INSTITUTO SUPERIOR
DE CIÊNCIAS DA SAÚDE
NORTE

CESPU

Master Thesis

ESTIMATING THE *POST MORTEM* INTERVAL BY CLINICAL CHEMISTRY

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Master Degree in Forensic Sciences

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Advanced Institute of Health Sciences – North

Sciences Department

2014

Dissertação de candidatura ao
Grau de Mestre em Ciências Forenses
Apresentada ao Instituto Superior de Ciências da Saúde do Norte

Dissertation thesis for the degree
of Master in Forensic Sciences
Submitted to the *Advanced Institute of Health Sciences – North (ISCS-N)*

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"The value of things is not in how long they last, but the intensity with which they occur. So there are unforgettable moments, inexplicable things and incomparable people."

Fernando Pessoa

ACKNOWLEDGMENTS

I would like to acknowledge all of people who directly and indirectly have made this project possible.

Firstly, to my supervisor, Prof. Ricardo Silvestre my most sincere, humble and profound THANK YOU SO MUCH for guidance at critical spirit, availability, understanding, caring and above all by the confidence, knowledge and wisdom that gave me throughout these months of work.

To my co-supervisor, Professor Ricardo Dinis Oliveira my sincere and humble THANK YOU SO MUCH for ever and full willingness, for guidance at critical spirit, for the commitment, caring, attention and patience always provided.

At Master Isabel Costa, who worked with me in the lab over the last months, the true and humble thanks for my full and relentless availability, patience, companionship and sharing of knowledge demonstrated. THANK YOU SO MUCH

For Isabel, Nuno and Vanessa huge thank true friendship, affection, dedication, total availability, patience, and support the company in good times and bad times. Basically I appreciate the fact that they are part of my life. VERY SAME THANK YOU FOR EVERYTHING

Finally, A VERY HIGHLY THANKS TO ALL THE PEOPLE WHO ARE REALLY IMPORTANT TO ME AND FUNDAMENTAL PART OF MY LIFE

THANK YOU MY GRANDPA FOR BEING GUARDIAN ANGEL

ABSTRACT

The post mortem interval (PMI) is the period of time between the occurrence of death and the time the body is found. An accurate estimation of PMI is a major focus of research in forensic pathology since the knowledge of the PMI is central to the forensic investigation. This estimate is based almost exclusively on observations of macroscopic changes that occur during the cadaverous decomposition, especially in the first hours after death. The physical changes (*algor mortis*, *livor mortis*) and physicochemical (*rigor mortis*) are wholly related to biochemical changes and only visible to the naked eye. The autolysis process begins shortly after the term of cellular metabolism and leads to loss of morphological integrity, physics and chemistry of the corpse. Thus, these changes are useful for estimating the PMI in the first hours after death. Physical changes are related to the activity of arthropods and the corpse decomposition and are therefore more significant in the later *post mortem* states.

Ideally, researchers aim to uncover parameters that change linearly over time towards a precise PMI determination. To achieve this goal, several studies have quantitatively evaluated the biochemical changes in the body after death. Thus, various lipids, substrates, electrolytes, enzymes and proteins have been studied in different biological matrices, from cerebrospinal fluid, blood, and most commonly the vitreous humor. These studies have shown very promising and expected results demonstrating the potential of biochemical analysis for the accurate determination of the PMI.

This study aimed to evaluate the *post mortem* changes in different biochemical parameters (e.g. lipids, substrates, electrolytes, enzymes and proteins) in human blood. Human blood samples were collected from 14 volunteers donors and subjected to a temperature decrease (from 37 at 21 °C), which mimics the decline in body temperature after death. The kinetics of 35 biochemical parameters were evaluated in the human blood samples for each time point set for a period of 10 days.

It was necessary to establish a decision criterion in data analysis to check the most relevant parameters for future use in estimating the PMI. Thus, our selection criteria aimed to evaluate the degree of variability of each parameter and the extent which these correlate with PMI. Overall, our study allowed the identification of relevant parameters with potential for accurate PMI estimation. Thus, sodium, calcium, potassium, transferrin and ferritin can play an important role in estimating the PMI.

