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Postmortem Biochemistry – Analysis of Metabolic Imbalance



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Postmortem biochemistry - analysis of metabolic imbalance

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ACADEMIC DISSERTATION

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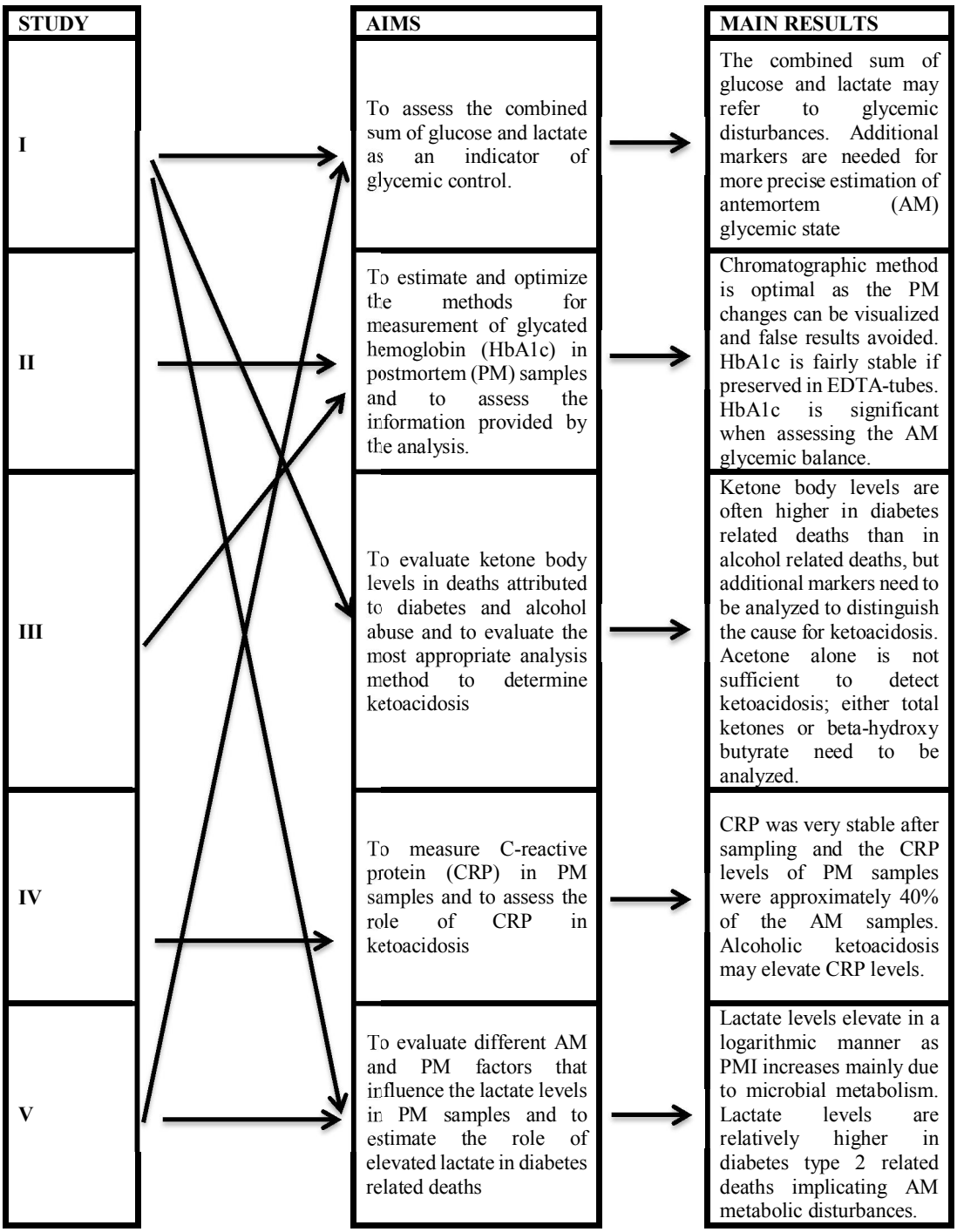
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ABBREVIATIONS

1,5- AG	1,5-anhydroglucitol
AcAc	acetoacetate
AGE	advanced glycated end product
AKA	alcoholic ketoacidosis
AM	antemortem
AUD	alcohol use disorder
BHB	beta-hydroxy butyrate
BHBD	beta-hydroxy butyrate dehydrogenase
CF	cerebrospinal fluid
CRP	c-reactive protein
CoA	coenzyme A
CoD	cause of death
G6P	glucose 6-phosphate
GC	gas chromatography
DKA	diabetic ketoacidosis
DM	diabetes mellitus
DM1	diabetes mellitus type I
DM2	diabetes mellitus type II
EDTA	ethylenediaminetetraacetic acid
HbA1c	glycated hemoglobin
HHS	hyperglycemic hyperosmolar state
INT	iodonitrotetrazolium
KPDM2	ketosis-prone diabetes mellitus type II
LDH	lactate dehydrogenase
MeG	methylglyoxal
$NADH/NAD^+$	nicotinamide adenine dinucleotide
NaF	sodium fluoride
PCA	perchloric acid
PF	pericardial fluid
PM	postmortem
PMI	postmortem interval
RI	reference interval
VH	vitreous humor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-V:

I Keltanen T, Sajantila A, Palo JU, Partanen T, Valonen T, Lindroos K. Assessment of Traub formula and ketone bodies in cause of death investigations. Int J Legal Med. 2013 Nov;127(6):1131-7.

II Keltanen T, Sajantila A, Valonen T, Vanhala T, Lindroos K. Measuring postmortem glycated hemoglobin - A comparison of three methods. Leg Med (Tokyo). 2013 Mar;15(2):72-8.

III Keltanen T, Sajantila A, Valonen T, Vanhala T, Lindroos K. HbA1c method evaluation for postmortem samples. Forensic Sci Med Pathol. 2015 Mar;11(1):35-9.

IV Lindroos-Jokinen K, Keltanen T, Vanhala T, Valonen T, Sajantila A. Postmortem measurement of C-reactive protein and interpretation of results in ketoacidosis. Leg Med (Tokyo). 2012 May;14(3):140-6.

V Keltanen T, Nenonen T,Ketola RA, Ojanperä I, Sajantila A, Lindroos K. Post-mortem analysis of lactate concentration in diabetics and metformin poisonings. Int J Legal Med. Published Online 2015 Sep.

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ABSTRACT

Postmortem (PM) biochemistry is utilized in cause of death (CoD) investigations. The challenges in PM biochemistry are caused mainly by the PM changes. Analytes may be degraded or denatured leading to methodological difficulties. In addition, the re-distribution may cause falsely elevated values when compared to clinical samples. Analytes may also be consumed or produced by microbial metabolism. The sample matrix is often different from that in clinical biochemistry and the reference levels need be obtained through research. The actual death process and agonal period can also lead to unique changes in the analyte concentrations. Despite these challenges, with a sufficient number of samples and adequate research it is possible to optimize biochemical methods for PM samples and obtain reference values for certain conditions.

The biochemical results can provide supporting information in cases where the death is caused or associated with metabolic disturbances for instance in diabetes mellitus (DM) or alcohol abuse. Hyperglycemia and ketoacidosis in diabetes can be fatal if not treated. The macroscopical, histological and toxicological findings in autopsy may be scarce or even absent. Determination of vitreous humor (VH) glucose and ketone bodies are very informative in these cases. As ketoacidosis can be present also after heavy alcohol use, the determination of glycemic status prior to death is essential in distinguishing between these two conditions. Glycated hemoglobin (HbA1c) provides information on the glycemic balance in previous weeks.

In our studies, we have assessed the results, methods and interpretation provided by analysis of glucose, lactate, ketone bodies, HbA1c and C-reactive protein (CRP) in PM samples. Optimization has been performed in routine casework according to our findings providing more accurate information to the medico-legal pathologists in their CoD investigation work. In addition, our results have provided some insights into the pathology of severe diabetic emergencies as well as to ketoacidosis due to alcohol.

According to our results, we recommend that in DM and alcohol abuse related deaths VH glucose, total ketone bodies or beta-hydroxy butyrate and blood HbA1c should always be analyzed. Lactate levels may provide additional information, when the results are interpreted considering the PM interval (PMI). These analyses are recommended also in cases where no suspicion of metabolic disturbances exists, but the findings in autopsy are scarce.

The collaboration between the medico-legal pathologists and the laboratory is very important for the development and understanding of PM biochemistry and to include novel analyses to support the CoD investigation. The CoD determination is very important not only on the individual level, but also nationally, since Finnish Statistics collects the CoD data, which can be than further utilized in recommendations concerning health and primary prevention.

INTRODUCTION

Forensic biochemistry can be defined as "the study of the biochemical parameters in the cadaver, to solve the problems that outline the diagnosis of the cause and circumstances of the death" [1]. Coe et al. have described a comprehensive range of different biochemical analytes that can be useful in forensic medicine [2]. These and others have been utilized in cause of death (CoD) investigations or in postmortem (PM) research concerning for instance evaluation of glycemic status in suspected diabetic deaths [3-9], sepsis or inflammation [10-15], metabolic disturbances caused by alcohol abuse [16-19], long term alcohol abuse [20-22], sudden cardiac deaths [23-29] and deaths caused by hypo- or hyperthermia [30-34]. In addition to diagnostic purposes, biochemical markers have been utilized in time of death estimations [35,36].

Despite the various biochemical assays available, PM biochemistry is not very often included as a routine process in forensic autopsies [37]. The application of biochemical methods for analysis of PM samples is rarely straightforward [38], which may lead to suspicion about the reliability of the results. PM changes, such as autolysis and putrefaction, may complicate the analysis and cause erratic results [39]. The extent of PM changes depends mostly on the PMI, but also on the antemortem (AM) and PM environmental circumstances and pathological conditions prior to death [40,41].

