indicator] of an anaphylactic death. High levels of tryptase have also been obtained three days after a suspected anaphylactic death (Fisher & Baldo, 1998).

Yet, despite its seemingly prominent role as an indicator of anaphylactic reaction, high levels of serum tryptase obtained post-mortem may not provide sufficient proof of an anaphylactic death, absent other diagnostic criteria.

This result is borne out by the published research, and at best, although MCT can be utilised as an indicator of a possible anaphylactic event, caution needs to be exercised as its presence or absence often serves merely as a confounder. This phenomenon has been eloquently addressed by Edston et al.(Edston, Eriksson & van Hage, 2007)who also found that mast cell tryptase levels can be affected by sampling site. They also found that position, resuscitation efforts and the presence or absence of conjunctival or facial petechiae have no relevance to MCT levels. However femoral blood was the preferred sample, as it is less prone to show false results.

Interestingly, Kounis in a long series of communications has shown that cardiac involvement is also prevalent in anaphylactic/anaphylactoid/hypersensitivity

reactions, and, not surprisingly, elevated levels of mast cell tryptase in cardiac tissue and increased numbers of mast cells have been demonstrated - as have increased numbers of basophils and eosinophils. (Kounis et al., 2011a; Kounis et al., 2011b; Kounis et al., 2012; Kounis, 2013; Kounis, Soufras & Hahalis, 2014). This led to his sobriquet of anaphylactic cardiac shock [2013 *op. cit.*]

Prior to this research, Randall Butts & Halsey (Randall, Butts & Halsey, 1995) examined a relatively large [27 and 22 subjects] population of post-mortem tryptase levels determined routinely at autopsy in an effort to assess the diagnostic efficacy of post-mortem tryptase levels. The sample cases selected by them represented two consecutive series of autopsies, where anaphylaxis was not suspected as a cause of death.

Whilst none of the subjects died as a result of anaphylaxis some showed an elevated level of mast-cell tryptase but only one subject showed a level that was significantly higher [106 µg/L] than the majority of the other observed levels. It must be noted that their selected threshold level was 1ng/ml. They postulated that although an elevated post mortem level of mast cell tryptase may be indicative of anaphylaxis, utilisation of a threshold value in excess of 10 ng/ml [thus10 µg/L] may provide more specificity. They concluded that an appropriate clinical history coupled with a finding of an allergen specific IgE and an elevated tryptase level is strong evidence pointing toward an anaphylactic dearth – but it is not conclusive. It is to be noted that this level of mast cell tryptase [10 ng/ml] is markedly lower than the level proposed by Mayer et al. [2011, *op.cit.*] of 45µg/L as indicative of an anaphylactic episode. Mayer

et al. accepted that levels in excess of 11.4 µg/L are within the normal range for living subjects.

Returning to the research of Randall et al. they [Randall, Butts & Halsey] thus concluded that firstly, a tryptase assay has high level of specificity for anaphylaxis provided that the specimen is obtained whilst the patient is still alive, secondly that false positives are a rare occurrence. Nonetheless, the *caveat* sounded above should be borne in mind at all times – MCT is as often as not a confounder and confuser in the post-mortem diagnosis of anaphylactic death; particularly as it can be elevated in traumatic deaths, more especially chest trauma, possibly as a result of the traumatic lysis of mast cells followed by degranulation .

This research, although valuable, does not go very far in assisting pathologists in determining the cause of death at autopsy where a paucity of diagnostic criteria exists. The difficulties encountered in determination of anaphylactic death at post-mortem are elegantly and succinctly stated by Da Broi & Moreschi (Da Broi & Moreschi, 2011):- "*coroners and pathologists are well conscious that a post-mortem diagnosis of an anaphylaxis hides mores problems and technical difficulties than a clinical investigation on a living, atopic patient."*

In 2001, Fineschi et al. (Fineschi et al., 2001) sampled pulmonary tissue in anaphylactic, trauma-related and heroin-related deaths. Samples consisted of perihilar tissue and a sample from each lobe of the lung. Mast cells were counted by quantitative morphometry using an area equivalent to 117.7 mm^2. The mast cell

populations were visualised by immuno-histochemical staining using anti-tryptase antibody as a mast cell specific marker.

As regards the tryptase levels, these were measured in samples of post-mortem blood of the selected cases. Their results indicated a significantly raised tryptase level in the group diagnosed as anaphylactic deaths, as well as the heroin-related death group, compared to the trauma cases [the control group].

Their conclusion was that tryptase levels are a good indicator of mast-cell degranulation, but they took this postulate no further.

Shen et al., (2009) then published work, in a retrospective study, dealing with a review of anaphylactic deaths in Maryland, USA and Shanghai, China, covering autopsies from 2004 to 2006. One of their conclusions [which was consistent with other published studies – (Edston& van Hage-Hamsten, 2003); (Randall, Butts & Halsey, 1995)] was that elevated mast-cell tryptase, although a good indicator thereof, is not an absolute indicator for a diagnosis of anaphylactic death at autopsy.

In 2006 Schwartz, (Schwartz, 2006)theorised that, as tryptase was abundantly and selectively produced by mast cells, tryptase levels should provide a more precise measure of local or systemic involvement of mast cells than was possible by documenting IgE. Discussing the fact that elevated β-tryptase levels also occurred in non-anaphylactic deaths, he stated that, as mast cell activation occurs with anaphylactic reactions and hypersensitivity to COX [cyclooxygenase] inhibitors, an elevated mast-cell tryptase level found in one case of salicylate overdose could be attributable to aspirin sensitivity. In common with other authors he was thus of the

opinion that reliance could not be placed solely on mast-cell tryptase levels in determining that the cause of death was due to systemic anaphylaxis, but that regard must be had to other diagnostic criteria and aids.

Horn et al. (Horn, Halsey & Zumwalt, 2004) stated that although serum mast cell tryptase was a reliable indicator of mast cell activation, combining it with a more specific form of assay, such as allergen-specific serum IgE assays might prove to be more conclusive. Citing the work by Randall Butts et al. [1995, *supra*], elevated mast cell tryptase levels were also found in cases of death attributable to cardio-vascular disease, as well as in deaths attributable to chest trauma. They were of the opinion that elevated serum tryptase levels in fatal chest injuries could be related to the relatively large numbers of mast cells present in lung and gastric tissue. In respect of cardio-vascular deaths they stated that they could not determine whether mast cell tryptase levels in both atherosclerotic and chest injury deaths were attributable to atherosclerotic plaques [as detailed in the work of Marone et al., 1999] or to traumatised mast cells.

Edston& van Hage-Hamsten (Edston& van Hage-Hamsten, 1998) examined β-tryptase measurements post-mortem and determined that firstly, an elevated level of β-tryptase provides a useful adjunct in the confirmation of a suspected acute anaphylactic death, secondly that the sampling site for blood used in the determination of tryptase levels was an important factor with femoral blood being the preferred option. A second study by the same authors in 2003(Edston & van Hage-Hamsten, 2003) attempted to confirm their previous work and further to determine if

the release of tryptase, after the occurrence of trauma, was acute or delayed. Concomitantly with this they attempted to determine the importance of direct trauma to, and lysis of, mast cells. Their results showed that tryptase levels increase shortly after trauma and, naturally, that tryptase levels are elevated in circumstances of traumatic death. They thus opined that mast cell degranulation occurs relatively early on in response to trauma [this would support and satisfactorily explain our observations of elevated mast cell tryptase levels in two of the three case traversed above, but which were excluded from the study for various other reasons. The causes of death being determined as traumatic head injury and drowning in a bucket of urine respectively. The case involving a myocardial infarct was, in fact, included in our study.].

In a departure from the investigations into mast cell tryptase as an indicator in anaphylactic, traumatic or cardio-vascular death, Nishio et al. (Nishio et al., 2005) examined serum chymase levels in eight cases of anaphylactic death using 104 cases as controls. Building on the research already published that mast cell tryptase levels were known to be a diagnostic indicator of anaphylaxis they explored the relationship between serum tryptase and serum chymase levels. Their results indicated a significant positive correlation of 0.826 [$p < 0.05$] indicative of the fact that serum chymase levels measurements might well be an additional tool in the armamentarium of post-mortem diagnostic tools for the determination of anaphylactic death.

Shanmugam et al. (Shanmugam, Schwartz & Khan, 2006) in a letter to the editor documented a case of elevated mature tryptase obtained some twenty-six hours after symptom onset. Their patient had been diagnosed with a single episode of idiopathic anaphylaxis.

Lastly, Mayer et al (Mayer et al., 2011) reported on the post-mortem levels of tryptase histamine and diamine oxidase as indicators supporting a diagnosis of anaphylactic death. Succinctly stated their research showed [in a study involving three cases of anaphylactic death compared to 55 controls] that whilst a moderately elevated level of mast cell tryptase was common in post-mortem sera, levels in excess of 45 µg/l might well be supportive of a diagnosis of fatal anaphylaxis. Conversely serum diamine oxidase was singularly unhelpful, whereas a highly elevated level of histamine might provide an additional clue pointing toward a fatal anaphylaxis. This latter statement, however, may not be of significant value as the authors themselves state that [citing the work of Schwartz et al. 1989(Schwartz et al., 1989)] histamine's validity as a marker for living patients is limited owing to its short half-life[+/- 15 minutes]coupled with a possibility of non-specific histamine release during blood drawing.

To summarise, therefore, despite mast cells being regarded as the primary source of mediators released on allergen challenge, and although mast cell tryptase would seem to be a useful indicator, the very fact that a plethora of triggers can result in elevated levels of mast cell tryptase militates against its use as a pathognonomic indicator of anaphylaxis. Some other method that is relatively rapid, cost-effective and simple is thus necessary as an aid to routine diagnostic measures in determining anaphylaxis at post-mortem. Here it must be clearly stated that this

search for a rapid first line test is purely as a diagnostic aid. The forensic pathologist is still required to have due regard to both anatomical and other histological indicators in supporting a diagnosis of anaphylactic death – particularly as mast cells, basophils, neutrophils and eosinophils are also implicated as will be seen below.

In seeking this diagnostic aid, regard must be had to the work of Trani et al (Trani et al., 2008).The authors in the course of their investigation into four antibiotic related cases of anaphylactic death, departed radically from the main stream school of mast cell tryptase levels, and focused on the *sequelae* of mast cell activation, namely degranulation and eosinophilia. Again they departed from the use of conventional stains for histological examination of their selected tissue [the spleen] such as H&E and Toluidine blue, and also steered away from expensive immunohistochemical assays, preferring to explore the use of a commonly available fabric dye, Pagoda Red, marketed by DYLON.

Although this would appear to be a radical and novel departure from the norm, previous research had already explored the use of fabric dyes in eosinophil staining [(Kiyono et al., 1982); (Yanagihara, Mehregan & Mehregan, 1984); (Battaglia et al., 1985)].

In this study they [Trani et al. 2008] focused primarily on splenic hypereosinophilia as a predominant feature for the differential diagnosis of anaphylactic death. This, apparently, was in accordance with a number of isolated previous studies. Their

results indicated that tissue sections stained with Pagoda Red exhibited “a diffuse perifollicular, cordonal and endosinusal splenic eosinophilia”, together with mast cells and degranulated mast cells. Visualisation of the mast cells, mast cell granules and eosinophils was facilitated by the use of Pagoda Red stain, showing up as brilliant red on a pale blue counterstained background. In contrast thereto, non-anaphylactic deaths demonstrated only scattered numbers of eosinophils together with mast cells exhibiting no degranulation. In their discussion and conclusion the authors also posited a possible eosinophilia in pulmonary and hepatic tissue, as reported by Delage & Irey (Delage & Irey, 1972) and by Vance and Stassman [1942], which they had also found in two of their cases.

A fundamental hypothesis of Trani et al.’s research was that in humans the spleen acted as a “shock organ”. Some five years later, Marone et al. (Marone et al., 2014) postulated the heart as a shock organ.

This research naturally begs the question as to whether the demonstration of a diffuse eosinophilia is pathognomonic of anaphylaxis, or whether the combination of eosinophilia and degranulated mast cells is the preferred indicator, ever bearing in mind that the post-mortem diagnosis of anaphylactic death is fraught with difficulty

Turning to Marone’s research [cited *supra*], his researchers, stating that it was trite that the human heart is implicated directly or indirectly in anaphylaxis, and citing the work of Brockow & Ring [2011]; Criep & Woehler [1971; Hanashiro & Weil [1967]; Matucci et al. [2011]; and others [Kounis, *op.cit*.], went on to show in various articles that cardiac mast cells and their mediators play a not insignificant role in both systemic and anaphylactic cardiac shock in humans.