This study may provide a new paradigm for estimating the PMI becoming a complementary procedure methodologies already used. The data will be used to develop a mathematical model with predictive value in order to estimate the PMI. Confirmatory studies will be conducted in *post mortem* blood samples.

Keywords: *Post mortem* interval (PMI); Biochemical changes; Lipids; Substrates; Electrolytes; Enzymes; Proteins; Estimate PMI.

RESUMO

O intervalo *post mortem* (PMI) é o período de tempo decorrente entre a ocorrência da morte e o momento em que o corpo é encontrado. A estimativa precisa do PMI é um dos principais focos de investigação em Patologia Forense, uma vez que o conhecimento do PMI é fulcral para a investigação forense. Esta estimativa baseia-se quase exclusivamente na observação de alterações macroscópicas que ocorrem durante a decomposição cadavérica, principalmente nas primeiras horas após a morte. As alterações físicas (e.g. *algor mortis* e *livor mortis*) e físico-químicas (*rigor mortis*) estão inteiramente relacionadas com as alterações bioquímicas, sendo observadas unicamente a olho nu. O processo de autólise começa logo após o término do metabolismo celular e leva à perda da integridade morfológica, física e química do cadáver. Desta forma, estas alterações são úteis para estimar o PMI nas primeiras horas após a morte. As alterações físicas estão relacionadas com a atividade de artrópodes e com a decomposição cadavérica, sendo por isso, mais relevantes em estados *post mortem* mais tardios.

Idealmente, para uma determinação precisa do PMI os investigadores pretendem descobrir parâmetros que se alterem de forma linear ao longo do tempo. Para alcançar este objetivo, vários estudos têm avaliado quantitativamente as alterações bioquímicas ocorridas no organismo após a morte. Assim, vários lípidos, substratos, eletrólitos, enzimas e proteínas têm sido estudados em distintas matrizes biológicas, desde o líquido cefalorraquidiano, ao sangue e mais comummente ao humor vítreo. Estes estudos têm apresentado resultados bastante promissores e expectáveis demonstrando o potencial da análise bioquímica para a determinação precisa do PMI.

Este estudo teve como principal objetivo avaliar as alterações *post mortem* de diferentes parâmetros bioquímicos (e.g. lípidos, substratos, eletrólitos, enzimas e proteínas) no sangue humano. Recolheu-se amostras de sangue humano de 14 dadores voluntários que foram submetidas a um decréscimo da temperatura (de 37 a 21 °C), que mimetiza o declínio da temperatura do corpo

após a morte. As alterações cinéticas de 35 parâmetros bioquímicos foram avaliadas nas amostras de sangue humano para cada ponto de tempo definido, durante um período de 10 dias.

Foi necessário estabelecer um critério de decisão na análise dos dados para se verificar os parâmetros mais relevantes para serem usados futuramente na estimativa do PMI. Assim, os nossos critérios de seleção tiveram como objetivo avaliar o grau de variabilidade de cada parâmetro e de que forma estes se correlacionam com PMI. No geral, o nosso estudo permitiu a identificação de parâmetros relevantes com potencial para a estimativa precisa PMI. Assim, o sódio, o cálcio, o potássio, a transferrina e a ferritina podem desempenhar um papel importante na estimativa do PMI.

Este estudo pode proporcionar um novo paradigma para a estimativa do PMI tornando-se num procedimento complementar às metodologias já utilizadas. Os dados obtidos serão utilizados para desenvolver um modelo matemático com valor preditivo de modo a estimar o PMI. Estudos confirmatórios serão realizados em amostras sanguíneas *post mortem*.

Palavras-chave: Intervalo *post mortem* (PMI); Alterações bioquímicas; Lípidos; Substratos; Eletrólitos; Enzimas; Proteínas; Estimativa PMI.