Because of PM changes and potential body destruction the sample matrices needed are not always available. Plasma or serum is often the matrix of choice in clinical biochemistry, whereas PM samples do not always allow the separation of these from whole blood. Blood is also prone to alterations caused by bacterial metabolism and autolysis. Therefore, alternative sample matrices for blood (or plasma/serum), such as the vitreous humor (VH) of the eye, cerebrospinal fluid (CF) and pericardial fluid (PF), are preferred. If the matrix differs from the one used in clinical biochemistry, the correlation of the concentrations in different matrices needs to be evaluated. The distribution of the analyte or biomolecule in tissues and body fluids is relevant. The PM redistribution is better described in forensic toxicology regarding drugs [42]. It is also relevant in PM biochemistry if the analyte is for instance a marker of tissue disruption such as the liver enzymes, alanine aminotransferase and aspartate aminotransferase [43], when autolysis greatly influences the concentration in blood.

Also the interpretation of PM results often differs compared to clinical biochemistry. The reference intervals (RIs) of clinical assays are usually set so that 95 % of healthy individuals are within the reference range [44]. No public databases for PM biochemical RIs exist, so each laboratory needs to set their own based on the particular sample material that is analyzed and the methods that are used in the unit [45]. RIs need to be set so that in addition to the population and the method used, the effects of PM changes are taken into account. In addition, the purpose of the analysis should be addressed; for example is it relevant to know any slight changes in blood

glucose, or is the aim only to find cases where the glycemic control is so unbalanced that the condition may be fatal?

The PM changes cause difficulties in analyzing samples as well as the interpretation of the results. In addition, there are different opinions on which markers are the relevant ones to support the diagnosis of the CoD. Therefore, a suitable combination of metabolic markers is needed [6,8]. The challenges of PM biochemistry can be addressed by research and co-operation between the laboratory and forensic pathologists performing the autopsies. By using a multidisciplinary approach, the most suitable combination of biochemical markers can be found providing essential information for the CoD investigation [37].

This thesis focuses on metabolic acidosis in diabetes and alcohol abuse caused by elevated ketone bodies, lactate or both. Glycemic disturbances in diabetes mellitus (DM) are also addressed. The preferable combination of metabolites to be analyzed, the analysis methods and interpretation of results are presented and discussed.

REVIEW OF THE LITERATURE

1. Derangements in carbohydrate metabolism causing acidosis

In the normal physiological state, glucose is used as a source of energy in the cells or it can be stored in the form of glycogen in the liver and skeletal muscle cells when energy is abundant [46]. In DM the glucose metabolism is impaired causing hyperglycemia if not treated. Ketoacidosis can result if fats are used for energy in the absence of intracellular glucose [47]. Another cause for ketoacidosis and possible concomitant lactic acidosis is long-term alcohol abuse [48]. These derangements are often severe and can be fatal when untreated.

1.1 Diabetes and its acute complications

DM is a range of metabolic diseases, where the secretion or the effect of insulin is insufficient leading to elevated blood glucose [49,50]. Most of the patients belong to either type 1 diabetes (DM1) or type 2 diabetes (DM2). Other categories of diabetes include gestational diabetes and other specific types caused for instance by genetic defects in insulin secretion or action, diseases of the exocrine pancreas, endocrinopathies, drugs or chemicals and infections [50,51]. In DM1 the insulin deficiency is caused by the autoimmune destruction of the β-cells in the pancreas and subsequent lack of excretion [52], whereas in DM2 the effect of insulin is diminished causing relative deficiency [53]. Consistently elevated blood glucose in DM causes several macro- and microvascular complications, such as retinopathy [54], neuropathy [55] and cardiovascular disease [56,57].

Acute complications of diabetes or its treatment are hypoglycemia [58], diabetic ketoacidosis (DKA) and hyperglycemic hyperosmolar state (HHS) [59-61]. Severe hypoglycemia is diagnosed when 1) blood glucose is less than 3.9 mM, the symptoms are typical to hypoglycemia and they are resolved after glucose administration and 2) there is a need for medical or non-medical external help [58]. DKA results from inadequate insulin amount or effect and the increased action of counterregulatory hormones (glucagon, growth hormone, catecholamines and cortisol) that lead to hyperglycemia and ketoacidosis [60]. HHS is caused by the same factors as DKA. The differences between these are that in HHS the dehydration is greater than in DKA, but insulin secretion is not as diminished preventing ketosis [62]. Typical diagnostic laboratory findings in DKA and HHS are presented in Table 1 [60].

Table 1. Clinical laboratory findings in diabetic ketoacidosis (DKA) and the hyperglycemic hyperosmolar state (HHS)

	Severe DKA	HHS
Plasma glucose (mM)	>14	>33
Arterial pH	< 7	>7.30
Serum bicarbonate (mM)	< 10	> 15
Urine/Serum ketones	positive	small
Serum osmolality (mOsm/kg)	variable	> 320
Anion gap (mM)	> 12	< 12

1.2 Alcohol abuse and metabolic disturbances

Alcohol abuse and dependence predict markedly elevated risk of mortality [63]. In a recent study by Westman et al. it was found that mortality among patients suffering from alcohol use disorder (AUD) was significantly higher than in the general populations in Finland, Sweden and Denmark [64]. The causes for premature deaths were alcohol related diseases and medical conditions, suicide and other external causes. The bias in their study was that it included only hospitalized patients with AUD, who are likely to have more severe health issues than patients outside of medical care. In 2013 in Finland 15% of all deaths of working-age (15-64 years) people were due to alcohol related diseases and accidental poisoning by alcohol [65]. Alcohol related deaths were the third most common CoD in this age group, which makes it a significant public health issue. Common CoD's due to alcohol are liver diseases, pancreatic diseases, cancer and cardiovascular diseases [66] in addition to alcohol poisonings [67]. Alcohol is also often a contributing CoD in accidents [67].

In addition to idiopathic, autoimmune and genetic factors, also alcohol may cause acute and chronic pancreatitis [68,69]. The acute form can further evolve to the chronic form after recurrent episodes [70]. Chronic pancreatitis is the cause in almost 80% of the cases of secondary diabetes due to pancreatic diseases (type 3c diabetes; DM3c) [71]. The impairment of glucose metabolism in DM3c is variable from mild to severe and the condition is complex due to the destruction of exocrine and glucagon producing cells in addition to insulin producing cells causing a "brittle" disease, in which the glycemic balance is difficult to sustain [71,72]. These large swings in blood glucose level predispose individuals to the acute metabolic emergencies of DM.

Alcohol abuse can cause metabolic disturbances that are not related to DM, although the manifestations are similar [73]. Ketoacidosis in non-diabetic adults after prolonged alcohol consumption was first described by Dillon et al. in 1940 [74]. This syndrome was later defined as alcoholic ketoacidosis (AKA) by Jenkins et al. [75]. It is typically presented after binge drinking and poor nutrition, and may be worsened with concomitant lactic acidosis [76]. In addition to AKA and lactic acidosis [77], heavy alcohol use may cause severe hypoglycemia [78].

1.3 Formation of ketone bodies and ketoacidosis

Ketone bodies are formed in the liver as a result of fatty acid oxidation in low carbohydrate conditions. Normally, fatty acids and glucose are metabolized to acetyl coenzyme A (acetyl CoA), which enters the citric acid cycle by condensing with oxaloacetate [79]. If glycolysis is insufficient the acetyl CoA molecules from fatty acid oxidation are used for ketogenesis. Two acetyl CoA molecules condense into acetoacetyl CoA, which forms the ketone body acetoacetate (AcAc) through 3-hydroxy-3-methylglutaryl CoA. AcAc can be further reduced to another ketone body, beta-hydroxybutyrate (BHB), in nicotineamide adenine dinucleotide (NADH; reduced form, NAD^+; oxidized form) dependent reaction catalyzed by beta- hydroxybutyrate dehydrogenase (BHBD). AcAc can also be decarboxylated to form acetone. In ketosis, the excess acetone may further form isopropyl alcohol in some cases [80,81]. The main components of ketone body formation are presented in Figure 1.

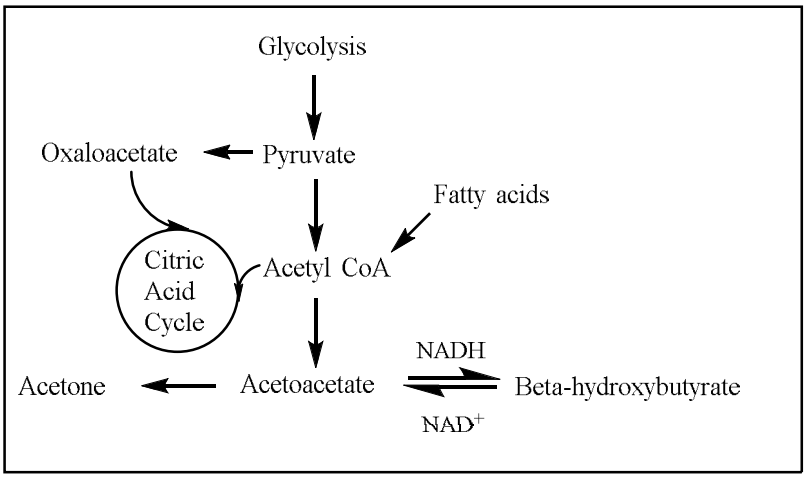


Figure 1 Sources of ketone bodies; acetyl CoA is formed in glucose or fatty acid metabolism. If abundant, acetyl CoA is not shuttled into the citric acid cycle but instead forms acetoacetate.