Co-incidentally in the same year Edston(Edston, 2013) published work where he utilised eosinophil, mast cell and basophil counts in the spleen as diagnostic adjuncts in determining anaphylactic deaths. His results demonstrated a significant [$p < 0.05$] increase of eosinophil granulocytes and mast cells in sample tissue versus the control tissue. He also demonstrated that there was no significant difference in the mast cell and eosinophil count in pulmonary tissue in anaphylactic deaths when compared to tissue from the controls. This would appear to negate the theory proposed by Trani et al. [2008] insofar as eosinophils as a primary indicator of anaphylaxis were concerned. It is however to be noted that Edston restricted his examination to splenic and pulmonary tissue only and did not consider hepatic tissue. Edston did note, however, that basophils did not feature prominently in his examination of tissue. We therefore discounted the use of basophils as a supplementary indicator in our study

Of interest is the fact that he, too, utilised Pagoda Red as a stain according to the method proposed by Trani et al. [2008] and also Haematoxylin-Eosin-saffron stain according to the method of Edston (Edston 2013). His observation showed that mast cells in pulmonary tissue were located primarily in the bronchial walls and peri-vascular spaces, exhibiting moreover, an uneven distribution. Very few eosinophils were observed. In the spleen, however, the number of mast cells and eosinophils varied widely from case to case, being located mainly in the red pulp. Eosinophil counts showed a significant difference compared to control numbers with a mean of 26.16 +/- 17.8/SD/ per 40x field in the samples versus a mean of 7.0 +/- 10.5 in the controls.

A further factor of interest was his finding that opiate associated deaths demonstrated no significant difference in eosinophil counts. Our records however show one instance of an opiate-related death [following the use of self-administered continuous ambulatory pain control protocol utilizing intravenous Morphine] where the levels of mast-cell tryptase were not significantly elevated [33.2 µg/L], a finding somewhat at odds with Fineschi et al.'s research [*op.cit.].* This case, unfortunately, did not satisfy the criteria for inclusion in our study; nevertheless, we deemed it prudent to examine the spleen and heart tissue.

As an aside to this seemingly anomalous observation, regard must be had to the published work of Maurer et al. (Maurer et al., 2014), where it was found that the symptoms of heroin intoxication combined with asphyxia and/or cardiac involvement resemble anaphylaxis. Speculatively they determined that such drug–related deaths may be attributable to an allergic reaction.

Stepping back two years to 2012, we find that Stone & Brown (Stone & Brown, 2012)] state that direct activation of mast cells by opiates [specifically mentioned are morphine and codeine] can occur. That being the case, these two published articles offer an elegant explanation of the death mentioned above, but a closer histological examination of our case mentioned above would thus seem to be in order.

Returning to the research of Edston [*op cit. 2013*], we find that one of his principal findings was that Pagoda Red did not differentiate between eosinophils and mast cells, secondly that it was not as sensitive as immunohistochemical staining with

monoclonal antibodies- those utilised being tryptase antibody [supplier Dako] and chymase antibody [supplier Nordic Biosite], for the visualisation of connective tissue mast cells; major basic protein “MBP “ for eosinophil visualisation and pro MBP1 [both supplied by Nordic Biosite] for basophil visualisation.

An excursus on Anaphylaxis

Anaphylaxis is a severe systemic allergic reaction that can involve multiple systems of the body. It is often unpredictable, can have a rapid onset and, if serious, can have life-threatening and often fatal consequences.

If the reaction is attributable to, or precipitated by, an antigen antibody response, it is considered anaphylaxis, if however, it is caused by a non-antibody trigger, it is considered to be anaphylactoid in nature.

Notwithstanding this subtle difference, clinically, both responses present with the same symptoms and require the same management and treatment, and obviously both reactions are potentially fatal.

It is common knowledge, and has been for decades, that histamine is a primary mediator of anaphylaxis, further that challenge by means of a histamine infusion can reproduce the signs and symptoms of anaphylactic reaction.

Histamine, as is now well-known, triggers a cascade of inflammatory mediators and modulates its own release. H1-antihistamines are used as adjunctive treatment for acute anaphylaxis and anaphylactoid reactions, in which many mediators of inflammation are involved.

Research soon led to anaphylaxis being understood as a hyper-acute allergic syndrome, which is systemic in nature. Since then research has sought to establish which mechanisms, unique or not, were able to adequately explain the paradoxical response of the human immune system generating a potentially fatal disease state when challenged with, and reacting to, seemingly innocuous environmental substances such as animal hair [cats seem to be the favoured species!],certain foodstuffs [for example nuts], and house dust.

At this stage, a brief historical examination of the disease state is apposite.

In 1910, histamine was first found to reproduce anaphylactic symptoms (Dale & Laidlaw, 1910). Later, in the 1950s, histamine was found in cells present as a minor population in tissues (RILEY, 1953) and, in the 1960s, these cells, now known as mast cells, were shown to degranulate and release histamine when challenged with an antibody and antigen (Prouvost-Danon, Javierre & Lima, 1966)

1966 saw the discovery of a unique "*Erythema–wheal reaction-inducing Immunoglobulin"* [which led to the mnemonic *IgE*], present in minute amounts in plasma (Ishizaka, Ishizaka & Hornbrook, 1966). Shortly thereafter it was found to be secreted in large amounts by a rare plasmacytoma (Johansson & Bennich, 1967). Then in 1969 it was described, and allergic reactions were attributed to it. (Bennich

et al., 1969).This substance [IgE] was found to be *cytotropic*, (Evans & Thomson, 1972). In the same decade a receptor with a uniquely high affinity for IgE was described by Kulczycki et al. as occurring on mast cells and basophils (Kulczycki, Isersky & Metzger, 1974). This was then described and labelled FcεRI, by Blank et al., (Blank et al., 1989) and cloned by them. The 3D structure of this substance was published in1998 by Garman et al. (Garman, Kinect & Jardetzky, 1998). The allergen-IgE-FcεRI-mast cell-histamine-clinical-symptoms sequence subsequently constituted the paradigm of allergic reactions.

Antibodies other than IgE have also been shown to contribute to allergic responses, and similarly evidence has accumulated that mast cells, basophils, eosinophils, neutrophils, monocytes, T-cells and NK cells are implicated in allergic reactions. (Jonsson & Daeron, 2012).

In particular, Jonsson et al [(Jonsson et al., 2011), cited by Lowell [*supra*], implicate IgG1, IgG2 antibodies and their recognition by FcɣRIII and FcɣRIV receptors on neutrophils. Lowell [*op cit.*] presents an elegant simplified diagrammatic representation of the pattern of mediator release from mast cells via the commonly accepted mast cell – FcξRI pattern, as well as the sequence for the FcɣRIII/ IgGI – Basophil Platelet Activating Factor pathway and the FcɣRIV/IgG2-Neutrophil Platelet Activating Factor pathway.

The involvement of eosinophils in the anaphylactic process, coupled with published research that posited that the human spleen as a shock organ demonstrated a diffuse eosinophilia in cases of anaphylactic death, gave rise to the interest in this

particular study in rendering eosinophils more readily visible, particularly when the pathologist is faced with determining cause of death by autopsy.

The use of eosinophils as a diagnostic adjunct in preference, alternatively in addition, to the use of mast cell tryptase would appear to be a departure from mainstream thought on the applicable diagnostic criteria.

The problems arising in diagnosis in the living are multiplied exponentially in the deceased; hence any adjunct that would facilitate the rapid, reliable differential diagnosis of anaphylaxis at autopsy would obviously be most welcome.

The Role of Eosinophils

The role of IgE in mediating eosinophil activation has been somewhat controversial. It has been found that eosinophils can potentially express three types of IgE receptors: a low-affinity IgE receptor, a lectin-type IgE-binding molecule, and a high-affinity IgE receptor (Truong et al., 1993). Claims have been made that the latter, high affinity IgE receptor, FcεRI, is present on eosinophils from patients with eosinophilia and that eosinophilic degranulation [and parasite cytotoxicity], is mediated through this receptor (Gounni et al., 1994).

Conversely, it has been found that the number of high-affinity receptors expressed on the surfaces of eosinophils from patients with allergic diseases or airway eosinophilia was minimal or undetectable (Seminario et al., 1999).

Then, yet again, the research of Lowell and Jönsson [2011, *supra*] indicates the involvement of the FcγRIII and FcγRIV receptors on both neutrophils and basophils. Clearly more research in this field is needed, however, this study is concentrating on the role and accumulation of eosinophils in the spleen as an indicator of anaphylactic death, when examined post-mortem and research into the former is thus beyond the scope of this study.

Complicating the role of eosinophils in anaphylaxis is the fact that, although eosinophils comprise part, and are formed elements, of the peripheral circulation, they are primarily tissue-dwelling cells, and in the healthy individual, most eosinophils are found in the gut (but not in the oesophagus), mammary gland, uterus, thymus and bone marrow. It has also been found that the predominant population of eosinophils is found in the gastro-intestinal tract (Mishra et al., 1999).

The baseline state of eosinophils present in the gastrointestinal tract is independent of adaptive immunity and enteric flora; their levels being regulated by the constitutive expression of eotaxin-1 and eosinophil chemokine receptor, CCR3 (Pope et al., 2005; Humbles et al., 2002). Eosinophils also settle in, or more specifically home into, the thymus, mammary gland, and uterus (Gouon-Evans, Rothenberg & Pollard, 2000).

Thus the article by Trani et al [2008] postulating the spleen as a shock organ in humans, and that it will exhibit a marked eosinophilia in anaphylaxis is novel, and if

confirmed by this research will materially assist local pathologists in determining anaphylaxis at autopsy.

Confounding the issue though, is the published research of Maurone et al. [2013] and the report of Matucci et al. [2010], when read with the research of Kounis et al. [2013, 2014]. This latter research traversed the postulate that owing to the presence of mast cells in cardiac tissue [intimal, interstitial, perivascular and adventitial mast cells], an IgE or non IgE mediated event will trigger the massive degranulation of these mast cells with predictable results. Kounis [2013] opines that although cases of sudden death are often attributed to cardiac involvement, unrecognised anaphylaxis may accompany up to 13% of such deaths. Although he goes further to state that an increased presence of mast cells visualised with Toluidine blue stain was demonstrated in the coronary arteries, particularly the left anterior descending artery, he ends his discussion with the statement that histologic examination of the heart particularly the coronary arteries may well demonstrate mast cells and eosinophils. This latter observation is obviously important for our study

Historical overview of Eosinophils.

The eosinophil granulocyte was awarded this appellation by Paul Ehrlich in 1879 owing to the intense staining of its granules with the acidic dye eosin (Gleich & Adolphson, 1986) – staining the cell an intense red thereby making it readily visible on examination.

Since then the eosinophil has been the subject of extensive investigation, and as a result thereof its prevalence in widely disparate conditions such as parasitic

infections and hypersensitivity diseases (including anaphylaxis) has become better understood.

In Th2-type immune responses, eosinophils are recruited into sites of inflammation where they then produce an array of cytokines and lipid mediators, and also release toxic granule proteins.

From this it can be seen that the Eosinophil plays a significant role in a variety of settings in the human body, and therefore in forensic autopsy setting, fast reliable methods of visualising eosinophils in various tissues should markedly speed up the differential diagnosis of an anaphylactic/anaphylactoid death.

It is trite that the complicating factor in forensic autopsy cases is the exact determination of the cause of death. Was this death anaphylactic, anaphylactoid or attributable to some other disease process, or was it indeed an unnatural death? A question that should always be posed by the forensic pathologist.

Diagnosis of anaphylactic type reactions [a generic use of the word to cover both anaphylactic and anaphylactoid reactions] is, as a rule, difficult. In the Forensic Pathology setting at autopsy this difficulty is markedly increased.