ABREVIATION LIST

ALAT	Alanine aminotransferase
ALP	Alkaline phosphatase
ASAT	Aspartate aminotransferase
CSF	Cerebrospinal fluid
CK	Creatine kinase
CNP-G3	2-chloro-4-nitrophenyl-α-maltorioside
CRP	C-reactive protein
C3	Complement C3
C4	Complement C4
DGKC	Deutsche Gesselschaft fur Klinische Chemie
GGT	Gamma Glutamyltranspeptidase
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFCC	International Federation of Clinical Chemistry
LDH	Lactate dehydrogenase
O-krezoloft	O-cresolophthalein
ONPG	O-nitrophenyl - ϑ-D-galactopyranose
PMI	*Post mortem* interval

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PART I: THEORETICAL BACKGROUND

Introduction

The PMI is the period of time between the occurrence of death and the moment that the body is found. An accurate estimation of the *post mortem* interval is the main focus of research in Forensic Pathologic, since the knowledge of the PMI is essential to the forensic investigation (Romanelli et al., 2012; Sampaio-Silva et al., 2013). Currently, the expertise developed by legal medicine is quite limited since it has been based on the observation of macroscopic changes that occur during the cadaverous decomposition in short periods of time. Therefore, the development of new assessment methods are urgently needed allowing a more reliable and accurate determination of PMI especially in the first hours after death (Lendoiro et al., 2012; Munoz Barus et al., 2008; Sachdeva et al., 2011; Sampaio-Silva et al., 2013).

The cadaverous changes are grouped succinctly into six types: physical, physicochemical, biochemical, microbiological, entomological and botanical. All of which are currently used in medicolegal expertise, however these only provide a rough estimate of time of death (Madea, 2005a; Poloz and O'Day, 2009; Sachdeva et al., 2011). Physical changes (*algor mortis*, *livor mortis*) and physicochemical (*rigor mortis*) are entirely related to biochemical changes. These macroscopical changes have been helpful for the PMI estimation during the first hours after death (Munoz Barus et al., 2008; Poloz and O'Day, 2009; Sampaio-Silva et al., 2013). Microbiological and entomological changes are related to the arthropods' activity and the cadaverous decomposition being, however, more relevant in the later *post mortem* states (Poloz and O'Day, 2009; Sachdeva et al., 2011; Sampaio-Silva et al., 2013). The autolysis process begins shortly after the end of the energetic metabolism and leads to the loss of morphological, physic and chemical integrity of the corpse (Henssge and Madea, 2007; Madea, 2005a; Querido, 1990a; Singh et al., 2005; Singh et al., 2006). However, these cadaverous changes can be extremely sensitive and susceptible altered by numerous extrinsic factors, such as climatic conditions, temperature, humidity and the particular characteristics of the place where the corpse lies; or intrinsic, such as gender, age, physical constitution, physiological and pathological states of the corpse (Madea and Musshoff, 2007; Poloz and O'Day, 2009; Singh et al., 2005). In this way, an accurate determination of the

PMI is still a complex and subjective process that depends on the experience of the pathologist. Thus, research is needed to provide precise tools that will improve the PMI determination in an objective manner (Coe, 1993; Henssge and Madea, 2007).

The biochemical changes in the body after death have been the subject of numerous studies for the development of a more accurate methodological determination of the PMI. Biochemical studies have been conducted on various biological matrices, from cerebrospinal fluid, blood, and most commonly the vitreous humor, since it presents several advantages over the others (facility to harvest and access as well as biological preservation) (Coe, 1993; Lendoiro et al., 2012; Passos et al., 2009; Querido, 1990a; Singh et al., 2006; Tumram et al., 2011). Among the biochemical parameters explored, several lipids, substrates, electrolytes, enzymes and proteins have shown to be very promising (Coe, 1993; Naumann, 1959; Tumram et al., 2011; Uemura et al., 2008).

Lipids

Lipids are insoluble organic substances in water but soluble in nonpolar solvents. They are present in all tissues and act as hormones or hormone precursors, metabolic fuels, structural and functional components of biological membranes, which allow isolating nerve conduction and prevent heat loss. The main lipids in human plasma are cholesterol, triglycerides, cholesterol esters, phospholipids and non-esterified fatty acids (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). It is important to note that only 25% of cholesterol is derived from the diet, the remainder is primarily synthesized in the liver from acetyl CoA. Triglycerides are derived from the liver and intestine but are crucially dependent on nutritional status of the individual. The liver is the primary provider of plasma triglycerides. In this study, we focused only on cholesterol and triglycerides since it appear to be most promising and important lipid for this type of study, are also the most studied, the most abundant and easily measured in the human body.