Common causes for ketoacidosis are diabetes, alcohol abuse and starvation. In DKA the deficiency of insulin impairs the carbon hydrate metabolism, which in turn leads to hyperglycemia followed by lipolysis and ketogenesis induced by counterregulatory hormones, such as glucagon, cortisol, growth hormone and catecholamines. DKA was thought to be associated mainly with DM1 and it occurred only rarely in DM2 during acute illness where the counterregulatory hormones are elevated. Later it has been found that ketoacidosis is also often associated with DM2 [82,83]. In addition, in a subtype of diabetes called ketosis-prone type 2 diabetes (KPDM2), DKA may occur without the precipitating factors. KPDM2 was originally

found among African-Americans, but later also in several other populations [84]. Linfoot et al. [85] compared DM2 and KPDM2 groups and found that the hormonal levels in both groups were similar. They suggested that ketoacidosis in KPDM2 is caused by impaired insulin secretion that can be resolved later during treatment.

The elevation of ketones after heavy alcohol consumption is caused primarily by an elevated NADH/NAD^+ ratio [86,87]. The metabolism of alcohol and following elevated NADH/NAD^+ ratio reduce gluconeogenesis and aerobic oxidation in the citric acid cycle. Counterregulatory hormones induce lipolysis and fatty acid oxidation and the production of acetyl CoA. The blood glucose level is often normal or even low, especially if the prolonged alcohol abuse is associated with malnourishment and the glycogen storages are depleted [76,88,89]. In addition to an elevated NADH/ NAD^+ ratio, excess acetate is formed in alcohol metabolism. Acetate could serve as a precursor for ketone bodies as presented in Figure 2, although there have been some controversial findings about this. For instance, Yamashita et al. concluded in their *in vitro* study that although acetate is formed in the liver due to alcohol metabolism, it is not further metabolized, but utilized in peripheral tissues [90].

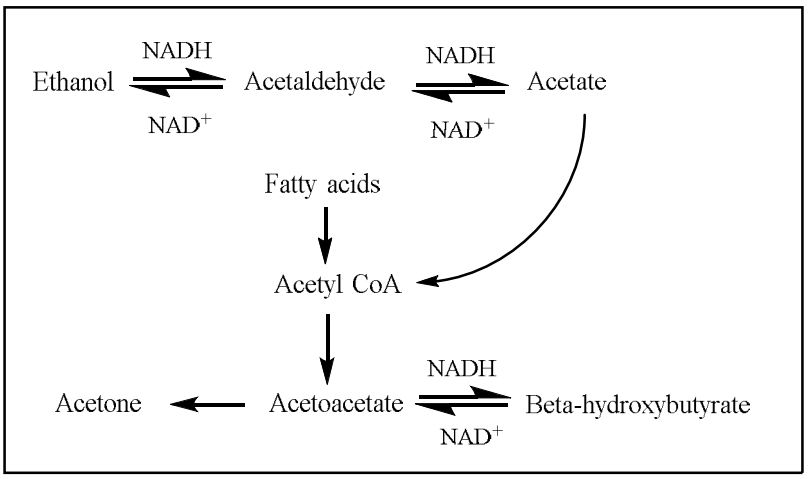


Figure 2 Formation of ketone bodies in alcoholic ketoacidosis; enhanced lipolysis and possibly the acetate from alcohol metabolism form excess acetyl CoA, which further forms acetoacetate.

In diabetes care the method for ketone testing has traditionally been a test based on the reaction of AcAc in urine or blood in the presence of alkali with nitroprusside (nitroferricyanide) to produce a colored complex [86]. As this method relies on the AcAc concentration and not the more predominating BHB, it is not as sensitive a marker in DKA as BHB [91]. The recommended assay in DM for ketones is therefore a test that quantifies blood BHB [92]. In AKA the

BHB/AcAc ratio is even higher than in DKA [93], which would suggest that BHB is the optimal marker for ketoacidosis.

1.4 Lactic acidosis in diabetes and alcohol abuse

Lactate is produced in glycolysis from pyruvate under anaerobic conditions. Pyruvate is reduced by lactate dehydrogenase (LDH) and the cofactor NADH is oxidized to NAD+. Normally most of the pyruvate is transformed into acetyl CoA, which enters the citric acid cycle. An elevation of NADH/NAD^+ ratio and the accumulation of pyruvate favor the formation of lactate. Lactate is converted back to pyruvate in the liver or kidneys and used for the formation of glucose in the Cori cycle [94]. Small amounts can be excreted in urine. Lactic acidosis occurs if the production of lactate exceeds its utilization. Cohen and Woods [95] classified lactic acidosis into two subtypes A and B. Type A lactic acidosis is caused by insufficient oxygen delivery and type B by inadequate utilization of oxygen e.g. in diabetes, the systemic inflammatory response syndrome (SIRS), mitochondrial myopathies, malignancy and in some intoxications [96].

Lactic acidosis in DM2 has been thought to be associated with the biguanides (phenformin and metformin) used in the therapy of diabetes. Phenformin was withdrawn as it caused severe lactic acidosis [97], and since metformin belongs to the same drug class it has also raised questions about the safety of the treatment [98]. However, a systematic Cochrane review by Salpeter et al. [99] stated that the incidence of lactic acidosis is similar with the metformin treated patients and patients treated with other anti-hyperglycemic medications. Therefore, the lactic acidosis may rather be coincidental due to other precipitating causes then metformin medication [100,101].

As ethanol metabolism elevates the NADH/NAD^+ ratio (Figure 2) the conversion of pyruvate to lactate is enhanced and may lead to lactic acidosis in alcoholics. If the alcohol abuse has caused malnourishment, an additional risk for lactic acidosis exists due to thiamine deficiency [102]. Thiamine is an essential cofactor for several enzymes and its deficiency leads to impaired aerobic glucose metabolism and subsequent lactic acidosis [103].

1.5 D-lactate in diabetes

Under normal conditions, lactate in humans is mainly in the L-isoform and only a small amount of D-lactate is formed from methyl glyoxal (MeG) in the glyoxalase system in cytosol and cell organelles, especially mitochondria [104]. D-lactate can also be formed in the bowel as a result of microbial fermentation. D-lactic acidosis is a rare neurologic syndrome most commonly caused by malabsorption (e.g. in short bowel syndrome) of relatively large amounts of fermentable carbohydrates [105,106]. Elevated D-lactate levels have been found also in diabetics due to hyperglycemia and/or ketoacidosis, which elevate MeG amounts that in turn are metabolized into D-lactate [107,108]. MeG is a significant factor in diabetic complications, since it is a very

efficient glycating agent and serves as a precursor for advanced glycated end products (AGEs) that cause microvascular complications [109]. D-lactate measurements have been evaluated for assessing the AGE load and also for diagnosis of D-lactic acidosis [110,111]. The evaluation of D-lactate measurements has not been performed for PM samples.

2. Postmortem analysis of metabolic disturbances

Biochemical markers are informative in deaths due to metabolic disturbances in DM and alcohol abuse, since these conditions may not be detectable in autopsy by other means. In histological examination, diabetic nephropathy or fatty liver may be present [112]. Also glycogen containing cytoplasmic vacuolizations of the renal tubular cells (Armanni-Ebstein lesions) caused by hyperglycemia can exist. Closely similar basal vacuolizations containing lipids and triglycerides can be formed in ketoacidosis even without concomitant hyperglycemia [113]. Although significant, these findings can be more specified by combining the biochemical results of glucose and fat metabolism [114-117]. Tormey and Moore stated that to identify the role of diabetes in sudden deaths, biochemical markers should be evaluated routinely [118].

2.1 Evaluation of glycemic balance by analyzing PM samples

VH glucose concentration can be assessed when hyperglycemia prior to death is suspected [119]. Glucose concentration in VH correlates with the (AM) blood glucose levels being approximately 0.4-0.5 times the blood concentration [3,5]. Urine glucose is not the optimal choice, since it is not necessarily elevated even in moderate hyperglycemia due to individually variable renal threshold for glucose [6]. VH glucose concentration decreases in the early PM phase. This decrease is partially caused by anaerobic glycolysis in the hyalocytes [5] where one molecule of glucose turns into two molecules of lactate. Traub suggested that in estimating AM hyperglycemia the combined sum of glucose and lactate should be used to include the effect of glycolysis [120]. This value has been commonly used in interpretation of hyperglycemia in PM biochemistry [3,4,6,121]. However, there have been questions about the reliability of the combined sum. Factors other than glycolysis can be the source of lactate, such as autolysis, bacterial metabolism, AM lactic acidosis and prolonged agony prior to death [5,122]. Therefore, the Traub value may overestimate hyperglycemic deaths and currently glucose alone is preferred as a marker for AM hyperglycemia [5,112,123].

There is no reliable way to diagnose hypoglycemia by analyzing the glucose concentration of PM samples [2], since the glucose level decreases after death. To aid in the interpretation of hypoglycemia it can be more informative to analyze human insulin and its synthetic analogs, although there are also challenges due to the instability of insulin in hemolyzed PM blood, this is why the vitreous is preferred [124-126].

Glycated hemoglobin (HbA1c) is formed as a result of non-enzymatic addition of glucose to hemoglobin [127]. It is formed after prolonged (~12 hrs.) glucose exposure [128,129] and reflects the average blood glucose level during the preceding 1-2 months. Because of methodological variability the results may differ significantly [130], but after successful international standardization [131,132], HbA1c is recommended as one of the markers for diagnosis and follow-up of diabetes [133]. The usefulness of HbA1c in PM samples was has already been assessed over 30 years ago [4,134]. The conclusion of these studies was that HbA1c is a useful marker and also very stable even in PM samples. Valenzuela concluded in a study of fructosamine and HbA1c in PM samples that HbA1c measurement is an important advantage in PM diagnosis of diabetes, although the methods for determining HbA1c were technically demanding, time consuming and expensive at the time [135]. Later as the instrumentation had improved, studies were performed on estimating the stability and optimal preservation of the PM samples to attain reliable results [136- 138]. HbA1c has been confirmed as a reliable PM marker for long term hyperglycemia, and it is used for estimating the glycemic status in diabetic deaths and also for distinguishing ketoacidosis caused by diabetes from other possible reasons [139-141].