Typically in the Forensic pathology setting, a detailed history may well be unavailable, reference levels of ante-mortem biochemical markers may not have been taken or may be unavailable, alternatively no witnesses to the fatal event may be present, and clinical symptoms may be unknown. Testimony to the comment of Da Broi & Moreschi [*supra, op. cit.*]

Criteria for the diagnosis of the disease state have been elegantly formulated as follows [(Khan et al., 2013) citing (Sampson et al., 2006)]:

“Anaphylaxis is highly likely when any one of the following three criteria is fulfilled:

Acute onset of an illness (minutes to several hours) with involvement of the skin, mucosal tissue, or both (e.g. generalized hives, pruritus or flushing, swollen lips–tongue–uvula). AND at least one of the following:

(a) Respiratory distress (e.g. dyspnoea, wheeze-bronchospasm, stridor, reduced Peak Expiratory Flow (PEF), hypoxaemia)

(b) Reduced blood pressure [“BP”] or associated symptoms of end-organ dysfunction (e.g. hypotonia, syncope, incontinence).

Two or more of the following that occur rapidly after exposure to a possible allergen for that particular patient (minutes to several hours):

(a) Skin–mucosal tissue Involvement (e.g. generalized hives, itch-flush, swollen lips–tongue–uvula).

(b) Respiratory distress as outlined above

(c) Reduced BP or associated symptoms (e.g. hypotonia [collapse], syncope, incontinence).

(d) Persistent gastrointestinal symptoms (e.g. cramps, abdominal pain, vomiting).

Reduced BP after exposure to a known allergen for that particular patient (minutes to several hours):

(a) For Infants and children: low systolic BP (age specific) or >30% decrease in systolic BP.

(b) For adults: systolic BP of <90mmHg or >30% decrease from that person's baseline."

As can readily be seen these diagnostic criteria [conveniently summarised as History; Known exposure to a trigger; and Clinical signs and Symptoms], require input from the patient or a detailed history both of which are, more often than not, sorely lacking at the stage of a forensic autopsy.

A further complicating factor is that a significant amount of research has demonstrated that mast cell tryptase ["MCT"] is a significant biomarker that can support a diagnosis of anaphylactic death, but not necessarily so, and would seem not to be pathognonomic. Elevated MCT levels are thus not diagnostic on their own as they are seen in both general and forensic medicine

[The various published explanations for these seeming anomalies observed with mast cell tryptase have been traversed above under the heading of mast cells.]

It is thus hypothesised that MCT levels, although sometimes providing a useful guideline to the cause of death, can be a significant confounder and confuser in the post-mortem determination of the cause of death.

Furthermore, in the forensic autopsy setting, baseline levels taken ante-mortem, are more often than not unavailable, hence reliance [possibly unwarranted] at autopsy, is placed on a generally accepted “normal level “of MCT as the baseline value [postulated variously as 10ng/ml or 45µg/l *supra*] might well be misplaced, when in fact the normal value for a particular patient may well be markedly above this artificially determined “normal level”.

Intriguingly recent studies have shown that although an elevated post-mortem MCT level can be indicative of an anaphylaxis related death, so too can an elevated level of MCT be found in cardio-vascular disease related deaths, in trauma and in drug related deaths as stated above.

On the positive side, MCT has been shown to be relatively stable and with a long half-life, making it suitable as a post-mortem marker, in contrast to its shorter lived analogue histamine (Edston, 2013).

Evaluation& Concluding remarks

Trani et al. (2008) had already stated in their research that both eosinophils and mast cells showed up as brilliant red when stained with Pagoda Red, and had accumulated in the spleen, in cases of anaphylactic death. This was confirmed by Edston (Edston, 2013), in a study of 43 cases.

Kounis and Matucci [*supra*] subsequently theorised that cardiac tissue is involved in anaphylaxis and demonstrates increased levels of mast cells and degranulation,

ergo, eosinophils should also be found in cardiac tissue as a pointer to "Kounis syndrome" or similar anaphylactic death.

Returning to Trani et al.'s research, it would thus appear that a correct interpretation of their work is that the presence of large numbers of both eosinophils and degranulated mast cells in splenic tissue would be indicative of a possible anaphylaxis. At no stage did they claim that their method differentiated between eosinophils and mast cells.

As regards immunohistochemical staining, this is generally accepted as the "gold standard" for staining, yet its cost remains prohibitive in certain resource challenged environments, such as ours.

Relating these various published articles to the situation found in Cape Town, it can be stated that:

Firstly, the problems experienced world–wide in the post mortem diagnosis of anaphylaxis are also found locally.

Secondly, although it is recognised that immuno-histochemical staining is the gold standard for diagnosis as set out above; in the local situation costs of obtaining such slides are prohibitive [*supra*] and in consequence thereof unsuitable for routine day to day use. The budget for forensic pathology services in the local setting is severely constrained, and is combined with a heavy workload of cases. Simply put the local pathologists do not have the twin luxuries of time and funds to obtain routine

immuno-histochemical staining of tissue to aid their possible diagnosis of anaphylactic death. By way of example, the South African costs for mast cell specific immuno-histochemical staining of tissue are some ZAR900.00 per slide, [written quote, National Health Laboratory Services, 26 August 2014], whereas, if proved to be efficacious, the use of a local dye reduces the cost to approximately R75.00per slide. [Histology Section of Division of Forensic Medicine and Toxicology, oral communication, 2014]. Its use as a diagnostic criterion would thus be of immense value.

Thus, if this research confirms the postulates of Trani et al. [2008], and if the local dye shows no statistically significant variation of eosinophil counts when compared with Pagoda Red dye, then local pathologists can utilise this low cost local dye secure in the knowledge that it replicates the published research using Pagoda Red in a statistically valid fashion.

It was determined that the dye Pagoda Red was not readily available locally or in South Africa, hence a local dye was selected. The local dye selected is manufactured by Lady Dye, apparently a South African company but very little detail is available. The dye selected is marketed as Lady Dye “Magenta”.

Nonetheless should the local dye prove inefficacious, the use of Pagoda Red dye, despite difficulties in obtaining it locally, may well prove to be a powerful tool in the visualisation of eosinophils in splenic hypereosinophilia.

SECTION C: PUBLICATION READY ARTICLE (Incorporating method, results & discussion. For submission to *Journal of Forensic Sciences*)

TITLE: A follow-up study using fabric dye to visualise Eosinophils as an aid to diagnosing Anaphylactic Death in Forensic Autopsies.

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ABSTRACT

A retrospective study of anaphylactic deaths was undertaken, with a view to determining if splenic eosinophilia was present, as well as if it was easily visualised using a cheap fabric dye as previously published.

Two common fabric dyes; one local, cheap and readily available, Magenta™ and Pagoda Red ™ manufactured by DYLON ™, imported as it was not locally available; as stains to visualise eosinophils were used. Haematoxylin/Eosin [H&E] was used as a control.

Simultaneously the study sought to determine if post-mortem mast cell tryptase levels were a reliable diagnostic indicator of anaphylactic death.

It was found that there was no significant difference in eosinophil counts between anaphylactic cases and controls at the $p < 0.05$ level.

No significant difference in eosinophil counts between the various stains used was

demonstrated. Pagoda Red rendered eosinophils more easily visible, resulting in a reduced amount of time spent in counting.

Lastly there was no significant difference [$p<0.05$] between the mast cell tryptase levels of anaphylactic deaths and control samples.

Keywords: Forensic science; Anaphylaxis; splenic eosinophilia; mast cell tryptase; histological stains

Anaphylaxis is an intriguing entity. It may manifest in a protean number of situations: idiosyncratic, manifesting after previous exposure to an allergen, as a *de novo* occurrence in procedure-related events, and also as undocumented [in the sense that no hospital records, or detailed history is available], unwitnessed deaths. Clinical diagnosis in survivors requires some effort in documenting the cascade of physiological events, particularly if the causative agent must be identified for future desensitisation or avoidance of a repeat challenge.

Diagnosis of anaphylaxis as the cause of death at autopsy is even more complicated as the normal diagnostic armamentarium is significantly impoverished, the death commonly being undocumented, and unwitnessed. Furthermore the many ante-mortem clinical baselines and usual indicators of anaphylaxis change or may disappear. Diagnosis thus relies mainly on the exclusion of other factors coupled, where possible, with patient history, histological examination and with appropriate biochemical testing of appropriate tissue samples. These diagnoses are thus notoriously difficult [1-4], and it is commonly known that these diagnoses cannot rely on any one single factor, but on a combination of factors.

The role of mast cells in inflammatory reactions and anaphylactic shock has been well established for a considerable period of time [5-9].

It is also well understood that histamine, being the main mediator of anaphylaxis, presents special difficulties for post-mortem analysis/determination owing to its manifestly short half-life [10]. The same however is not true for mast cell tryptase, it having been shown to be relatively stable in post-mortem blood [11,12] This naturally provides a useful post-mortem indication of mast cell activation, possibly indicative of an anaphylactic, [or anaphylactoid] death.

Frustratingly though, it has been shown that elevated levels of mast cell tryptase at post-mortem have occurred where the cause of death has been shown as being unrelated to anaphylaxis [12]. It is thus unsafe to rely solely on mast cell tryptase as the only indicator of anaphylactic death. The use of I_gE levels to assist in the determination of anaphylactic death does not seem to be widely used.

Eosinophilia and oedema in the upper airways has been shown to be a common, but not omnipresent, finding in anaphylactic deaths [2]. It has been shown that there would appear to be no demonstrable eosinophilia in pulmonary tissue in anaphylactic deaths, but a confirmed splenic eosinophilia [3, 6, 7, 13], it was also shown that a high level of mast cell tryptase was linked to an increased number of eosinophils and mast cells in splenic tissue. [6]

This short study attempts to replicate the results obtained by Trani et al. [7], but using a locally available fabric dye, as DYLON ™ Pagoda Red is locally unavailable, and to determine if splenic eosinophilia could be demonstrated in all anaphylactic death cases considered. A superficial examination of a possible correlation between mast cell tryptase levels anaphylaxis, and splenic eosinophilia was also performed.

Methods

Ethics approval for the study having been granted by the Health Sciences Faculty of the University of Cape Town ethics committee under the reference HREC/REF 088/2015, a retrospective study of forensic autopsy files at the Salt River Forensic Pathology Laboratory, Division of Forensic Medicine at the University of Cape Town, in Cape Town was conducted, covering the period 2002 to 2015. Cases where anaphylaxis was diagnosed as the cause of death were identified. A cohort of non-anaphylactic death cases was also identified.

Selection criteria for both test and control cases were: similar age and gender [as far as was practically possible; availability of spleen tissue and mast cell tryptase [MCT] levels. Sample and control cases were scrutinised by a senior forensic pathologist.

The selection took place in accordance with the following 5 step protocol:

1 Cases where a mast cell tryptase level was available were identified from a spreadsheet of forensic autopsy cases with mast cell tryptase levels recorded by the UCT Lung Institute;

2 Thereafter, the hard copy documents [archive records] of all identified cases were retrieved for scrutiny by the researchers;

3 Documentation was carefully examined to determine if appropriate histological specimens had been kept.

4 Histological samples were then examined to ensure that a spleen sample was in fact available.

5 Each case was exhaustively reviewed by a Senior Forensic Pathologist to determine if the diagnosis was one of anaphylaxis or one positively excluding anaphylaxis. These identified cases then formed the basis of our study.

A variety of clear-cut non-anaphylactic causes of death were noted in the control cases, including drowning, air embolism, cardiac disease and gunshot wounds and

complex disease profiles. As mentioned before the availability of mast cell tryptase levels and a spleen section were pre-requisites. On this basis it was possible to select nine cases where anaphylaxis was determined to be the cause of death, ten cases where cause of death was not anaphylaxis, plus four other cases included as controls owing to complex pathology, but where anaphylaxis as the cause of death had been excluded. One of the nine cases had two different paraffin embedded sections of spleen [listed as Block 6 and Block 7 respectively, samples D & K] as a further internal control, giving a total of ten sample slides, and a total of fourteen control slides. The master data table of the cases selected for both samples and controls is reflected in table 1.

Blood for MCT levels was femoral blood in accordance with standard operating protocols in the Salt River Forensic Pathology Laboratory. Post-mortem intervals varied between less than five hours to seven days after death. These survival times are also detailed in table 1. Table 2 is a key to the study samples, detailing the alphabetic character identifying each sample, the pathology services case number, the diagnosis, cause of death and the mast cell tryptase level.

Tissue samples of spleen were processed in our histology laboratory and each sample stained with, variously: H&E, Pagoda Red (according to the method of Trani et al.) [7] and a locally manufactured fabric dye called “Magenta”. Allergic nasal polyps, rich in eosinophils and mast cells were used as an internal control.

In the case of the sections stained with Magenta, the method we developed was as follows:

Sections of paraffin wax embedded splenic tissue of 5, 3, and 1.5 micron thickness

were stained to determine the optimum tissue section thickness. It was found that the standard 1.5 micron section gave the best results as far as dye uptake, colour and contrast were concerned.