The studies developed by Uemura *et al.* (Uemura et al., 2008) showed *post mortem* changes in the triglycerides, namely a significantly decrease up to three days *post mortem*. The authors also investigated total cholesterol from three sampling sites (left cardiac blood, right cardiac blood, and femoral vein blood). No significant differences were found in the triglycerides levels by sampling site, although femoral blood showed a lower value. As for total cholesterol, it is convenient to use left cardiac blood since it reaches higher levels in this matrix than in femoral blood. Sarkioja *et al.* (Sarkioja et al., 1988) evaluated the stability of plasma triglycerides and total cholesterol at different *post mortem* times, although it was not correlated with the PMI. The results clearly show that fluctuations occur in plasma lipid concentrations during the first 24 hours after death. *Post mortem* plasma triglycerides concentrations showed the greatest fluctuations (Sarkioja et al., 1988). According to these authors, the variation in lipids concentrations occurs due to the disintegration of the cell membrane and the osmotic changes in the blood (Sarkioja et al., 1988). However, in other studies the lipid concentrations stayed relatively stable after death (Enticknap, 1961; 1962; Glanville, 1960).

Up to date, an absence of consensus is observed on the kinetics of lipid concentrations *post mortem*. Therefore, further studies are needed with better control on the arrays and sample packaging for a conclusion to be taken implying a potential importance of the lipid group in the accurate estimation of PMI.

Substrates

Biochemical substrates are chemical compounds that undergoes a reaction catalyzed by enzymes (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997).

Coe *et al.* analyzed the *post mortem* serum uric acid concentration (Coe, 1993). In their study, the authors observed increased serum levels of uric acid although this finding was not consistent. Uemura *et al*. investigated the modification of total bilirubin and creatinine in a PMI up to three days (Uemura et al., 2008). Total bilirubin showed a time-dependent increase after *post mortem*, although not significant. For this parameter femoral blood showed a significantly lower value than the cardiac blood. The latter seems more appropriate for creatinine evaluation as demonstrated by time-dependent increase *post-mortem* (Uemura et al., 2008). The PMI estimation by creatinine concentrations was also studied in human muscle. A stronger correlation with PMI was found at 11°C up to 30 days and at 20 °C up to 15 days (Brion et al., 1991). Madea *et al*. (Madea et al., 2001) reported a high spread of creatinine concentrations that were higher in synovial fluid than in vitreous humor. A high spread of *post mortem* concentrations in both compartments was found for urea (Madea et al., 2001). The increase of urea levels was also found in other body fluids such as cerebrospinal fluid (CSF), blood almost to the same extent (Naumann, 1959). However, in a study of Tumram *et al*. the creatinine and urea concentration in synovial fluid and vitreous humor failed to significantly correlate these with PMI up to 36 hours ($p > 0.05$) (Tumram et al., 2011). Identical results for determining the creatinine and urea concentration in vitreous humor samples were obtained in other studies (Munoz Barus et al., 2002; Munoz et al., 2001). In these, in order to improve the precision of results, the authors excluded the cases with creatinine ≥0.5 mg/dL and urea <30 mg/dL. Similar limitations were previously taking into consideration by others authors (Coe, 1969; Madea and Henssge, 1996; Madea et al., 1989). Munoz Barus *et al*. observed urea concentration in vitreous constant over the PMI (range 1 to 29 hours) (Munoz Barus et al., 2008). Madea *et al*. also reported that urea is stable in the PMI up to 120 hours *post mortem* in the 170 cases studied using mean concentrations of both eyes (Madea et al., 1989). The results showed an increase of urea in some cases, but this increase was attributed to *ante mortem* urea retention. A

previous study, Coe *et al.* compared urea concentrations obtained from each eye at different time intervals after death (up to 24 hours) and conclude also that high urea concentrations were due to *ante mortem* retention and not to *post mortem* changes (Coe, 1969). Urea levels showed a statistically significant inverse relationship with PMI (decreases with time) in the cortical and medullar zones. The urea it could be easily degradable by microorganisms and gradually disappear from bone. However, urea did not show a high correlation for cortical zone. This is probably due to the fact that this not only comes from proteins but also from amino acids (Prieto-Castelló et al., 2007).