Fructosamine is formed when blood proteins are glycated non-enzymatically as HbA1c, although the formation is faster reflecting the glycemic control of the previous 10-20 days [142]. Fructosamines have also been evaluated in PM context for estimation of glycemic disturbances. John et al. measured fructosamine in PM plasma with a colorimetric method from 26 non-diabetics and 3 diabetics [143]. The high total protein concentrations of the PM samples compared to clinical samples required a correction to the results. After correction, they found higher fructosamine concentrations in diabetics, although the sample set was relatively small. Osuna et al. evaluated fructosamine concentrations in VH with a colorimetric assay [144]. They analyzed the samples of 49 diabetics and 43 non-diabetics and found fructosamine levels to be significantly higher in diabetics. In a later study by Osuna et al. the fructosamine levels were compared to glucose and lactate and a significant correlation was detected between glucose and fructosamine [145]. Hess at al. presented 6 cases of hyperglycemic deaths, where fructosamine from VH, CF and blood was measured in addition to glucose, lactate, ketones, HbA1c and 1,5-anhydroglucitol (1,5-AG). Contrary to Osuna et al. [144], they did not detect significant differences between diabetics and non-diabetics when VH or CF was analyzed. When blood samples were used, a difference between these groups was seen. Their conclusion was that fructosamine can provide additional information, but the essential markers for hyperglycemic disturbances are glucose and ketones.

1,5-AG is a monosaccharide naturally occurring in many foods. It competes with glucose in renal reabsorption resulting in lower serum concentration in hyperglycemia [146]. Hess et al. have measured 1,5-AG from PM samples [9,112]. They concluded that 1,5-AG is stable in PM samples and the concentrations are similar in PM and clinical samples. However, although the 1,5-AG reflects the glycemic control in a shorter period of time than HbA1c or fructosamine, it did not correlate with glucose levels in VH. They suggested that 1,5-AG can be used as an additional marker with VH glucose.

2.2 Analysis of PM samples to reveal DKA and AKA

Elevated ketones are readily analyzed from PM samples. Ketone body levels are fairly stable even if advanced PM changes have occurred [19,147] allowing the ketoacidosis to be reliably diagnosed. The causes for ketoacidosis need additional analyses. As diabetes and alcohol abuse are the most common causes for ketoacidosis, glycemic status is important to distinguish between these two. In clinical medicine, a history of diabetes or alcoholism and blood glucose levels are the significant factors, as differences in hormonal levels and metabolites other than glucose are either not significantly diverse or cannot be readily analyzed in a timely manner [93]. DKA is commonly associated with hyperglycemia, but some exceptions exist. Glucose levels may be relatively low in DKA as a result of reduced gluconeogenesis caused by e.g. alcohol or liver failure, or if food intake is reduced and/or insulin received [148,149]. In addition, as the glucose level is decreased in the early PM phase due to glycolysis [5], the vitreous glucose may not always be elevated in DKA related deaths. Therefore, the measurement of HbA1c provides additional information when estimating the cause for ketoacidosis [136].

DKA can be diagnosed by analyzing the vitreous glucose and ketone bodies in VH or blood [8]. Péclet et al. found blood acetone measurements valuable in addition to vitreous glucose and lactate in determining diabetic disturbances as the CoD. In ketoacidosis due to AKA, DKA, malnutrition or hypothermia, the ketone body measurements of different fluids have been described by several authors [16-19,141,150-156]. Total ketones have been analyzed from blood and also the correlation of blood to VH, spinal fluid and urine have been evaluated [16,151,152]. The analysis of BHB has been described in PM blood, serum, VH, urine, cerebrospinal and pericardial fluids, liver homogenate and synovial fluid [19,141,150,153,155-158]. In addition, acetone measurements in PM blood [17] and VH [159] have been described.

2.3 PM analysis of C-reactive protein

C-reactive protein (CRP) is an acute phase protein synthetized in liver in infection, inflammation and trauma [160]. The CRP levels of PM blood have been assessed in sepsis and non-septic fatalities [11] and it was shown that the CRP levels in early PM phase decreased log-linearly up to 140 hours PM. In another study the correspondence of AM and PM levels of CRP were evaluated by analyzing 26 samples where CRP was measured at 24 hours prior to death and 1-6 days PM. In this study the average reduction of PM levels compared to AM was 35-42% depending on the method used [161]. CRP levels have been analyzed in PM blood with emphasis on the CoD and survival time in traumatic deaths [162]. In traumatic deaths the CRP reflected the survival time and to some extent the severity of the tissue injury. In addition, the CRP could be used as in clinical medicine to evaluate possible infection. The routine use of CRP measurements of blood, serum and liver samples in CoD investigations has been assessed [12]. In the study, no significant correlation was found between PMI and CRP levels. It was also found that CRP levels were stable in blood and serum samples for at least one month or in liver samples for one week when stored in +5ºC. Two cases of AKA were identified with elevated CRP levels.

No other reason than acidosis was found that could cause the elevation of the CRP. The elevation of CRP had previously been described in DKA even in the absence of concomitant infection [163,164], but not in AKA.

2.4 Lactate measurement and interpretation of results of PM samples

The lactate concentration of vitreous has mainly been used in evaluation of PM glycemic balance [3,4,6,165]. In addition, Sturner et al. analyzed the lactate levels to evaluate any differences in traumatic asphyxic deaths of infants from sudden infant death syndrome [166]. Their conclusion was that the lactate level is lower in traumatic asphyxia where the agonal period is shorter. The effect of prolonged agony on lactate levels was also shown by De Letter et al. [122]. In clinical medicine, the definition of lactic acidosis may vary, but most accepted definition for diagnosis is when the lactate level exceeds 5-6 mM and the pH of blood is less than 7.35 [94,167]. In PM samples, the lactate levels elevate as the PMI increases causing difficulty in evaluation of the AM lactate levels and possible lactic acidosis [168]. Therefore, careful evaluation is needed in the interpretation of the results. Issues to be considered in addition to findings in autopsy are the PMI and PM conditions, manner of death and medical history prior to death [169].

AIMS OF THE STUDY

The aims of the study were:

1. to assess the combined sum of glucose and lactate as an indicator of disturbances in glycemic control (**I, V**)
2. to estimate and optimize the methods for measurement of glycated hemoglobin (HbA1c) in postmortem (PM) samples and to assess the information provided by the analysis (**II, III**)
3. to evaluate ketone body levels in deaths attributed to diabetes and alcohol abuse and to evaluate the most appropriate analysis method to determine ketoacidosis (**I**)
4. to measure C-reactive protein (CRP) in PM samples and to assess the role of CRP in ketoacidosis (**IV**)
5. to evaluate different AM and PM factors that influence the lactate levels in PM samples and to estimate the role of elevated lactate in diabetes related deaths (**I, V**)

MATERIALS AND METHODS

1. Samples

VH and blood samples were collected in medico-legal autopsies performed at the Department of Forensic Medicine, University of Helsinki, Finland. Autopsies were performed with a standardized procedure and the CoD was determined by a medico-legal pathologist. The study was approved by the HUCH Ethical Committee, the National Institute of Health and Welfare, and the National Supervisory Authority for Welfare and Health.

According to Finnish law (Act 1.6.1973/459), the police may order a medico-legal autopsy as a part of the CoD investigation under following circumstances:

1. if the deceased has died from an unknown disease or did not receive medical treatment during the person's last illness or the death has occurred under medical treatment,
2. if the death was or was suspected to be the result of an accident, criminal offence, suicide, intoxication or occupational disease,
3. if the death was otherwise unexpected.

2. Autopsy data

Data was collected from the autopsy records of the National Institute of Health and Welfare and from the Laboratory Information Management Software (LIMS) used at the Unit of Forensic Chemistry in the Department of Forensic Medicine, University of Helsinki, Finland.

3. Statistical methods

IBM SPSS Statistics 21 (International Business Machines Corp., USA) and Microsoft Excel (Microsoft Corp., USA) software were used for data management and statistics. Pearson's chi-squared test was used to evaluate the statistical significance in study **IV**. Pearson's correlation was calculated for linear correlations in studies **II**, **III** and **IV**. Kendall's tau correlation coefficient was calculated to assess associations between groups in study **V.** The agreement between methods was evaluated by using the Bland-Altman plot in study **III**.

4. Glucose and lactate measurements

Glucose was measured from perchloric acid (PCA) precipitated VH samples with the hexokinase/glucose-6-phosphate dehydrogenase method. Glucose in the sample is

phosphorylated to glucose-6- phosphate (G6P) by hexokinase. G6P is then further oxidized with concurrent reduction of cofactor NADP to NADPH. The amount of NADPH is detected as a change in absorption of light at 340 nm wavelength and it is directly proportional to the amount of glucose in the sample. The upper reference limit for PM samples was 7 mM.

Lactate was measured from PCA precipitated VH with the LDH assay. Depending on the isoform (L-lactate or D-lactate) quantitated, either L-LDH or D-LDH was used. L-lactate is the predominant isoform, and is denoted as "lactate", whereas the D-isoform is denoted as "D-lactate" later in the text. Lactate is oxidized to pyruvate by LDH with concurrent reduction of cofactor NAD^+ to NADH. The amount of NADH is detected as a change in absorption of light at 340 nm wavelength. Pyruvate is further incorporated to a reaction catalyzed by glutamate-pyruvate transaminase to ensure the proceeding of the first reaction. The amount of NADH is directly proportional to the amount of lactate in the sample. The upper reference limit for L-lactate was 35 mM and for D-lactate 3 mM.