Thereafter the pH of the Pagoda Red solution was measured and the pH of the Magenta solution adjusted to match [pH 4.15].

Sections were then stained in the aqueous Magenta solution [1%] for a period of one hour at 55^0 C, washed in water for 1 minute, counter stained with Harris Haematoxylin solution for one minute, again washed for one minute in water, followed by Scott's solution for one minute. A further wash in water for a one minute period was performed, whereafter the slides were washed in successive alcohol baths and clarified with Xylol as per the H&E staining method.

No immunohistochemical staining was performed as in our case costs are prohibitive and only one laboratory in the country has the capability to perform this type of processing. The laboratory concerned also cannot perform the specific test mentioned in the literature, but can perform a generic type of mast cell immunohistochemical staining [CD 117, cost ZAR 900.00 per slide]]

At this stage the triplicate sets of spleen slides [comprising H&E, Magenta & Pagoda Red stains], were randomly coded alphabetically by the histotechnologist, Ms Y. Davies, to anonymise the case numbers. This was done in order to blind the analysts as to whether a specific slide/section originated from a sample case, or control case.

Eosinophil counts were performed in ten randomly selected x 40 fields that had been digitally captured and recorded with an Olympus BX43F photo microscope.

One analyst preferred to count the cells directly using a standard binocular microscope [LL], the other analyst preferring to use the photo-micrographs. [PB]

The two analysts [Dr. Liebenberg “LL”, forensic pathologist; P Burgers “PB”, student] [PB] were then given the slides and eosinophils visible were counted as set out below.

Once the counting of eosinophils had been completed the slides were de-anonymised to reveal the alpha-numerical details of each set, and these data were then used to draw up the tables of results.

The data was statistically analysed using Wilcoxon rank-sum method. Means and standard deviations were routinely calculated. Significant differences were defined as $p < 0.05$. Where applicable, Spearman correlation co-efficients were also calculated.

Results

Median cell counts per analyst were statistically evaluated using the Wilcoxon rank sum test. The total cell counts [10 random fields] per slide per stain are shown in table 3.

Statistically significant discrepancies in inter-observer cell counts were found as follows:

The experienced analyst, “LL”, using a microscope field for counting, demonstrated a significant [$p < 0.05$] difference in cell counts as set out in table 4.

The inexperienced analyst, “PB”, counting from the photomicrographs, demonstrated a greater consistency in cell counts, but demonstrated significant differences in cell counts as set out in table 5

Bearing in mind that there were a total of twenty two different cases, it is apparent that the experienced analyst [in excess of twenty years’ experience] demonstrated significant differences in a far greater number of cases than the inexperienced [one year’s experience] analyst. The experienced analyst showed a bias in favour of cell counts with H&E, followed by Pagoda Red, and trailed by the Magenta stain.

The Magenta stain proved disappointing in its application, it being difficult to identify eosinophils in congested splenic tissue. In a few of the study samples it was reported that the use of Magenta, despite it not being as effective as Pagoda Red, certainly rendered the eosinophils more readily visible than the H&E stained samples. Generally these were samples where congestion was absent

Thereafter, and once the various diagnoses of the cases were revealed, the anaphylaxis cases were compared to the control cases and secondary control cases by means of a Wilcoxon rank-sum test, using the cell counts obtained with the three different stains.

As before the results were separated into those obtained by the experienced analyst and those obtained by the inexperienced analyst and grouped appropriately. Without exception we demonstrated that there were no significant differences between test, control and secondary control cases' eosinophil counts, across all three stains.

Lastly, we determined the Spearman correlation co-efficients between the eosinophil counts and mast cell tryptase levels, for the sample cases [anaphylactic deaths] and the control cases, per analyst.

We determined that there was no statistically significant correlation [rho = -0.13374 for the H&E sample cases; -0.13982 for the Pagoda Red sample cases; 0.139 and 0.182 for the control cases, respectively] between mast cell tryptase levels and a diagnosis of anaphylactic death.

In all cases it was found that the number of eosinophils present was significantly lower than previously reported [6, 7]. The summary of means, medians etc. is reflected in tables 6 and 7.

Our results indicate that whilst mast cells may not be clearly differentiated from eosinophils in a routine stain [H&E], the use of Pagoda Red particularly, and to a lesser extent our local dye Magenta, renders mast cells far more easily distinguishable from eosinophils than is possible with a routinely stained slide [H&E].thereby rendering both mast cells and eosinophils highly visible and capable of being rapidly counted. With Pagoda Red we noted that the granules from degranulated mast cells appeared as brick red clumps similar in appearance to

caviar, and thus readily identifiable and distinguishable from eosinophils.

Elevated Mast Cell Tryptase level in Anaphylaxis.

In two confirmed cases of anaphylaxis, ante-mortem sera could be obtained as base-line values. In these two hospital-setting deaths, the time frames of anaphylaxis as well as the causative agents were documented. Mast cell tryptase levels were 5.7 µg/l ante-mortem, and 19.5 µg/l post-mortem [sample N, table 1]. The ante-mortem baseline value in the second case was 10.4 µg/l, post arrest, 18.1 µg/l and at autopsy 39.5 µg/l [sample U, table1]. In both cases the mast cell tryptase level did not exceed the 45 µg/l level [12].

On an individual basis our findings did not seem to support the theory that an elevated mast cell tryptase level went hand in hand with anaphylaxis. When combined the mean values of MCT in the anaphylactic samples were 278.85µg/l with an SD of 151.21 µg/l, with a range from 16.4 to 1530 µg/l, whereas with the control group the mean value was shown to be 38.758 µg/l with an SD of 18.35 µg/l, and a range from 3.68 to 200 µg/l. Our combined results would seem to support the previous research [6] that elevated MCT levels are frequently, but not consistently, found with anaphylactic deaths. Because of the extreme variability of the ranges we deem it prudent to continue this research in an effort to expand diagnostic parameters, rather than relying on MCT alone.

Discussion.

Limitations.

A retrospective study has inherent limitations. Autopsies cannot be performed again;

tissue cannot be resampled, the particular pathologist who performed the autopsy may no longer be available, and the rationale for the obtaining of mast cell tryptase levels, in itself a specialised test, may not be reflected in the documentation. Hospital records may not be available and owing to the effluxion of time may not be traceable.

Anaphylactic deaths are notoriously difficult to diagnose, and generally rely on the exclusion of other causes of death coupled with exhaustive testing and histological examination. Numbers of anaphylactic deaths in the records are generally low.

Splenic eosinophilia

Having examined the results obtained by both analysts, we would cautiously conclude as follows.

That in the subjective opinion of the experienced analyst Pagoda Red rendered the eosinophils more easily visible than traditional H&E stain, thereby markedly reducing time spent in counting [although this was not measured or recorded].

In the hands of the inexperienced analyst, the variations were far less numerous, but his opinion mirrored the opinion of the experienced analyst as regards visibility of eosinophils. That is: H&E followed by Pagoda Red and again trailed by Magenta. This analyst echoed the sentiments of the experienced analyst that the Pagoda Red stain rendered eosinophils far more easily visible. We would suggest that, based on the number of significant differences exhibited in the sample counts in the hands of the inexperienced analyst, an inexperienced analyst would benefit more by the use of Pagoda Red or H&E stain, rather than the local Magenta stain we tested; secondly

that in the case of experienced analysts the stains of choice would be H&E or Pagoda Red. It is noted that the demonstrated efficiency of Pagoda red mirrors the findings of Trani et al. and Edston [6, 7].

We could not confirm, statistically, a splenic eosinophilia in anaphylactic deaths as reported [6, 7]. Only one of the nine sample cases, sample V in table 1, [comprising ten mounted sections of tissue] demonstrated an eosinophil count of greater than ten in each field.

At this point it must be mentioned that shortly before the completion of the study, we evaluated the following cases:

Case 1: This particular case was unique as it proved to be a case of nematode infestation, possibly Anisakis simplex, in a fish factory worker who was otherwise healthy. This case was reflected as sample V in the study. The subject had returned home from work with no complaints, went outside, and was found dead twenty minutes later. Toxicology screen demonstrated no alcohol in the blood, and paracetamol on the urine only. Apart from a massive splenic eosinophilia, eosinophilic granulomata with Charcot Leyden crystals around remnants of nematode cuticle were visible in the liver. Total IgE was 124, MCT was 28 µg/L, and Anisakis specific IgE was 0.12. [levels of<0.1kU/l are regarded as below the reliable detection limit; 0.1-0.35 kU/l very low and >100kU/l extremely high. A low specific IgE is apparently often found in allergy cases. A positive test is regarded as conclusive, a negative test is inconclusive. [Personal communication, Ms. B Fenimore, Medical Technologist, Allergology, Diagnostic & Clinical Research Unit, UCT Lung Institute, 6 June 2012]

Case 2: was that of a 24 year old female who died under suspicious circumstances, whilst engaged in sexual activity. At autopsy she was found to have swollen pale tracheal mucosa, and puffy lungs. She also exhibited a congested spleen, with a marked eosinophilia in the stomach mucosa, a normal mast cell tryptase level, but a markedly elevated IgE level [Total IgE 436; MCT 20.4µg/L; Latex specific IgE 0.06kU/l]. Toxicology screen demonstrated no drugs of abuse or alcohol in the blood. This case proved to be an anaphylactic death most likely attributable to latex allergy from the condom that was used. A sample of spleen tissue was stained as per the protocols above, was disappointing as it was too congested to permit cell counting.

Eosinophilia and Mast Cell Tryptase

Our results could not confirm a correlation between MCT levels and splenic eosinophilia. Spearman coefficients demonstrated no significant correlations.

Differentiation of Mast Cells and Eosinophils.

The result that eosinophils and mast cells are rapidly distinguishable when stained with Pagoda Red, and to a lesser extent Magenta is significant in that analysts performing cell counts can quickly distinguish between the two and cell counting is markedly facilitated. With Pagoda Red we noted that the granules from degranulated mast cells appeared as brick red clumps similar in appearance to caviar, and thus readily identifiable and distinguishable from eosinophils. A considerable amount of time saved in cell counting is achieved.

Conclusions

Based on our research we concluded that there is most definitely a place for the use of rapid dye staining techniques such Pagoda Red [6, 7, 13] and to a lesser extent, our local Magenta dye. The significance of this in a resource-challenged environment where routine immunohistochemical staining cannot be achieved should not be under-estimated.

Our results could not confirm the findings of Trani et al. and Edston [6, 7], that a splenic hypereosinophilia was present in anaphylactic deaths. Nonetheless we determined that Pagoda Red staining of tissue samples has a number of other possible applications.

Apart from anaphylaxis, a number of disease processes elicit eosinophilia.

Apart from Forensic Pathology, we suggest a number of applications of Pagoda Red in the fields of surgery and medicine, which may be of particular value and applicability in a resource challenged environment:

- Bronchial aspirates with eosinophilia may indicate allergic asthma;
- renal biopsy eosinophilia may indicate drug reaction;
- parasitic diseases such as nematode infestations and schistosomiasis are frequently encountered and eosinophilia is a typical hallmark of such infestations.

A further possible example is that the use of Pagoda Red staining could be useful in evaluating oesophageal biopsies where eosinophils are searched for as an indication of gastro-oesophageal reflux disease. We foresee that Pagoda Red stains may have useful application in other disciplines.

Secondly we concluded that a count of less than 10 splenic eosinophils per x40 field OR a MCT below 45 does NOT exclude anaphylactic death.

In a small cohort (cases A, B, I etc.) IgE levels were sometimes more conclusive in documenting the allergic nature of the death than were splenic eosinophils or MCT (sample V – case 1 *vide supra*) and case report 2.

It thus appears that the diagnosis of anaphylaxis at autopsy remains a challenge to the forensic pathologist. We also sound a *caveat* regarding the non-linearity of human systems [14], and we are of the opinion that this line of research involving MCT levels, eosinophilia and anaphylactic death should be pursued vigorously, particularly in the light of the interesting questions posed by various authors [15-18].