Analysis of glucose concentrations in synovial fluid and vitreous humor after death showed a decrease with increasing PMI up to 36 hours (Madea et al., 2001; Schoning and Strafuss, 1980; Tumram et al., 2011). Glucose concentrations decrease rapidly after death due to continuing tissue glycolysis (Naumann, 1959), and a corresponding increase of lactate concentration is concomitantly observed. Importantly, the decrease of glucose concentration seems to be independent of the biological matrix analyzed and *post mortem* temperature (Naumann, 1959). On the other hand, Ith *et al*. identified some metabolites by H-MRS for a preliminary estimation of the PMI. In an early *post mortem* phase, the lactate concentration increased (Ith et al., 2001).

In the group of substrates is also observed the absence of consensus on the kinetics concentrations of creatinine and urea *post mortem*. So, it is necessary to conduct further studies with better control on the arrays and sample packaging, in order to reach a consensus on the potential importance of this group in the accurate estimation of PMI.

Eletrolytes

The role of electrolytes in estimating the PMI has been widely studied by several researchers (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997).

Nauman *et al.* (Naumann, 1959) verified an increase of phosphorus with longer PMI in the vitreous humor and CSF in 211 *post mortem* cases studied. This increase was considerably less in vitreous humor than in the CSF and can be explained by *post mortem* esterase activity. The concentrations of electrolytes have also been correlated with PMI in other *post mortem* specimens such as bone. Prieto-Castelló *et al.* analyzed phosphorus electrolyte in medullar and cortical bone zones to establish the PMI (Prieto-Castelló et al., 2007). The study of the mean concentrations of the biochemical parameters in function of two PMI (up to and including 10 years, and more than 10 years) shown an increase in the mean phosphorus concentrations in function of PMI, but statistically significant correlation was not observed (Prieto-Castelló et al., 2007). In *post mortem* investigations have also been demonstrated that vitreous humor magnesium do not have an important role in estimating PMI, since no correlation was found with PMI. Moreover, the magnesium concentrations are easily affected by external factors such as ambient temperature (Farmer et al., 1985).

Several *post mortem* investigations have demonstrated that vitreous humor calcium, chloride and sodium do not have an important role in estimating PMI (Dufour, 1982; Farmer et al., 1985; Jashnani et al., 2010; Madea et al., 1989; Naumann, 1959). These electrolytes did not change significantly following death and were not statistically significant (Coe, 1969; Jashnani et al., 2010; Madea et al., 1989; Madea et al., 1990). Tumram *et al*. demonstrated that calcium and sodium concentration in synovial fluid and vitreous humor does not change significantly after death without any significant correlation with PMI up to 36 hours (Tumram et al., 2011). Chloride decreases after death and does not show significant correlation with PMI (Tumram et al., 2011). Jetter *et al.* was the first to report that human plasma chloride concentration decreased at 24 hours *post mortem* (Jetter, 1959). Later Querido *et al.* also observed that the chloride concentration decreased rapidly during the first 24 hours following death (Querido, 1990b). Highly significant linear relationship was observed between the *post mortem* rat plasma chloride concentration and PMI up to 96 hours. The authors obtained similar concentrations to those previously reported for PMI up to 24 hours. The study also demonstrated that because of similarities in rat and

human blood, the relationship between plasma chloride concentrations can be applicable to humans. The same was observed in study by Singh *et al*. that found a significant relationship between chloride concentration and PMI, during 3 to 58 hours after death, when analyzing 474 samples of human plasma (Singh et al., 2003). However, chloride concentration was found highly dependent on the factors like age, gender, cause of death (due to trauma, burn and poison), and environmental temperature. The authors concluded that rate of fall of plasma chloride concentration was higher in cold than in hot climate (Singh et al., 2003). Tumram *et al.* demonstrated a decrease on chloride concentration after death, although it failed to correlate significantly with PMI (Tumram et al., 2011). In other studies, the serological chloride concentration was significantly correlated with PMI, decreasing linearly in the first 3 to 58 hours (Jetter, 1959; Querido, 1990b; Singh et al., 2003). However, the serological chloride concentration of the *post mortem*, depends on many factors, such as age, gender, ambient, temperature and cause of death (trauma, burns and poisoning) (Singh et al., 2003). In a study of Coe *et al.* (Coe, 1974) the chloride concentration decreases linearly in blood for the 0 to 24 hours *post mortem* period with PMI. Time and temperature (4, 20, and 37 °C) effects on *post mortem* vitreous humor were studied. Chloride and sodium concentration were stable at 4 °C for 48 hours, but were less stable at higher temperatures (Schoning and Strafuss, 1980). In vitreous humor, a *post mortem* fall in sodium and rise of potassium concentration proportional to PMI has been reported (Balasooriya et al., 1984; Jashnani et al., 2010; Singh et al., 2005). The *post mortem* vitreous humor potassium concentration and sodium/potassium ratio can thus play an important role for PMI estimation (Singh et al., 2002). Balasooriya *et al*. found a gradual linear decrease in sodium concentration during the first 85 hours after death (Balasooriya et al., 1984). In a study of Jashnani *et al*. vitreous sodium up to 50 hours after death showed little change (Jashnani et al., 2010).