5. Ketone body measurements

Ketone bodies were analyzed with gas chromatography (GC). The sample material was PCA precipitated VH or blood. First the BHB in the samples is transformed to AcAc enzymatically by adding BHBD (Roche, Switzerland). After this the AcAc in the samples is transformed to acetone by decarboxylation achieved by boiling the samples for 1 hour. The acetone is then analyzed with the GC using an Elite-BAC 1 column (Perkin Elmer, USA) and the amount consists of the total ketones in the sample. Values exceeding 1 mM indicated ketosis and 3 mM ketoacidosis.

BHB alone was analyzed enzymatically with the BHBD/diaphorase method, adapted from methods described by Laun et al. [170] and McMurray et al. [171]. The BHB in the sample is transformed to AcAc by BHBD in the presence of cofactor NAD^+ which is reduced to NADH. The NADH is further oxidized by diaphorase sigma-Aldrich, USA) with concomitant reduction of iodonitrotetrazolium chloride (INT; Sigma-Aldrich, USA) to formazan. The amount of formazan is directly proportional to the amount of BHB and can be measured with the change in absorption of light at a wavelength of 505 nm.

6. Methods for analyzing glycated hemoglobin

HbA1c was analyzed from peripheral blood samples stored either in sodium fluoride or EDTA. The methods used were the following:

- Mono S 5/50 GL cation exchange column (GE Healthcare Europe GmbH, Germany) using Agilent 1100 HPLC machinery and ChemStation software (Agilent Technologies, USA)

- BioRad In2it® point-of-care analyzer, the boronate affinity method (Bio-Rad Laboratories Ltd., USA)
- Diazyme Direct Enzymatic HbA1c Assay™ (Diazyme Europe GmbH, Germany)
- BioRad D-10 HPLC analyzer, the cation exchange method (Bio-Rad Laboratories Ltd., USA)

The reference range for PM samples was 20-67 mmol/mol. The measurements with BioRad In2it® point-of-care and BioRad D-10 HPLC analyzer were performed as instructed by the manufacturer. The Diazyme Direct Enzymatic HbA1c Assay™ was adapted for a microplate according to Penttilä et al. [172]. The Mono S cation exchange HPLC method was modified from the method described by Jeppsson et al. [173]. Briefly, the erythrocytes in whole blood were washed with 0.9% NaCl and centrifuged. After washing the erythrocytes were disrupted with citrate-phosphate hemolysis buffer including Triton-X. The labile intermediate form of HbA1c was eliminated by incubating the samples in hemolysis buffer for 30 minutes at +37°C. The supernatant was cleared by centrifugation and transferred into vials for injection to the Mono S column. Sample loading and elution was performed with 20 mM malonate buffer with a NaCl gradient.

7. CRP measurements

CRP values of the PM samples were measured using the QuikRead 101-CRP instrument (Orion Diagnostica Oy, Finland). The assay is an immunoturbidometric test in which the CRP in the sample reacts with microparticles coated with anti-human CRP F(ab)2 fragments. The change in turbidity of the solution is measured by the QuickRead Instrument photometrically. The samples analyzed were peripheral blood samples stored in EDTA.

RESULTS

1. Traub value in predicting glycemic disturbances (I, V)

We evaluated the Traub formula **(I)** by assessing 70 cases where the CoD was DM (insulin dependent, non-insulin dependent or other/unspecified). Among the 70 cases there were 12 cases where the CoD was specified to be hyperglycemic coma due to diabetes and 31 cases with diabetes-caused ketoacidosis as CoD. In two of the hyperglycemic coma cases glucose levels did not exceed the 7 mM threshold indicating hyperglycemia, but in both cases the Traub value exceeded 35 mM. In the DKA cases, the glucose was elevated in 19 of the 31 cases and the Traub value in 27 cases. Glucose level was elevated in 54.3% and the Traub value in 85.7% of the 70 cases. When cases other than DM related deaths were assessed (N = 910) glucose was elevated in 7.1% of the cases and the Traub value in 35.9% of the cases. Ketone bodies were elevated in all DM related cases (N = 70).

For assessing the glucose and lactate levels in relation to PMI, 1865 cases were evaluated where the PMI was 1-10 days **(V)** The average glucose level for all cases was 1.9 mM and lactate level 32.0 mM, so both the lactate level and the Traub value remained under the previously defined 35 mM threshold. The relations of glucose and lactate levels to PMI evaluated in 1865 cases are presented in Figures 3a and 3b. The glucose is fairly stable PM. Lactate on the contrary elevates rapidly approximately up to 4 days PM, after which the elevation slows and a plateau is reached around 8-10 days PM.

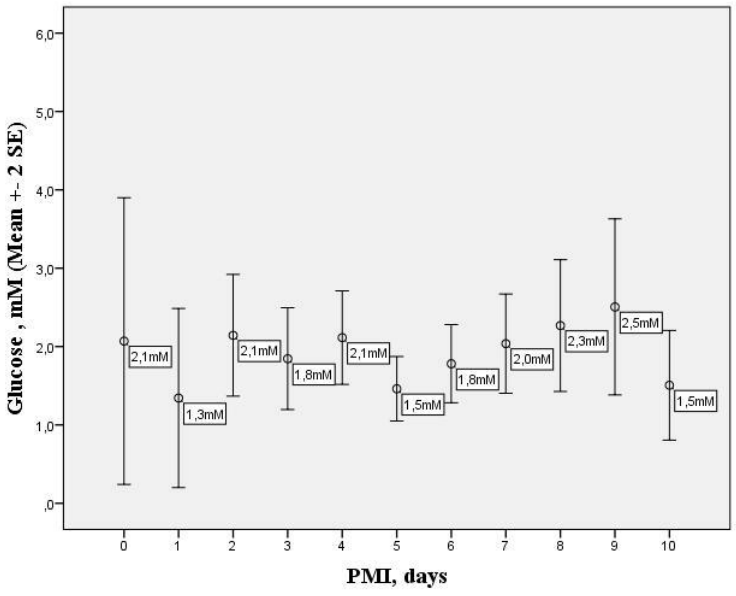


Figure 3a Glucose levels in relation to PMI

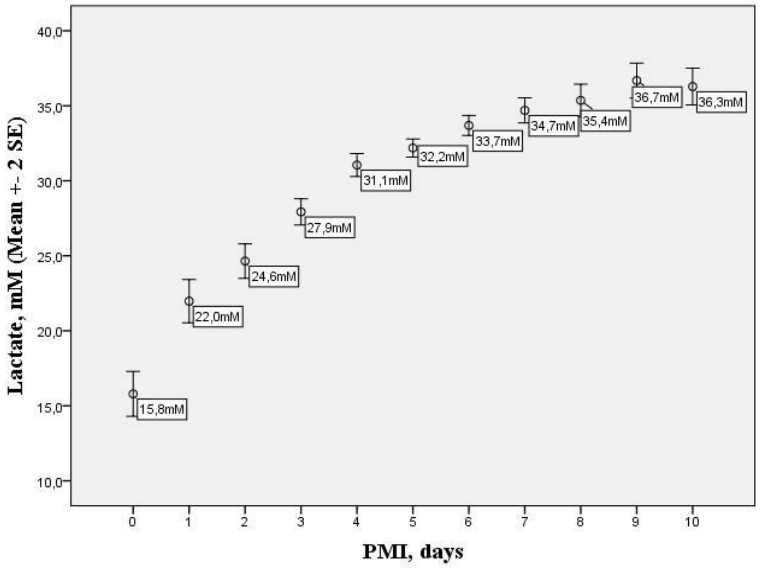


Figure 3b Lactate levels in relation to PMI

The following subgroups from the 1865 cases evaluated were formed based on the CoD; DM1, DM2 and metformin poisoning. The mean glucose and lactate levels for all cases and for these subgroups at the 95% confidence interval (± standard error, SE) are presented in Table 2.

Table 2 Mean glucose and lactate levels in PM samples

Cause of death	Glucose (mM) ± SE of mean	Lactate (mM) ± SE of mean
All cases (N = 1865)	1.9 ± 0.1	32.0 ± 0.2
Diabetes mellitus type 1 (N = 32)	17.5 ± 1.8	32.0 ± 1.3
Diabetes mellitus type 2 (N = 41)	8.3 ± 1.3	37.2 ± 1.2
Metformin poisoning (N = 9)	1.3 ± 0.7	36.8 ± 2.6

To reduce the effect of the PMI, lactate values for the subgroups were normalized against the mean value for the particular PMI in all cases (Fig. 3b). In the DM1group the normalized value was 0.98, in the DM2 group 1.13 and in the metformin group 1.16

2. HbA1c methods in PM sample analysis (II, III)

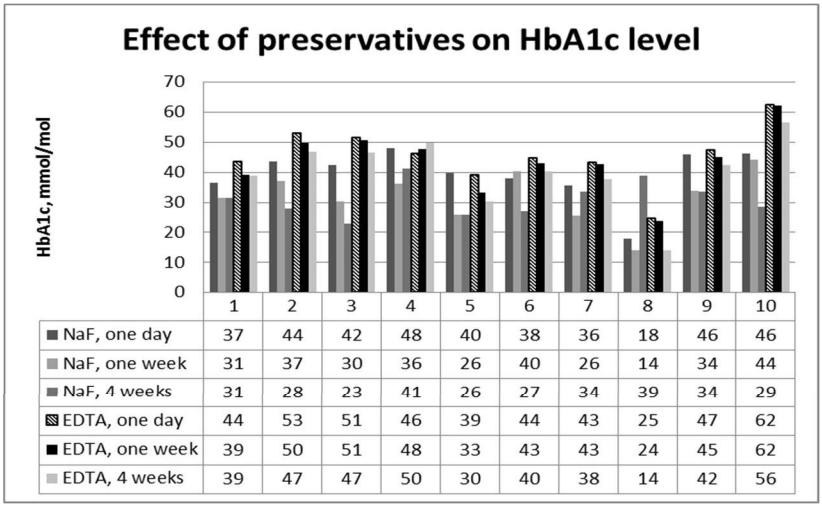
Three different methods were evaluated for measurement of HbA1c in 20 PM samples (**II**). The methods compared were cation exchange chromatography (Mono S HPLC), an enzymatic method (Diazyme Direct Enzymatic HbA1c Assay™) and a POC method based on boronate affinity (BioRad In2it®). The Mono S HPLC was the reference method used previously in the laboratory. The correlations of the enzymatic method and the boronate affinity method compared to cation exchange HPLC were $R^2 = 0.68$ and $R^2 = 0.84$, respectively. Mono S HPLC gave results for all the samples but one where the deceased had been embalmed. The other two methods gave a false result for the embalmed sample. In addition, the boronate affinity and enzymatic assay failed to give a result in 5 cases due to PM deterioration of the samples.