Tables for upload

Study ID	Sex	Age	Presentation	Trigger	Time to death	Resus. Done	P.M. Findings	MCT	Known Allergies	Additional
G	F	54	Angioedema after ACE inhibitor administered. Known sensitive, but hospital folder was locked away.	ACE inhibitor	1 hour	Yes	Marked oedema and pallor of tongue and laryngeal mucosa. Co-morbidities	16.4	ACE inhibitors	
N	F	71	Post hip replacement	Tramacet [Tramadol/Para-cetamol]	30 minutes	No	No mucosal swelling. Hypostatic congestion. Nephrosclerosis	AM 5.7 PM 19.5	Penicillin	Drug screen: paracetamol.
U	F	72	Chest infection Rx. Intravenous Avelon. Collapsed in 15 minutes. Urticaria. Hypoxic brain injury.	Avelon IV	15 minutes	Yes	No mucosal swelling. Subpleural atelectasis. . Ischaemic Heart Disease. Haemorrhagic gastritis. Scarred kidneys. Enlarged spleen.	AM Base: 10.4 Post arrest: 18.1 PM: 39.5	Penicillin	
D & K	F	22	Started fitting in her sleep.	Unknown	Unknown	No	No mucosal swelling. Pulmonary TB. Vegetables in stomach. Multiple small pale areas in spleen.	Blood 21 Eye fluid 11.9	Developed a rash after eating unidentified foodstuff	
R	F	66	New on ACE inhibitor Rx. ACE inhibitor anaphylactic reaction. Hypertension and Diabetes Mellitus	ACE inhibitor	unknown	Yes	Tongue swollen, prolapsed. Swollen face. General congestion. Subconjunctival haemorrhage. Pulmonary oedema. Airway mucosa swollen. Ischaemic Heart Disease Hypertension. Haemorrhagic gastritis	Blood 539 Eye fluid 6.01	Unknown	Photos
A	M	38	Aorto-iliac AS with acute leg ischaemia. Angiogram. Thrombectomies. Streptokinase administration. Immediate BP drop	Streptokinase	Immediate	Yes	Medical and surgical additions. Mural thrombus left ventricle. Extensive atherosclerosis. Shock kidneys.	139	Unknown	Total IgE 36kU/l

H	M	64	Fell ill immediately after ingesting Panamor AT50 for lower back pain. Severe rash. Collapsed 4 hours later.	Panamor [Diclofenac]	Immediate and died in 4 hours	Yes	Swollen laryngeal mucosa. General congestion. Congested lungs, subpleural haemorrhages Ischaemic Heart Disease & Atherosclerosis	436	Unknown	
O	M	2	Betapen syrup for respiratory infection. After administration child felt tired and was taken to bed. He turned blue and was DOA at clinic.	Betapen [Penicillin]	less than 1 hour	No	Vocal cords not swollen. Lungs oedematous.	1530	Had amoxicillin treatment in the week before death.	
V	M	32	Returned from night shift at fish factory. Girlfriend says he stepped out of the house at 07h40. Found passed away outside the house at 08h00.	? Parasite ? Anisakis	Unknown	No	Hypertensive heart, oedematous lungs. Pale conjunctivae and oral mucosa. Laryngeal oedema not seen. Granulomatous lesions in the liver. Follicular spleen.	28.1	NO	Anisakis antibody weak positive. IgE 126. Blood alcohol 0. Drugs: paracetamol in urine, not in blood.
E	M	71	Total hip replacement. Co-morbidities: IHD with pacemaker. Follicular lymphoma. Obese. Hypotension and oliguria day after op. ARDS. ?sepsis ?drug reaction. Died 2 days after op.	? Drug	within 24 hours	Yes	Heart large and flabby. Liver fatty and nutmeg. Shock kidneys. Spleen diffluent.	21	Unknown	
L	F	32	Septic wound hand, cellulitis whole arm. IV Antibiotics and analgesics. Started 00h45. Cardiac arrest at 13h30. ?Gas gangrene	?Gas gangrene	within 24 hours	Yes	Septic wound R hand, arm swollen with blisters. Pus swab: staph aureus and strep pyogenes.	3.68	Unknown	
S	F	88	Gradual development skin rash 2 weeks. Developed Toxic epidermal necrolysis, uncertain which drug triggered it. Sepsis: Klebsiella, E coli, Proteus, coagulase negative Staph. 11 day hospital stay.	Toxic Epidermal Necrolysis	more than 3 weeks	Yes	Skin and mucosal desquamation. Mitral valve stenosis, Left Ventricular Hypertrophy; Atherosclerosis	51	Unknown	toxicology negative

B	M	67	SCUBA diving accident. Drowning and injuries	Drowning	±1 hour	Yes	Resus trauma. Bruising caused by being washed onto rocks. Bruising of neck due to violent dragging of diving mask. Swelling and bruising larynx and one vocal cord. Irregular aeration lungs. Bruised stomach.	13.4	Unknown	Alcohol and toxicology negative. IgE 32.
I	M	69	Endoscopic eye operation. Fatal air embolism	Air embolism	Immediate	Yes	Air embolism demonstrated by X-ray and PM testing.	14.1	Unknown	IgE to Suxamethonium less than 0,35kU .Chlorhexidine 0,13
P	F	40	Obese. Polycystic ovarian syndrome. DM. LV function 47% Mitral valve replacement. Unsuccessful weaning from bypass. Crashed after protamine. Died on table.	Cardiac failure	Minutes	Yes	Advanced cardiac pathology: Hypertension; Atherosclerosis, Valvular heart disease.	13.5	Unknown	Protamine IgE: 0.00kUA/l
W	M	35	Fit Nigerian soccer player. Was queuing at Home Affairs. Sudden collapse, grabbing his throat as if choking.	HOCM	Minutes	No	Pale conjunctiva. Pulmonary oedema. Enlarged heart.	18.3	Unknown	Sulfadoxine, possibly malaria prophylaxis
F	F	37	Started making funny noises in her sleep. Had been complaining of chest pain and shortness of breath. DOA	Acute MI	Minutes	No	Acute on chronic MI, 90% LAD occlusion. Tongue bitten. Pulmonary oedema.	More than 200	Unknown	
M	M	22	Gunshot wound through the chest	GSW chest	Minutes	No	Haemothorax: 1300 L, 900 R. Lungs collapsed, pale.	24.2	Unknown	Alcohol 0.00
T	M	47	Suicide by drowning in the sea	Drowning	Minutes	No	Froth in airways. Lungs hypervoluminous, puffy and frothy oedema present. Hypertensive and atherosclerotic cardiac disease.	28.4	Unknown	Alcohol 0.00 .Lidocaine in therapeutic range. Benzodiazepine and Amitriptyline
C	F	5 months	Sudden unexpected infant death while sleeping at a crèche.	Bronchiolitis	Uncertain	No	Healthy looking. Mottled oedematous lungs.	17.2	Unknown. Viral?	Blood culture - mixed growth.

J	F	75	Descending colon and rectum resection for bowel obstruction. DIC, sepsis. Multiple blood transfusions	Complications of bowel obstruction	5 hours	Yes	Sepsis, DIC	67.6	Unknown	
Q	M	28	Still's disease, haemophagocytic lymphohistiocytosis, ARDS, Sepsis. ?reaction to cloxacillin.	Complex immune disease	1 day	Yes	Bronchopneumonia, hypercellular bone marrow, splenic follicles non-reactive.	12.7	?cloxacillin allergy	
X	M	5 months	Sudden unexpected infant death while sleeping at a crèche. Room recently painted - strong paint fume presence.	Strong vapours, underlying upper and lower respiratory tract infection.	Minutes	No	Dark, puffy lungs.	52.6		

Table1: Master Datasheet indicating history, cause of death, MCT levels & histological findings on which study is based

Sample Reference	Case Number	Diagnosis	Cause of death	Mast Cell Tryptase Level
A	DR 2046/2006	Anaphylaxis	Streptokinase allergic reaction	139
D *	DR 313/2006 block 6	Anaphylaxis	Unknown	21
G	DR 1520/2002	Anaphylaxis	ACE inhibitor reaction	16.4
H	WC 11/1548/2007	Anaphylaxis	Panamor [Diclofenac p.o.] reaction	436
K *	DR 313/2006 block 7	Anaphylaxis	Unknown	21
N	DR 830/2005	Anaphylaxis	Tramacet [tramadol/Paracetamol p.o.] reaction	19.5
O	WC 11/676/2011	Anaphylaxis	Betapen [penicillin p.o.]- penicillin allergy	1530
R	DR 359/2006	Anaphylaxis	ACE inhibitor reaction	539
U	DR 982/2005	Anaphylaxis	Avelon IV [Moxifloxacin] -reaction to	39.5
V	WC 11/873/2013	Anaphylaxis	Nematode infestation - possibly Anasakis	28.1
B	WC 11/2712/2008	Control	Drowning at sea	13.4
E	DR 2032/2002	Control	Sepsis	21
F	WC 11/1348/2012	Control	Acute MI	200 +
I	WC 11/2385/2009	Control	Air embolism during surgery	14.1
L	DR 827/2005	Control	Gas gangrene	3.68
M	WC 11/1372/2012	Control	Gunshot chest	24.2
P	WC 11/1325/2011	Control	IHD & valvular heart disease	13.5
S	DR 2147/2006	Control	Toxic epidermal necrolysis	51
T	WC 11/1670/2012	Control	Drowning at sea	28.4
W	WC 11/720/2012	Control	Hypertrophic obstructive cardiomyopathy	18.3
C	WC 11/1389/2012	Sec. Control	Bronchiolitis	17.2
J	WC 11/1452/2012	Sec. Control	Sepsis	67.6
Q	WC 11/1456/2012	Sec. Control	Septic Shock	12.7
X	WC 11/1628/2012	Sec. Control	Bronchiolitis	52.6

Table 2. Key to samples with abbreviated diagnoses, MCT levels and case numbers
Asterisked samples represent two different sections of spleen from the same case

Experience-ed analyst "LL"					Inexperienced Analyst "PB"			
Diagnosis	**Sample**	**H&E**	**Magenta.**	**Pagoda Red**	**H&E**	**Magenta**	**Pagoda Red**	**Abbreviated diagnosis**
Anaphylaxis	A	87	19	20	90	34	28	Drug reaction- Streptokinase
Anaphylaxis	D	32	7	2	2	2	0	Unknown
Anaphylaxis	G	29	29	66	54	61	82	Drug reaction -ACE inhibitor
Anaphylaxis	H	23	6	3	2	3	10	Drug reaction- Diclofenac
Anaphylaxis	K	24	13	37	16	14	15	Unknown
Anaphylaxis	N	128	31	33	11	8	16	Drug reaction -Paracetamol/Tramadol
Anaphylaxis	O	79	83	92	41	23	28	Drug reaction - Penicillin
Anaphylaxis	R	10	9	2	20	12	0	Drug reaction -ACE inhibitor
Anaphylaxis	U	38	2	45	36	21	19	Drug reaction - Moxifloxacin
Anaphylaxis	V	484	413	537	226	221	181	Nematode infestation - ? Anasakis
Control	B	19	13	15	52	31	55	Drowning at sea
Control	C	64	29	67	33	33	71	Bronchiolitis
Control	E	124	23	4	10	4	6	Sepsis
Control	F	92	22	23	116	69	24	Acute MI
Control	I	9	0	20	3	0	27	Air embolism during surgery
Control	J	20	5	8	24	0	0	Sepsis
Control	L	45	7	1	2	1	0	Gas Gangrene
Control	M	38	32	53	24	10	34	Gunshot wound - chest
Control	P	62	31	25	55	72	6	IHD & valvular heart disease
Control	Q	25	15	18	9	13	12	Septic Shock
Control	S	23	7	15	6	15	8	Toxic epidermal necrolysis
Control	T	25	9	13	61	37	8	Drowning at sea
Control	W	118	92	195	42	14	52	Hypertrophic obstructive cardiomyopathy
Control	X	91	59	83	31	20	25	Bronchiolitis

Table 3. Table of total cell counts in ten 40x fields, per sample, per analyst, per stain, plus abbreviated diagnosis

Sample reference	Sample Type	Stains compared	P values	Stain exhibiting the Highest cell count
A	Anaphylaxis	H&E vs Pagoda Red	0.0022	H&E
D	Anaphylaxis	H&E vs Magenta	0.0015	H&E
D	Anaphylaxis	H&E vs Pagoda Red	0.0001	H&E
G	Anaphylaxis	H&E vs Pagoda Red	0.0042	Pagoda Red
G	Anaphylaxis	Magenta vs Pagoda Red	0.0074	Pagoda Red
H	Anaphylaxis	H&E vs Magenta	0.0011	H&E
H	Anaphylaxis	H&E vs Pagoda Red	0.0006	H&E
K	Anaphylaxis	H&E vs Pagoda Red	0.0484	Pagoda Red
K	Anaphylaxis	Magenta vs Pagoda Red	0.0011	Pagoda Red
N	Anaphylaxis	H&E vs Magenta	0.0003	H&E
N	Anaphylaxis	H&E vs Pagoda Red	0.0002	H&E
R	Anaphylaxis	H&E vs Pagoda Red	0.0017	H&E
R	Anaphylaxis	Magenta vs Pagoda Red	0.0017	Magenta
C	Control	H&E vs Magenta	0.0026	H&E
C	Control	Magenta vs Pagoda Red	0.0034	Pagoda Red
E	Control	H&E vs Magenta	0.0002	H&E
E	Control	H&E vs Pagoda Red	0.0001	H&E
E	Control	Magenta vs Pagoda Red	0.0034	Magenta
F	Control	H&E vs Magenta	0.0003	H&E
F	Control	H&E vs Pagoda Red	0.0003	H&E
I	Control	H&E vs Pagoda Red	0.0242	Pagoda Red
I	Control	Magenta vs Pagoda Red	0.0002	Pagoda Red
J	Control	H&E vs Magenta	0.0454	H&E
J	Control	H&E vs Pagoda Red	0.0401	H&E
L	Control	H&E vs Magenta	0.0002	H&E
L	Control	H&E vs Pagoda Red	0.0001	H&E
L	Control	Magenta vs Pagoda Red	0.0212	H&E
M	Control	Magenta vs Pagoda Red	0.0129	Pagoda Red
P	Control	H&E vs Magenta	0.0091	H&E
P	Control	H&E vs Pagoda Red	0.0035	H&E
S	Control	H&E vs Magenta	0.0027	H&E
T	Control	H&E vs Magenta	0.0093	Pagoda Red
W	Control	H&E vs Pagoda Red	0.0028	Pagoda Red
W	Control	Magenta vs Pagoda Red	0.006	Pagoda Red
X	Control	H&E vs Magenta	0.0202	H&E

Table 4: Showing statistically significant comparison cell counts by the experienced analyst, per stain

Sample reference	Sample Type	Stains compared	P values	Stain exhibiting the Highest cell count
R	Anaphylaxis	H&E vs Pagoda Red	0.0019	H&E
R	Anaphylaxis	Magenta vs Pagoda Red	0.0049	Magenta
V	Anaphylaxis	H&E vs Magenta	0.0311	H&E
C	Control	H&E vs Pagoda Red	0.0192	Pagoda Red
C	Control	Magenta vs Pagoda Red	0.0361	Pagoda Red
F	Control	H&E vs Magenta	0.0301	H&E
F	Control	Magenta vs Pagoda Red	0.0017	Magenta
I	Control	H&E vs Pagoda Red	0.0006	Pagoda Red
I	Control	Magenta vs Pagoda Red	0.0002	Pagoda Red
J	Control	H&E vs Magenta	0.0024	H&E
J	Control	H&E vs Pagoda Red	0.0002	H&E
M	Control	Magenta vs Pagoda Red	0.0078	Pagoda Red
P	Control	H&E vs Pagoda Red	0.0001	H&E
P	Control	Magenta vs Pagoda Red	0.0001	Magenta
T	Control	H&E vs Pagoda Red	0.0018	H&E
W	Control	Magenta vs Pagoda Red	0.0025	Pagoda Red

Table 5: Showing statistically significant comparison cell counts by the inexperienced analyst, per stain

Experienced Analyst "LL"	**Mean cell count per field**	**Median cell Count**	**Std. Deviation**	**Range**
H&E sample cases	9.34	4	14.33	0-71
H&E control cases	5.22	4	4.601	0-21
Magenta sample cases	6.02	2	13.08	0-73
Magenta control cases	2.46	2	2.95	0-17
Pagoda Red sample cases	3.71	3	5.35	0-46
Pagoda Red control cases	3.86	2	6.16	0-37

Table 6: Mean cell counts per stain for the experienced analyst

Inexperienced Analyst "PB"	Mean cell count per field	Median cell Count	Std. Deviation	Range
H&E sample cases	4.98	2	7.82	0-33
H&E control cases	3.34	2	4.58	0-27
Magenta sample cases	3.99	1	7.03	0-34
Magenta control cases	2.27	1	3.59	0-21
Pagoda Red sample cases	3.8	1	5.79	0-28
Pagoda Red control cases	2.34	1	3.52	0-28

Table 7: Mean cell counts per stain for the inexperienced analyst

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PART D: APPENDICES

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Sundry Tables and Data

Anaphylaxis	16.4
Anaphylaxis	19.5
Anaphylaxis	21
Anaphylaxis	21
Anaphylaxis	28.1
Anaphylaxis	39.5
Anaphylaxis	139
Anaphylaxis	436
Anaphylaxis	539
Anaphylaxis	1530
Mean	**278.95**
Median	**33.8**
Control	3.68
Control	13.4
Control	13.5
Control	14.1
Control	18.3
Control	21
Control	24.2
Control	28.4
Control	51
Control	200
Control	12.7
Control	17.2
Control	52.6
Control	67.6
Mean	**38.40571**
Median	**19.65**

Table of Mast Cell tryptase levels: Anaphylactic cases versus Controls

	Experienced Analyst "LL"				Inexperienced analyst "PB"		
	H&E	Mag	P.R.		H&E	Mag.	P.R.
Anaphylaxis	87	20	19		90	28	34
Anaphylaxis	32	2	7		2	0	2
Anaphylaxis	29	66	29		54	82	61
Anaphylaxis	23	4	6		2	10	3
Anaphylaxis	24	37	13		16	15	14
Anaphylaxis	128	32	31		11	16	8
Anaphylaxis	79	99	83		41	28	23
Anaphylaxis	10	0	9		20	0	12
Anaphylaxis	38	45	0		74	64	21
Anaphylaxis					226	181	221
Mean	**50**	**33.9**	**21.9**		**53.6**	**42.4**	**39.9**
Median	**32**	**32**	**13**		**30.5**	**22**	**17.5**
Control	19	15	13		52	55	31
Control	128	4	23		10	6	4
Control	87	23	22		116	24	69
Control	9	20	0		3	27	0
Control	45	1	7		2	0	1
Control	38	53	32		24	34	10
Control	62	25	31		55	6	72
Control	21	16	7		6	8	15
Control	25	13	9		69	21	46
Control	118	193	92		42	52	14
Mean	**55.2**	**36.3**	**23.6**		**37.9**	**23.3**	**26.2**
Median	**41.5**	**18**	**17.5**		**33**	**22.5**	**14.5**
Control	64	67	29		33	71	33
Control	17	8	5		24	0	4
Control	25	18	15		9	12	13
Control	91	94	59		31	25	20
Mean	**49.3**	**46.8**	**27**		**24.3**	**27**	**17.5**
Median	**44.5**	**42.5**	**22**		**27.5**	**18.5**	**16.5**

Table of Cell counts per stain, per Analyst, Anaphylaxis versus controls

[Used for Statistical analysis: Wilcoxon Rank Sum test]

Slide ref.	Comments	Cell Count PB	Cell Count LL	Sample Key
AH	Autolytic	90	87	Anaphylaxis
AM		34	19	
AP		28	20	
BH	Follicular	52	19	Control
BM		31	13	
BP		55	15	
CH	Fragmentation	33	64	Control
CM		33	29	
CP		71	67	
DH	Well preserved	2	32	Anaphylaxis
DM		2	7	
DP		0	2	
EH	Autolytic	10	128	Control
EM		4	23	
EP		6	4	
FH	Congested ++	116	87	Control
FM		69	22	
FP		24	23	
GH	Hyperaemic; Beautiful pagoda	54	29	Anaphylaxis
GM		61	29	
GP		82	66	
HH	Follicular, Polis ++ cellular ++	2	23	Anaphylaxis
HM		3	6	
HP		10	4	
IH	Congestion +++,inactive white pulp	3	9	Control
IM		0	0	
IP		27	20	
JH	Pale Diffluent Autolytic	24	17	Control
JM		4	5	
JP		0	8	
KH	Hyperaemic, Pale	16	24	Anaphylaxis
KM		14	13	
KP		15	37	
LH	Congested	2	45	Control
LM	Nuclei of possible eos stain red	1	7	
LP		0	1	

MH	Very reactive, cellular	24	38	Control
MM		10	32	
MP		34	53	
NH	Very crowded, Reactive, Congested	11	128	Anaphylaxis
NM	Cell counts difficult due to ++ cellularity	8	31	
NP		16	32	
OH	Follicular	41	79	Anaphylaxis
OM		23	83	
OP		28	99	
PH	Congested; Autolytic; Non-reactive. Poor staining	55	62	Control
PM		72	31	
PP		6	25	
QH	Extreme congestion ; small follicles -	9	25	Control
QM		13	15	
QP		12	18	
RH	Moderate Autolysis ; bacteria ++; Poor staining	20	10	Anaphylaxis
RM		12	9	
RP		0	0	
SH	Well preserved; Follicular; Congested	6	21	Control
SM		15	7	
SP		8	16	
TH	Very cellular Polis ++	69	25	Control
TM		46	9	
TP		21	13	
UH	well preserved, Congested. Mod reactive	74	38	Anaphylaxis
UM		21	0	
UP		64	45	
VH	well preserved, extremely cellular	226	484	Anaphylaxis
VM		221	413	
VP		181	537	
WH	Congested, Diffluent	42	118	Control
WM		14	92	
WP		52	193	
XH	Very Reactive, follicles, pale staining	31	91	Control
XM		20	59	
XP		25	84	

Table of pathologist's comments per study slide examined, plus cell counts per 10 fields per analyst.

(H = H&E; M = Magenta; P= Pagoda red)

Sample	Description	Case Number
A	Anaphylaxis	DR 2046/2006
D	Anaphylaxis	DR 313/2006
G	Anaphylaxis	DR 1520/2002
H	Anaphylaxis	WC 11/1548/2007
K	Anaphylaxis	DR 313/2006
N	Anaphylaxis	DR 830/2005
O	Anaphylaxis	WC 11/676/2011
R	Anaphylaxis	DR 359/2006
U	Anaphylaxis	DR 982/2005
V	Anaphylaxis	WC 11/873/2013
B	Control	WC 11/2712/2008
C	Control	WC 11/1389/2012
E	Control	DR 2032/2002
F	Control	WC 11/1348/2012
I	Control	WC 11/2385/2009
J	Control	WC 11/1452/2012
L	Control	DR 827/2005
M	Control	WC 11/1372/2012
P	Control	WC 11/1325/2011
Q	Control	WC 11/1456/2012
S	Control	DR 2147/2006
T	Control	WC 11/1670/2012
W	Control	WC 11/720/2012
X	Control	WC 11/1628/2012

Key to the Samples

Ethics approval Letter

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27 March 2015

HREC/REF: 088/2015

Dr L Liebenberg
Forensic Pathology
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Dear Dr Liebenberg

Project Title: AN INVESTIGATION INTO THE USE OF COMMONLY AVAILABLE FABRIC DYE AS A ROUTINE STAIN FOR TISSUE SAMPLES TO BE USED AS A FIRST LINE LOW COST DIAGNOSTIC ADJUNCT FOR THE DIAGNOSIS OF ANAPHYLACTIC DEATH AT AUTOPSY IN A RESOURCE-CHALLENGED ENVIRONMENT

Thank you for your response letter dated 25 February 2015, addressing the issues raised by the Human Research Ethics Committee (HREC).

It is a pleasure to inform you that the HREC has **formally approved** the above mentioned study.

Approval is granted for one year until the 28 March 2016.

Please submit a progress form, using the standardised Annual Report Form, if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.