We compared the Mono S HPLC to a fully-automated BioRad D-10 analyzer that was also based on cation exchange chromatography (**III**). A set of 71 samples were analyzed in parallel with both assays, in which 3 of the samples were clinical and 68 PM blood samples. Both of the methods failed to give a result for three samples; one of the cases was embalmed, another in an advanced decomposition state and in the last case the proportion of fetal hemoglobin (HbF) exceeded 10%. The Mono S HPLC method did not give a result for four other samples due to sample decomposition. Correlation coefficient of the BioRad D-10 HPLC compared to Mono S HPLC was $R^2 = 0.91$. The agreement of the two methods was evaluated by using the Bland-Altman plot [174,175] after taking a natural logarithm (ln) of the measurements and performing a linear regression analysis. After the ln transformation and linear regression, the limits of agreement were reasonably narrow (Equation 1) and the results attained with the BioRad D-10 HPLC analyzer were comparable to Mono S HPLC results, especially in higher mean levels close to the 67 mmol/mol reference limit.

Equation 1. $\ln(\text{difference, mmol/mol}) = -0.141 \times \ln(\text{result, mmol/mol}) + (0.584 \pm 0.386)$

3. PM sample storage for HbA1c analysis with Mono S HPLC (II)

To evaluate the optimal storage and preservation the PM blood samples were stored in sodium fluoride (NaF) or ethylenediaminetetraacetic acid (EDTA). The storage temperature was +4°C and aliquots of the samples were frozen at -70ºC for evaluation of the effect of the freeze-thaw cycle. The correlation of 37 frozen and +4ºC stored sample results was $R^2 = 0.86$. EDTA and NaF were compared as preservatives by measuring 10 parallel samples at 1 day, 1 week and 4 weeks after sampling. All samples were stored at +4ºC. EDTA proved to be the optimal preservative compared to NaF, as the HbA1c levels in EDTA samples were higher even in the first measurement after 1 day (Figure 4). The correlation was good for EDTA samples after one week ($R^2 = 0.96$) and even after four weeks ($R^2 = 0.91$). NaF sample values showed markedly weaker correlation after 1 week ($R^2 = 0.66$) and no correlation was seen after 4 weeks of storage.



	1	2	3	4	5	6	7	8	9	10
NaF, one day	37	44	42	48	40	38	36	18	46	46
NaF, one week	31	37	30	36	26	40	26	14	34	44
NaF, 4 weeks	31	28	23	41	26	27	34	39	34	29
EDTA, one day	44	53	51	46	39	44	43	25	47	62
EDTA, one week	39	50	51	48	33	43	43	24	45	62
EDTA, 4 weeks	39	47	47	50	30	40	38	14	42	56

Figure 4 The effect of NaF and EDTA to HbA1c levels of 10 PM samples

4. Ketone body levels in DKA and AKA (I)

Ketone bodies (total ketones) of 980 cases were evaluated in context of the CoD. Ketones were elevated in 29% of the cases. When grouped into diabetes (N = 53), alcohol abuse (N = 73) and both diabetes and alcohol abuse (N = 33) related deaths, the ketones were elevated in 78%, 54% and 71% of the cases, respectively. The mean ketone body level in alcohol abuse related deaths was 6.5 mM (median 1.2 mM). In diabetic deaths and alcohol abuse combined with diabetes the mean levels were higher; 18.2 mM (median 10.0 mM) and 15.8 mM (median 6.3 mM), respectively.

5. BHB values compared to total ketones and acetone (I)

For comparison of BHB to total ketone levels and acetone, 36 samples were chosen with normal total ketone levels as controls and 25 samples where total ketones were elevated. The sensitivity of the BHB assay was 84% for predicting ketosis (≥ 1 mM) and 100% for predicting ketoacidosis (≥ 3 mM). The specificity was 88.9% for ketosis and 100% for ketoacidosis. Acetone alone was detected in 8 out of 12 cases where total ketones indicated ketoacidosis.

6. Ketoacidosis and CRP (IV)

For the evaluation CRP measurement of PM samples 41 cases were chosen where AM values of CRP were known. AM CRP analyses were performed less than 24 hours prior to death. CRP was found to reflect the AM state the PM value being approximately 42% of the AM value. CRP was also very stable in samples stored at +5ºC for even up to 75 days. The longest PMI of the samples analyzed in the study was 18 days.

To assess the relationship of CRP and ketoacidosis, 114 cases were chosen with a known alcohol abuse history. Of the 114 samples analyzed for glucose, lactate, ketone bodies and CRP there were 11 cases where the cause of elevated CRP was most likely ketoacidosis. In 4 of the 11 cases the deceased was diabetic, the remaining 7 had AKA. In all of the 11 cases hepatic steatosis and/or incipient liver cirrhosis was detected. The influence of these conditions on CRP was evaluated with another sample set of 17 cases without ketosis. In 3 of the 17 cases the CRP was elevated where one of these could be explained by pulmonary embolism

7. Correlation of lactate to metformin, blood alcohol concentration, metabolic markers and PMI (V)

Kendall's tau correlation was calculated for 319 PM samples of DM2 patients where metabolic markers (Glucose, lactate and ketone bodies), blood alcohol concentration (BAC) and metformin were analyzed. Lactate correlated with glucose, ketone bodies, metformin and PMI at the significance level $p = 0.01$ and with BAC at the significance level $p = 0.05$. In addition to lactate, metformin correlated with ketone bodies ($p = 0.01$). The mean lactate level in the cases ($N = 59$) where no metformin was detected was 37.8 ± 1.9 mM (95% CI, SE) and ketone body level 3.7 ± 2.1 mM. In the remaining 260 cases the mean metformin concentration was 17.2 ± 3.8 mg/l and range 1-280 mg/l, lactate level 37.4 ± 0.9 mM and ketone body level 2.8 ± 0.7 mM.

8. D-lactate levels of PM samples (V)

D-lactate was measured from 69 VH samples. Average D-lactate level was 0.42 mM ± 0.78 mM. No correlation of vitreous D-lactate to L-lactate, glucose or ketones was detected. PMI and D-lactate had a minor positive correlation ($R^2 = 0.13$) that was not statistically significant. In two samples the D-lactate concentration exceeded the 3 mM threshold. In the first case the deceased did not have diabetes and the VH sample was visibly putrefied. L-lactate was 35.7 mM, glucose 1.9 mM, ketones 0.71 mM and D-lactate 5.4 mM. In the second case the VH sample was not visibly deteriorated and the deceased was known to have DM1. L-lactate was 47.1 mM, glucose 13.6 mM, ketones 0.21 mM and D-lactate 3.5 mM.

DISCUSSION

1. Evaluation of the Traub value (I)

The Traub value [120] of PM samples has been used routinely in estimations of hyperglycemia prior to death [3,4,6,121,176]. Zilg et al. [5] analyzed glucose, lactate, potassium, sodium and chloride concentrations of 3076 PM samples. Their conclusion was that only vitreous glucose should be used for estimating severe AM hyperglycemia that could have caused or contributed to death. The conclusion was based on the continuous PM elevation of lactate, whereas the glucose drop is limited to the early PM phase after which the concentration is relatively stable. Hyperglycemic coma cases where only lactate would have been elevated without elevated glucose were not detected in their study. Palmiere et al. analyzed 470 cases to evaluate the accuracy of the Traub value [123]. Glucose, lactate, BHB, acetone and HbA1c were measured to determine possible fatal DKA. They found 11 cases where the glucose level exceeded 10 mM and in 8 of the cases the CoD was DKA. If the Traub sum had been used, the results would have falsely indicated glycemic disturbance in 15 cases. Their conclusion was that the Traub sum consists mainly of glucose in cases of glycemic disturbances and of lactate in other causes. Hess et al. [112] came to the same conclusion in their study, in which glucose, lactate, ketone bodies, HbA1c and 1,5- AG were analyzed. In a comparison of 80 cases the correlation of glucose in VH and CSF was good ($R^2 = 0.922$), whereas the addition of lactate values lowered it ($R^2 = 0.815$) showing that the concentrations of glucose and lactate are influenced differently after death.