Please note that the on-going ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the HREC REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

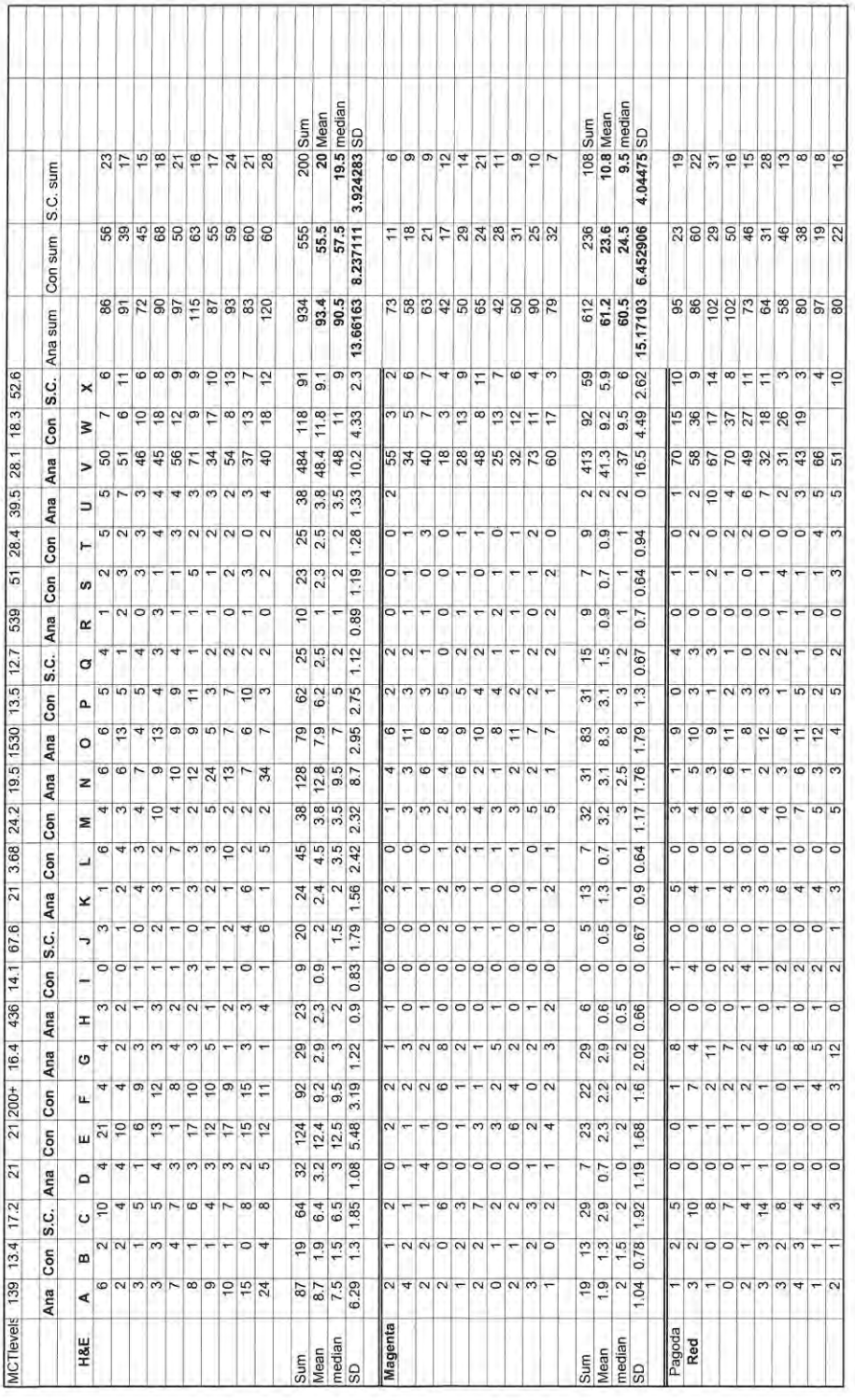
"Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

Hrec/ref:088/2015

Master data sheets showing MCT levels, Sample Identification letter and cell counts per field.

N.B. *Should it be required, a CD-ROM with the full size excel spreadsheets is available, alternatively the full size spreadsheets can be forwarded electronically*.

Key: Ana = Anaphylaxis sample; Con = Control sample; S.C.= Secondary Control sample



MCTlevel<	139	13.4	17.2	21	21	200+	16.4	436	14.1	67.6	21	3.68	24.2	19.5	1530	13.5	12.7	539	51	28.4	39.5	28.1	18.3	52.6					
	Ana	Con	S.C.	Ana	Con	Con	Ana	Ana	Con	S.C.	Ana	Con	Con	Ana	Ana	Con	S.C.	Ana	Con	Con	Ana	Ana	Con	S.C.	Ana sum	Con sum	S.C. sum		
H&E	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X					
	6	2	10	4	21	4	4	3	0	3	1	6	4	6	6	5	4	1	2	5	5	50	7	6	86	56	23		
	2	2	4	4	10	4	2	2	0	1	2	4	3	6	13	5	1	2	3	2	7	51	6	11	91	39	17		
	3	1	5	1	6	9	3	1	1	0	4	3	4	7	4	5	4	0	3	3	3	46	10	6	72	45	15		
	3	3	5	4	13	12	3	3	1	2	3	2	10	9	13	4	3	3	1	4	4	45	18	8	90	68	18		
	7	4	7	3	1	8	4	2	1	1	1	7	4	10	9	9	4	1	1	3	4	56	12	9	97	50	21		
	8	1	6	3	17	10	3	2	3	0	3	3	2	12	9	11	1	1	1	5	2	71	9	9	115	63	16		
	9	1	4	3	12	10	5	1	1	1	2	3	5	24	5	3	2	1	1	2	3	34	17	10	87	55	17		
	10	1	7	3	17	9	1	2	1	2	1	10	2	13	7	7	2	0	2	2	2	54	8	13	93	59	24		
	15	0	8	2	15	15	3	3	0	4	6	2	2	7	6	10	2	1	3	0	3	37	13	7	83	60	21		
	24	4	8	5	12	11	1	4	1	6	1	5	2	34	7	3	2	0	2	2	4	40	18	12	120	60	28		
Sum	87	19	64	32	124	92	29	23	9	20	24	45	38	128	79	62	25	10	23	25	38	484	118	91	934	555	200	Sum	
Mean	8.7	1.9	6.4	3.2	12.4	9.2	2.9	2.3	0.9	2	2.4	4.5	3.8	12.8	7.9	6.2	2.5	1	2.3	2.5	3.8	48.4	11.8	9.1	**93.4**	**55.5**	**20**	Mean	
median	7.5	1.5	6.5	3	12.5	9.5	3	2	1	1.5	2	3.5	3.5	9.5	7	5	2	1	2	2	3.5	48	11	9	**90.5**	**57.5**	**19.5**	median	
SD	6.29	1.3	1.85	1.08	5.48	3.19	1.22	0.9	0.83	1.79	1.56	2.42	2.32	8.7	2.95	2.75	1.12	0.89	1.19	1.28	1.33	10.2	4.33	2.3	**13.66163**	**8.237111**	**3.924283**	SD	
Magenta	2	1	2	0	2	2	1	1	0	0	2	0	1	4	6	2	2	0	0	0	2	55	3	2	73	11	6		
	4	2	1	1	1	2	3	0	0	0	1	0	3	3	11	3	2	1	1	1		34	5	6	58	18	9		
	2	2	1	4	1	2	2	1	0	0	3	6	6	3	1	1	0	3				40	7	7	63	21	9		
	2	0	6	0	0	6	8	0	0	2	2	1	2	4	8	5	0	0	0	0		18	3	4	42	17	12		
	1	2	3	0	1	1	2	0	0	0	3	2	3	6	9	5	2	1	1	1		28	13	9	50	29	14		
	2	2	7	0	3	1	1	0	0	1	1	1	4	2	10	4	2	1	0	1		48	8	11	65	24	21		
	0	1	2	0	3	2	5	1	0	1	0	1	3	1	8	4	1	2	1	0		25	13	7	42	28	11		
	2	1	2	0	6	4	2	0	0	0	0	1	3	2	11	2	1	1	1	1		32	12	6	50	31	9		
	3	2	3	1	2	0	2	1	0	1	1	0	5	2	7	2	2	0	1	2		73	11	4	90	25	10		
	1	0	2	1	4	2	3	2	0	0	2	1	5	1	7	1	2	2	2	0		60	17	3	79	32	7		
Sum	19	13	29	7	23	22	29	6	0	5	13	7	32	31	83	31	15	9	7	9	2	413	92	59	612	236	108	Sum	
Mean	1.9	1.3	2.9	0.7	2.3	2.2	2.9	0.6	0	0.5	1.3	0.7	3.2	3.1	8.3	3.1	1.5	0.9	0.7	0.9	2	41.3	9.2	5.9	**61.2**	**23.6**	**10.8**	Mean	
median	2	1.5	2	0	2	2	2	0.5	0	0	1	1	3	2.5	8	3	2	1	1	1	2	37	9.5	6	**60.5**	**24.5**	**9.5**	median	
SD	1.04	0.78	1.92	1.19	1.68	1.6	2.02	0.66	0	0.67	0.9	0.64	1.17	1.76	1.79	1.3	0.67	0.7	0.64	0.94	0	16.5	4.49	2.62	**15.17103**	**6.452906**	**4.04475**	SD	
Pagoda	1	2	5	0	0	1	8	0	1	0	5	0	3	1	9	0	4	0	1	0	1	70	15	10	95	23	19		
Red	3	2	10	0	1	7	4	0	4	0	4	0	4	5	10	3	3	0	1	2	2	58	36	9	86	60	22		
	1	0	8	0	1	2	11	0	0	6	1	0	6	3	9	1	3	0	2	0	10	67	17	14	102	29	31		
	0	0	7	0	1	2	7	0	2	0	4	0	3	6	11	2	1	0	1	2	4	70	37	8	102	50	16		
	2	1	4	1	1	2	2	1	4	0	3	0	6	1	8	3	0	0	0	2	6	49	27	11	73	46	15		
	3	3	14	1	0	1	4	0	1	1	3	0	4	2	12	3	2	0	1	0	7	32	18	11	64	31	28		
	3	2	8	0	0	0	5	1	2	0	6	1	10	3	6	1	2	1	4	0	2	31	26	3	58	46	13		
	4	3	4	0	0	1	8	0	2	0	4	0	7	6	11	5	1	1	1	0	3	43	19	3	80	38	8		
	1	1	4	0	0	4	5	1	2	0	4	0	5	3	12	2	0	0	1	4	5	66		4	97	19	8		
	2	1	3	0	0	3	12	0	2	1	3	0	5	3	4	5	2	0	3	3	5	51		10	80	22	16		