In our study **(I)** we analyzed the glucose, lactate and ketone body levels of 980 PM samples. In 70 of the cases, the death was caused by complications of DM. In these 70 cases, the CoD was DKA in 31 of the cases and diabetic coma in 12 of the cases. Glucose was elevated ($\geq$ 7 mM) in 10 of the 12 diabetic coma cases and the Traub sum in all 12 cases. In the DKA cases, the glucose level was elevated in 19 of the 31 cases and the Traub sum in 27 of the 31 cases. In the diabetic coma and DKA cases glucose would be expected to be elevated in all cases. However, our results are partly controversial compared to studies where glucose alone can be used for diagnosing AM hyperglycemia. One explanation could have been the relatively long PMI of the cases in our study; the mean time from the death to the arrival of the samples in our laboratory was 7 days in 2011 when the study was performed. The PMI in the study by Zilg et al. was less than 4 days and in the study by Palmiere et al. VH and CF samples were collected within 12 hours from the death. In our study, the Traub value was elevated more often in diabetics (85.7%, N = 70) than in cases where the CoD was not related to DM (35.9%, N = 910). For these reasons, we concluded that in cases of long PMI the Traub sum should be taken into consideration when glycemic disturbances are suspected and when it is elevated, other additional biomarkers such as HbA1c and ketone bodies should be analyzed. The diabetic coma in DM1 and DM2 (ICD-10; E10.01 and E11.01) is defined as "hyperglycemic coma", although the diagnosis includes ketoacidotic coma. If the presence of ketoacidosis is not additionally stated, any retrospective study based on CoD coding

information will have problems in distinguishing between HHS and DKA from only the laboratory data.

2. Lactate levels and interpretation of the results (I, V)

As can be seen from Figure 3b, the lactate levels are elevated in a logarithmic manner in the first days PM after which the elevation slows and a plateau is reached around 8-10 days PM. This reminds very much of a bacterial growth curve. Although the VH is better conserved than blood in cadavers, some bacterial contamination exists especially after longer PMIs. Brzeziński and Godlewski [177] studied pig eyeballs stored in +6-8ºC and sampled sequentially from 4 to 148 hours PM. No bacteria were detected in VH smears of samples where the PMI was less than 76 hours, but after 124 hours all the smears contained mainly gram-negative bacteria. At optimal temperatures the growth would probably be detected even earlier. This effect of bacterial metabolism as PMI increases causes difficulties in predicting the results. Therefore, we concluded that the lactate levels could be more informative if they are compared to reference levels defined for different PMIs. This could give a more reliable estimation of possible AM lactic acidosis than comparing all the results to a fixed value. Some uncertainty still remains even when a comprehensive reference set with known PMIs is used, since the PMI is an approximation in about half of the cases routinely analyzed in our institution. In addition, the bacterial growth may be different due to environmental factors, such as temperature and surroundings as well as pathological conditions or inter-individual diversities of microbiomes [178-180].

In our earlier study (**I**) we found that the Traub value is more often elevated than glucose alone when the CoD is diabetes related. For this reason, we evaluated the lactate levels in DM1, DM2 and metformin poisonings by normalizing to the values of 1865 cases with various CoDs and known PMI (**V**). The lactate levels in DM1 were close to the levels for all cases, whereas in DM2 and metformin poisonings there was a 13-16% elevation (Table 2). This could explain the correlation of the Traub value and glycemic disturbances in DM2. In DM1 the glucose level is markedly higher (17.5 mM) than in all the cases (1.9 mM) and also in DM2 (8.3 mM), which elevates the sum of glucose and lactate. Although the Traub sum is indicative of glycemic disturbance, we concluded that it should be omitted and the interpretation of glucose and lactate should be performed separately.

In addition to PMI, the correlation of lactate was significant at the level of $p = 0.01$ for glucose ketone bodies and metformin. Ketoacidosis and lactic acidosis can be concomitant [17,181] in both DKA and AKA, which could explain our finding. Cox et al. [181] found in their study that lactic acidosis is common with DKA patients. They suggested that in addition to hypoperfusion, the altered glucose metabolism in hyperglycemia and elevated counterregulatory hormones contribute to excess lactate formation. A case report by Feenstra et al. also emphasized the metabolic derangements in addition to hypoperfusion and hypoxia [182]. Brinkmann et al. looked into the AKA and lactic acidosis of alcoholics [17]. The study had separate groups for both conditions, but they found one case where the criteria for both were fulfilled. Their conclusion

was that these conditions may exist combined and lead to fatal outcome. Umpierrez et al. compared the metabolic profiles of DKA and AKA patients and found lactate levels to be more elevated in AKA [93]. The probable explanation was the elevation of $NADH/NAD^+$ ratio due to ethanol metabolism in AKA.

The correlation of metformin to lactate and ketone bodies in 319 DM2 cases was significant at the level of $p = 0.01$. It would be tempting to associate the elevated lactate levels with the use of metformin, as it is the most described oral therapy in Finland for DM2 patients according to Kela (The Social Insurance Institution of Finland). But when the DM2 cases were analyzed (**V**), the lactate level was similar in cases where no metformin was detected (N = 59, lactate 37.8 ± 1.9 mM) than in cases where the metformin ranged from 1-280 mg/l (N = 260, lactate 37.4 ± 0.9 mM). This leads to a conclusion consistent with the Cochrane review by Salpeter et al. [99], that DM2 and its complications rather than metformin are the cause for elevated lactate levels and possible lactic acidosis. Metformin is eliminated by secretion of the kidneys [183] and lactate is metabolized mainly in the liver [94]. In DM2 the metabolism in the liver is often disturbed [184] and especially in ketosis and DKA there are functional disturbances in the liver and in kidneys [185]. The functional problems caused by DM complications in the liver and kidneys could explain the concomitant accumulation of metformin with lactate and/or ketone bodies. This matter should be further evaluated with clinical sample material, where total ketones (or BHB), lactate and metformin should be systematically analyzed and the function of the liver and kidneys assessed, since in forensic biochemistry, the PM changes affecting the lactate concentration causes some uncertainty to the interpretation of results. A restriction of our results is that in this set, the actual lactate concentration was used and not values normalized to PMI-specific values, since in almost half of the cases the PMI was an approximation.

D-lactate is elevated in the urine and plasma of diabetics compared to non-diabetics [110,111]. We analyzed 69 VH samples in a preliminary trial to assess if the D-lactate levels would provide additional information of the metabolic status prior to death (V). No correlation to L-lactate, glucose or ketone bodies was detected and there was only a minor correlation with PMI. Two of the samples exceeded the 3 mM threshold, in which for one case the cause was most likely bacterial metabolism, since the sample was visibly putrefied and there were no other factors referring to diabetic complications. For the second case, diabetic complications might have caused the elevation, since the deceased had DM1 and the sample was well preserved. The measurement of D-Lactate needs further evaluation probably with a more precise assay method then the enzymatic assay we used. Urine could be an alternative sample matrix to VH.

3. Ketoacidosis, causes and measurement (I, IV)

DKA is known to induce the expression of pro-inflammatory cytokines and CRP [163]. Gogos et al. [186] showed in their study that 80% of 61 DKA or HHS patients had symptoms of SIRS. Although infection is often a precipitating factor in DKA, they also showed that the CRP levels in DKA patients with sepsis are significantly higher than in patients with SIRS with no infection.

The mechanism when no infection is present is probably due to the oxidative stress caused by the hyperglycemic crises [164]. Jain et al. [187] concluded in their review that in addition to hyperglycemia, the ketone bodies cause oxidative stress as well. The findings by Stentz et al. [164] were similar and they concluded that hyperglycemia and ketoacidosis influence oxidative stress and pro-inflammatory markers independently without synergistic effects. This would suggest that ketoacidosis without hyperglycemia, as in AKA, could also elevate markers of inflammation. Indeed, a study by Astrup and Thomsen [12] confirmed this as they found 2 cases where CRP was elevated due to AKA. We looked into this in our study (**IV**) where 114 cases were analyzed for ketone bodies and CRP. We found 11 cases where the probable cause for elevated CRP level was ketoacidosis. Of these, 7 were diabetics but for the remaining 4 the reason for ketoacidosis was most likely alcohol abuse. Therefore, our findings were consistent with Astrup and Thomsen [12] and we concluded that ketoacidosis alone can elevate the CRP level. DM and alcohol abuse may cause liver diseases [184,188] that can result in an inflammatory response. Since in all of our 11 cases some degree of liver disease was seen, another set of 17 samples was analyzed for CRP where the deceased had fatty liver changes or liver cirrhosis, but the ketone bodies here were under the reference limit. CRP was elevated in 3 cases and in one of those the cause was pulmonary embolism. Our conclusion was that the CRP levels are not markedly affected by steatohepatitis or incipient liver cirrhosis.

Ketoacidosis is most commonly caused by DM or alcohol abuse. In our study (**I**) we investigated whether there are differences in the ketone body levels between these two groups and a group where both, DM and alcohol abuse, contributed to death. Ketone body levels were lowest in the alcohol abuse group (N = 73) the median being 1.2 mM. The groups where the CoD was diabetes related (N = 53) or diabetes and alcohol abuse related (N = 33) the median ketone body levels were markedly higher; median 10.0 mM and 6.3 mM, respectively. Although the ketone body level is lower in the alcohol abuse group, sporadic extremely high ($\geq$ 20 mM) values were also detected. Our conclusion was that the ketone body level does not reflect the cause of ketoacidosis. For differentiation between DKA and AKA additional markers, such as glucose and HbA1c, need to be analyzed. Since glucose levels may be reduced in DKA patients e.g. after insulin intake, reduced food intake and in the presence of impaired gluconeogenesis [149] it is essential to measure the HbA1c in addition to glucose.

In PM context, the estimation of ketoacidosis can be performed by measuring acetone [4], BHB [140,150] and/or total ketones [152]. Elliott et al. [189] analyzed ethanol, acetone and BHB in 350 PM cases of alcoholics, diabetics and alcoholic diabetics. Their conclusion was that acetone can be used as an initial marker for ketoacidosis and the acetone positive cases (0.002 g/l, limit of detection in the study) can be further analyzed for BHB. The same conclusion was made by Hockenhull et al. [190] as their study showed that although the BHB is more precise marker, the preliminary screening can be done by analyzing acetone. In contrast, a study by Iten and Meier [19] found that acetone was elevated only in 64% of the cases in AKA cases where BHB was elevated. In clinical medicine, it has been shown that acetone and acetoacetate are not necessarily elevated even in moderate ketoacidosis and BHB should be preferred as the marker for ketoacidosis [191]. In our study (**I**) BHB and acetone were analyzed from PM samples where

total ketones had been previously measured. Based on the total ketone measurements we chose 36 samples as controls and 25 samples where the total ketones exceeded the 1 mM threshold for ketosis. The BHB assay showed 84% sensitivity and 88.9% specificity for samples referring to ketosis and 100% sensitivity and specificity for samples referring to ketoacidosis (≥ 3 mM total ketones). Acetone alone was detected in 8 out of 12 samples in which the total ketone and BHB measurements referred to ketoacidosis. Our findings were in contrast to the conclusions by Elliott et al. [189] and Hockenhull et al. who found [190] that acetone would be sufficient for primary screening for ketoacidosis. Our conclusion was that either BHB or total ketones need to be analyzed to detect ketoacidosis. The contrary results could be caused by different methodology and/or differences in sample quality and type, since these are specific for each laboratory and greatly influence the results and interpretation of the results.

4. Measurement and importance of HbA1c in PM samples (II,III)

We compared three different methods for HbA1c measurement of PM blood samples (**II**). The reference method was based on cation exchange chromatography and the other two methods were point-of-care (POC) assay based on boronate affinity chromatography [192] and an enzymatic assay [193]. We noticed that in the analysis of PM samples, it is necessary to be able to visualize the results as we can with chromatography to detect erroneous results due to PM changes, as one sample where the deceased had been embalmed gave a false result when analyzed with the enzymatic and POC assays. The cation exchange HPLC did not give a result and the abnormal matrix could be detected in the chromatogram. The POC assay correlated better than the enzymatic assay when compared to the cation exchange HPLC, but it failed to give a result in 5 out of 20 samples due to the PM deterioration of the samples. We concluded that the Mono S cation exchange HPLC was the optimal method for the laboratory to analyze PM samples. In our later study (**III**) we compared the BioRad D-10 fully-automated analyzer also based on cation exchange HPLC to the Mono S HPLC method to improve the measurement of HbA1c in PM samples. The correlation of the two methods was good ($R^2 = 0.91$) as were the limits of agreement analyzed by using the Bland-Altman method. The fully-automated analyzer was more cost-effective and it could be used for analyzing some samples that were too deteriorated to give a reliable result with Mono S HPLC. This was probably due to the pre-treatment needed for Mono S HPLC that might be too harsh for already slightly deteriorated samples. Our conclusion was that the previously validated Mono S HPLC method could be replaced with the BioRad D-10 analyzer.

PM samples are often stored in NaF to inhibit further bacterial growth and changes in the matrix after sampling [194]. Goullé et al. [136] compared the effect of preservatives (none, NaF and EDTA or heparin) on PM samples prior to HbA1c measurement. They found that the HbA1c peak can be detected in NaF stored samples up to 3 months and in samples stored without a preservative, or in EDTA or heparin, for up to one year when the storage temperature was +4ºC. When parallel measurements of samples stored with NaF, heparin or without a preservative were

performed they found no significant differences in the HbA1c levels. However, EDTA was the recommended preservative. Also in a study by Winecker et al. [137] no differences were found in parallel samples of NaF and EDTA stored blood. Our results on the contrary showed that NaF stored samples give lower values compared to EDTA stored samples even after one day of storage at +4ºC. After one week the NaF stored samples give markedly lower values, whereas the EDTA stored samples are fairly stable for at least up to 4 weeks. Our conclusion was that EDTA should be used as a preservative. If samples are stored in NaF, the results for samples frozen at -70ºC correlate better than those for samples stored at +4ºC for at least one week. In the study of Winecker et al. the samples used for comparison of preservatives were samples from healthy volunteers so presumably the samples were more stable than if PM samples were used. In the study of Goulle et al. no quantitative values were given for samples stored from 3 months up to one year, they only determined if the HbA1c peak could still be detected.

GENERAL DISCUSSION AND FUTURE AIMS

Aurelio Luna asked the question "Is postmortem biochemistry really useful? Why is it not used more widely?" in his review [1]. One of the challenges in PM biochemistry is the absence of reference values for analytes to aid in the interpretation of results. Published research can provide some guidelines, but each laboratory needs to define the RIs specific for the samples analyzed and for the methods used in that particular laboratory. This requires thorough research with sufficient amounts of samples and data. In addition, the methodology needs to be optimized for demanding sample materials. Pre-treatment of samples is often needed, which may cause difficulties when samples are at least partially deteriorated. In the assessment of the results and their importance, a knowledge of laboratory sciences alone is not sufficient but a knowledge of forensic medicine is also required making PM biochemistry a multidisciplinary scientific field. In addition, the results on their own are not of any value, but they need to be incorporated as part of all the other findings in CoD investigations [8].

Glycemic disturbances are difficult to diagnose in autopsy or histology alone, this is why biochemical methods are needed for additional information [6,37,195]. Hyperglycemia was long evaluated by analyzing glucose and the Traub sum where glucose and lactate are combined [3,4,6,121]. The Traub sum seemed to exaggerate the number of hyperglycemic deaths and after further research the conclusion by many is that it should be omitted [5,112,123]. In our research we found it to correlate with diabetic deaths (**I**) and concluded that a relatively long PMI in our institution may be one factor for our results diverting from those of others. However, after our later study (**V**) it seems that in DM1 glucose alone is more informative and the elevation of the Traub sum in DM2 is caused by metabolic impairment of the disease and not directly due to hyperglycemia. The importance of lactate in assessing glycemic disturbances needs further evaluation by using PMI-specific reference values. It would also be informative to include samples from medical autopsies where more often results from recent AM laboratory assays are obtained, which is rarely the situation in forensic autopsies.

Hypoglycemia still remains unidentified by biochemical means from PM samples. Low values of the Traub sum have been suggested to indicate hypoglycemia [3], but according to our study **V** it is evident that lactate levels are affected mainly by the microbial metabolism associated with PMI and glucose is very low even in normoglycemic cases. Therefore, the Traub sum is not reliable for diagnosis of hypoglycemia. In addition to insulin detection, hypoglycemia could be detected by means other than direct metabolite concentrations. Sharp et al. showed in their animal study, that a distinct gene expression profiles can be detected in ischemic and hemorrhagic stroke, hypoxia, and hypoglycemia [196]. The challenge in analyzing RNA levels as with other biological markers is the PMI related degradation. Blood may not be the optimal matrix [197], but for instance RNA in brain tissue could be more stable [198].

Glycemic balance in previous weeks can be evaluated by analyzing the HbA1c levels. HbA1c is readily measured from PM samples, although the methodology and sample preservation need to be optimized, as was shown in our studies **II** and **III**. HbA1c level reflects the long term glycemic balance and does not reveal the immediate blood glucose level prior to death, this is why it is not always seen as obligatory but more as an optional analyte [112]. Regardless of this, the information provided by HbA1c levels of PM samples in forensic medicine is significant when evaluating the causes for ketoacidosis, the glycemic balance of diabetics or possible undiagnosed diabetes. Therefore, in our opinion it should be included when assessing possible glycemic or acidotic disturbances.

DKA is a known complication of DM but AKA is often undiagnosed [81]. Analysis of ketone bodies in PM blood or VH is essential when metabolic disturbances due to diabetes or alcohol are suspected. Acetone is often measured in the analysis of alcohols. However, in our study **I** it was shown that acetone alone is not sufficient, but either total ketones or BHB need to be analyzed for the diagnosis of ketoacidosis. In our study **IV** we could confirm the finding of Astrup et al. [12] that CRP is elevated AKA. The inflammatory reaction had been described earlier in DKA [163,164] and it is suggested to associate with severe complications of DKA [199]. The association of CRP and AKA could be further assessed by including the analysis of cytokines. This could provide more information on the pathophysiology of AKA.

In our research (**I-V**) we have focused on the metabolic aspects in DM and alcohol abuse related deaths. Our results and findings have enabled the Laboratory of Forensic Biology to improve and adapt the methods as well as interpretations of the results used in routine casework. With continuous research and development of new methods PM biochemistry can provide important information for the medico-legal pathologists. It is easy to agree with the reply of Aurelio Luna to his question of the importance of PM biochemistry: “I think that the biochemical methods must be integrated in the autopsy routine with precautions in the fields with inconclusive data but with trust in the fields where the data are conclusive”.

CONCLUSIONS

Ketoacidosis can be detected by the quantitation of either BHB or total ketones in PM samples. To define the cause for ketoacidosis, additional markers such as glucose and HbA1c need to be analyzed. HbA1c is a reliable marker for poor glycemic control or undiagnosed diabetes and it can be used for distinguishing DKA from AKA. CRP level may be elevated in DKA and AKA without a concurrent infection. The elevation can be explained by the oxidative stress and following inflammatory state caused by hyperglycemia and/or hyperketonemia. The lactate levels of PM samples are relatively high due to PM changes and microbial metabolism. To evaluate any possible AM lactic acidosis, PMI-specific reference values may be more informative than a fixed value. Lactic acidosis is often caused by metabolic disturbances in DM and alcohol abuse. According to our studies of PM samples, the association between metformin and elevated lactate levels seems more likely to be concurrent than causal. For the assessment of metabolic disturbances, measurement of glucose, ketone bodies and HbA1c in PM samples is needed, while the information provided by lactate levels requires further evaluation. These markers should be analyzed in all cases where a death is caused or related to DM or alcohol abuse and also in cases where these conditions are not suspected, but there are few findings following autopsy.

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