Sum	20	15	67	2	4	23	66	3	20	8	37	1	53	33	92	25	18	2	15	13	45	537	195	83	837	364	176	Sum
Mean	2	1.5	6.7	0.2	0.4	2.3	6.6	0.3	2	0.8	3.7	0.1	5.3	3.3	9.2	2.5	1.8	0.2	1.5	1.3	4.5	53.7	24.4	8.3	**83.7**	**36.4**	**17.6**	Mean
median	2	1.5	6	0	0	2	6	0	2	0	4	0	5	3	9.5	2.5	2	0	1	1	4.5	54.5	22.5	9.5	**83**	**34.5**	**16**	median
SD	1.18	1.02	3.26	0.4	0.49	1.9	3.04	0.46	1.18	1.78	1.27	0.3	2	1.73	2.48	1.57	1.25	0.4	1.12	1.42	2.58	14.2	8.03	3.58	**14.73126**	**13.04761**	**7.255343**	SD
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X				
			Peters results																						Peters resu	Peters resu	Peters resu	Peters results
H&E	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X				
	24	7	2	1	0	12	6	0	0	1	0	0	1	10	2	6	2	0	5	9	4	24	6	13	71	46	18	
	10	2	3	1	0	5	6	0	0	3	4	0	7	0	8	5	0	4	0	25	7	19	0	6	59	44	12	
	23	6	3	0	0	7	4	0	1	3	2	1	1	0	5	4	0	0	0	3	12	33	2	1	79	25	7	
	11	27	2	0	2	8	4	0	1	2	4	0	3	0	6	3	1	1	0	8	4	30	8	0	60	60	5	
	7	3	1	0	0	13	0	0	0	6	0	0	4	0	2	6	2	2	0	2	0	23	2	1	34	30	10	
	7	0	0	0	0	13	1	0	0	1	0	0	3	1	2	4	0	4	0	3	3	26	8	4	44	31	5	
	2	3	3	0	4	18	4	0	0	5	4	0	4	0	4	6	0	0	0	4	3	13	3	0	30	42	8	
	0	3	8	0	1	12	21	0	0	2	0	1	1	0	2	12	2	2	1	1	0	18	1	3	43	33	15	
	0	1	6	0	2	9	8	0	1	0	0	0	0	0	10	6	2	3	0	5	3	7	4	1	31	28	9	
	6	0	5	0	1	19	0	2	0	1	2	0	0	0	0	3	0	4	0	1	0	33	8	2	47	32	8	
Sum	90	52	33	2	10	116	54	2	3	24	16	2	24	11	41	55	9	20	6	61	36	226	42	31	498	371	97	Sum
Mean	9	5.2	3.3	0.2	1	11.6	5.4	0.2	0.3	2.4	1.6	0.2	2.4	1.1	4.1	5.5	0.9	2	0.6	6.1	3.6	22.6	4.2	3.1	**49.8**	**37.1**	**9.7**	Mean
median	7	3	3	0	0.5	12	4	0	0	2	1	0	2	0	3	5.5	0.5	2	0	3.5	3	23.5	3.5	1.5	**45.5**	**32.5**	**8.5**	median
SD	8.09	7.59	2.28	0.4	1.26	4.29	5.78	0.6	0.46	1.8	1.74	0.4	2.11	2.98	2.98	2.46	0.94	1.61	1.5	6.8	3.5	8.09	2.93	3.75	**16.04244**	**10.173**	**4.00125**	SD
Magenta	2	1	0	0	2	3	1	1	0	0	0	0	0	4	3	5	2	2	0	0	11	22	3	1	46	14	3	
	3	3	0	0	0	7	4	1	0	0	0	1	0	1	1	5	1	0	2	16	0	34	0	0	44	34	1	
	1	0	2	0	0	10	3	1	0	0	0	0	3	2	0	15	1	0	0	5	0	30	0	3	37	33	6	
	6	21	0	0	0	7	7	0	0	0	1	0	0	1	1	2	2	2	2	5	1	21	0	0	40	37	2	
	10	3	1	0	0	9	3	0	0	0	0	0	1	0	0	6	0	2	0	7	3	26	0	2	44	26	3	
	3	0	4	1	2	5	8	0	0	0	3	0	1	0	2	5	2	1	4	0	0	20	0	2	38	17	8	
	1	0	12	0	0	3	4	0	0	0	4	0	2	0	0	13	2	2	2	1	0	10	2	2	21	23	16	
	1	0	4	1	0	13	21	0	0	0	4	0	0	0	4	6	3	3	2	3	0	23	5	2	57	29	9	
	3	0	4	0	0	3	6	0	0	0	2	0	1	0	9	5	0	0	3	0	3	21	1	5	44	13	9	
	4	3	6	0	0	9	4	0	0	0	0	0	2	0	3	10	0	0	0	0	3	14	3	3	28	27	9	
Sum	34	31	33	2	4	69	61	3	0	0	14	1	10	8	23	72	13	12	15	37	21	221	14	20	399	253	66	Sum
Mean	3.4	3.1	3.3	0.2	0.4	6.9	6.1	0.3	0	0	1.4	0.1	1	0.8	2.3	7.2	1.3	1.2	1.5	3.7	2.1	22.1	1.4	2	**39.9**	**25.3**	**6.6**	Mean
median	3	0.5	3	0	0	7	4	0	0	0	0.5	0	1	0	1.5	5.5	1.5	1.5	2	2	0.5	21.5	0.5	2	**42**	**26.5**	**7**	median
SD	2.65	6.11	3.52	0.4	0.8	3.24	5.34	0.46	0	0	1.62	0.3	1	1.25	2.61	3.89	1	1.08	1.36	4.78	3.24	6.62	1.69	1.41	**9.43875**	**8.013114**	**4.317407**	SD
Pagoda	3	1	7	0	1	1	15	0	4	0	0	0	3	0	0	1	0	0	1	1	2	23	4	2	43	17	9	
Red	2	3	3	0	2	2	13	5	3	0	1	0	1	0	3	1	0	0	1	2	1	28	2	2	53	17	5	

	5	28	7	0	0	1	6	1	2	0	3	0	4	2	0	1	2	0	1	3	3	14	6	1	34	46	10		
	0	15	4	0	0	3	11	0	2	0	0	0	2	0	1	0	3	0	1	0	1	13	9	4	26	32	11		
	5	2	3	0	0	3	2	0	4	0	1	0	10	0	5	0	1	0	0	0	1	21	3	1	35	22	5		
	5	0	7	0	0	5	3	2	2	0	1	0	1	1	1	1	2	0	1	0	3	12	7	3	28	17	12		
	1	1	15	0	2	2	12	0	2	0	3	0	2	2	2	2	1	0	2	1	2	14	2	1	36	16	17		
	4	2	9	0	0	1	6	0	0	0	4	0	2	1	2	0	1	0	0	1	1	18	5	3	36	11	13		
	2	0	13	0	0	3	9	1	4	0	0	0	4	4	7	0	1	0	0	0	2	20	7	4	45	18	18		
	1	3	3	0	1	3	5	1	4	0	2	0	5	6	7	0	1	0	1	0	3	18	7	4	43	24	8		
Sum	28	55	71	0	6	24	82	10	27	0	15	0	34	16	28	6	12	0	8	8	19	181	52	25	379	220	108	Sum	
Mean	2.8	5.5	7.1	0	0.6	2.4	8.2	1	2.7	0	1.5	0	3.4	1.6	2.8	0.6	1.2	0	0.8	0.8	1.9	18.1	5.2	2.5	**37.9**	**22**	**10.8**	Mean	
median	2.5	2	7	0	0	2.5	7.5	0.5	2.5	0	1	0	2.5	1	2	0.5	1	0	1	0.5	2	18	5.5	2.5	**36**	**17.5**	**10.5**	median	
SD	1.78	8.57	4.01	0	0.8	1.2	4.21	1.48	1.27	0	1.36	0	2.54	1.91	2.52	0.66	0.87	0	0.6	0.98	0.83	4.81	2.27	1.2	**7.751774**	**9.633276**	**4.190465**	SD	
MCTlevels	139	13.4	17.2	21	21	200+	16.4	436	14.1	67.6	21	3.68	24.2	19.5	1530	13.5	12.7	539	51	28.4	39.5	28.1	18.3	52.6					

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You CH, Lee KY, Chey RY, Menguy R. Electrogastrographic study of patients with unexplained nausea, bloating and vomiting. Gastroenterology 1980;79:311-4.

Goate AM, Haynes AR, Owen MJ, Farrall M, James LA, Lai LY et al. Predisposing locus for Alzheimer's disease on chromosome 21. Lancet 1989;1:352-5.

2. Organization as author

The Royal Marsden Hospital Bone-Marrow Transplantation Team.Failure of syngeneic bone-marrow graft without preconditioning in post-hepatitis marrow aplasia. Lancet 1977;2:742-4.

3. No author given

Coffee drinking and cancer of the pancreas [editorial]. BMJ 1981;283:628.

4. Article not in English

Massone L, Borghi S, Pestarino A, Piccini R, Gambini G. Localisations palmairespurpuriques de la dermatiteherpetiforme. Ann DermatolVenereol 1987;114:1545-7.

5. Volume with supplement

Magni F, Rossoni G, Berti F. BN-52021 protects guinea-pig from heart anaphylaxis. Pharmacol Res Commun 1988;20Suppl 5:75-8.

6. Issue with supplement

Gardos G, Cole JO, Haskell D, Marby D, Paine SS, Moore R.The natural history of tardive dyskinesia. J ClinPsychopharmacol 1988;8(4 Suppl):31S-37S.

7. Volume with part

Hanly C. Metaphysics and innateness: a psychoanalytic perspective. Int J Psychoanal 1988;69(Pt 3):389-99.

8. Issue with part

Edwards L, Meyskens F, Levine N. Effect of oral isotretinoin on dysplastic nevi. J Am AcadDermatol 1989;20(2 Pt 1):257-60.

9. Issue with no volume

Baumeister AA. Origins and control of stereotyped movements.Monogr Am AssocMentDefic1978;(3):353-84.

10. No issue or volume

Danoek K. Skiing in and through the history of medicine. Nord MedicinhistArsb 1982;86-100.

11. Pagination in roman numerals

Ronne Y. AnsvarsfallenBlodtransfusion till felpatient.Vardfacket 1989;13:XXXVI-XXVII.

12. Type of article indicated as needed

Spargo PM, Manners JM. DDAVP and open heart surgery [letter]. Anaesthesia 1989;44:363-4.

13. Article containing retraction
Shishido A. Retraction notice.Effect of platinum compounds on murine lymphocyte mitogenesis [Retraction of Alsabti EA, Ghalib ON, Salem MN. In: Jpn J Med SciBiol 1979;32:53-65]. Jpn J Med SciBiol 1980;33:235-7.

14. Article retracted

Alsabti EA, Ghalib ON, Sale MN. Effect of platinum compounds on murine lymphocyte mitogenesis [Retracted by Shishido A. In: Jpn J Med SciBiol 1980;33:235-7]. Jpn J Med SciBiol 1979;32:53-65.

15. Article containing comment

Piccoli A, Bossatti A. Early steroid therapy in IgA neuropathy: still an open question [comment] Nephron 1989;51:289-91. Comment on: Nephron 1988;48:12-7.

16. Article commented on

Kobayashi Y, Fujii K, Hiki Y, Tateno S, Kurokawa A, Kamiyama M. Steroid therapy in IgA neuropathy: a retrospective study in heavy proteinuric cases [see comments]. Nephron 1988;48:12-7. Comment in: Nephron 1989;51:289-91.

17. Article with published erratum

Schofield A. The CAGE questionnaire and psychological health [published erratum appears in Br J Addict 1989;84:701]. Br J Addict 1988;83;761-4.

Books and Other Monographs

18. Personal author(s)

Colson JH, Armour WJ. Sports injuries and their treatment. 2nd rev. ed. London: S. Paul, 1986.

19. Editor(s), compiler as author

Diener HC, Wilkinson M, editors.Drug-induced headache. New York: Springer-Verlag, 1988.

20. Organization as author and publisher

Virginia Law Foundation.The medical and legal implications of AIDS. Charlottesville: The Foundation, 1987.

21. Chapters in a book

Weinstein L, Swartz MN.Pathologic properties of invading microorganisms. In: Sodeman WA Jr, Sodeman WA, editors. Pathologic physiology: mechanisms of disease. Philadelphia: Saunders, 1974;457-72.

22. Conference proceedings

Vivian VL, editor. Child abuse and neglect: a medical community response. Proceedings of the First AMA National Conference on Child Abuse and Neglect; 1984 Mar 30-31; Chicago. Chicago: American Medical Association, 1985.

23. Conference paper

Harley NH. Comparing radon daughter dosimetric and risk models. In: Gammage RB, Kaye SV, editors. Indoor air and human health.Proceedings of the Seventh Life Sciences Symposium; 1984 Oct 29-31; Knoxville (TN). Chelsea (MI): Lewis, 1985;69-78.

24. Scientific or technical report

Akutsu T. Total heart replacement device. Bethesda (MD): National Institutes of Health, National Heart and Lung Institute; 1974 Apr. Report No.: NIH-NHLI-691 218514.

25. Dissertation

Youssef NM. School adjustment of children with congenital heart disease [dissertation]. Pittsburgh (PA): Univ. of Pittsburgh, 1988.

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Harred JF, Knight AR, McIntyre JS, inventors.Dow Chemical Company, assignee.Epoxidation process. US patent 3,654,317. 972 Apr 4.

Other Published Material

27. Newspaper article

Rensberger B, Specter B. CFCs may be destroyed by natural process. The Washington Post 1989 Aug 7; Sect. A:2 (col. 5).

28. Audiovisual

AIDS epidemic: the physician's role [videorecording]. Cleveland (OH): Academy of Medicine of Cleveland, 1987.

29. Computer file

Renal system [computer program].MS-DOS version. Edwardsville (KS): MediSim, 1988.

30. World Wide Web address or URL

http://www.uocf.edu/pharmacy/depts/drugdose/barbitutuates/index.html

31. Legal material

Toxic Substances Control Act: Hearing on S. 776 Before the Subcomm. on the Environment of the Senate Comm. on Commerce. 94th Cong., 1st Sess. 343 (1975).

32. Map

Scotland [topographic map]. Washington: National Geographic Society (US), 1981.

33. Book of the Bible

Ruth 3:1-18. The Holy Bible. Authorized King James version. New York: Oxford Univ. Press, 1972.

34. Dictionary and similar references

Ectasia. Dorland's illustrated medical dictionary. 27th ed. Philadelphia: Saunders, 1988;527.

35. Classical material

The Winter's Tale: act 5, scene 1, lines 13-16. The complete works of William Shakespeare. London: Rex, 1973.

Unpublished Material

36. In press
Lillywhite HD, Donald JA. Pulmonary blood flow regulation aquatic snake. Science.In press.

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