The potassium is a well-studied biochemical parameter for PMI estimation and promising results were achieved. It is the most known accurate and precise biochemical indicator of PMI prior to putrefaction (36-72 hours) (Coe, 1993; Vass et al., 2002). Although controversial, it is believed that factors like age,

gender, cause of death, season of death, and refrigeration do not significantly influence *post mortem* vitreous humor potassium concentration (Garg et al., 2004; Jashnani et al., 2010). Several studies have focused in the relation between the potassium concentration and the PMI and have found very promising results. Farmer *et al*. found a marked increase of potassium and, to a lesser extent, of magnesium and calcium in *post mortem* vitreous and despite the positive correlation, individual biological variability severely limits the usefulness of predictions of PMI (Farmer et al., 1985). Vitreous humor magnesium and potassium concentrations were quite affected by external influences, such as the elevated temperatures of fires which increase the rate of release of intracellular these electrolytes. After death, there is steady potassium leak through the cell membrane to approach equilibrium with the plasma, which helps to estimate the PMI (Jashnani et al., 2010). The increase of vitreous humor potassium concentrations after death (up to 104 hours) was first described by Sturner *et al.* (Sturner, 1963). Jashnani *et al*. evaluated 120 autopsy cases to determine the potassium concentrations for estimating the PMI (up to 50 hours *post mortem*) (Jashnani et al., 2010). Vitreous potassium represented a consistently linear rise with increasing PMI. Some authors found an increased in the potassium concentration starting shortly after death, which continues to increase up to 125 hours (Coe, 1969; Jaffe, 1962). Balasooriya *et al*. found a highly significant change in the potassium concentrations that were proportional to the PMI (Balasooriya et al., 1984). A gradual linear increase in potassium concentration occurred during the first 85 hours after death. Results of the other studies were similar and also showed an increased in *post mortem* potassium concentration with the PMI (Adelson et al., 1963; Adjutantis and Coutselinis, 1972; Blumenfeld et al., 1979; Choo-Kang et al., 1983; Gregora et al., 1978; Lange et al., 1994; Naumann, 1959; Prasad et al., 2003; Rognum et al., 1991). This increase may be explained by autolytic permeability changes of cell membranes (Naumann, 1959).

In electrolytic group there is great consensus among studies of the kinetics of concentration *post mortem*. In this case, optimization studies are needed on the arrays and sample packaging in order to conclude which parameters and arrays have greater potential importance in the accurate estimation of PMI.

Table 1 – Alterations of electrolytes with post mortem interval in different matrices.

Eletrolytes	Biological Matrices	Kinetic Alteration Of Concentration	References
Phosphorus	Vitreous Humor and CSF	Increases	(Naumann, 1959)
	Medullar And Cortical Bone	Increases	(Prieto-Castelló et al., 2007)
	Rat Skeletal Muscle Tissue	Not Change Significantly	(Dogan et al., 2010)
Zinc	Rat Skeletal Muscle Tissue	Not Change Significantly	(Dogan et al., 2010)
Iron	Rat Skeletal Muscle Tissue	Increases	(Dogan et al., 2010)
Magnesium	Vitreous Humor	Increases	(Farmer et al., 1985)
	Rat Skeletal Muscle Tissue	Not Change Significantly	(Dogan et al., 2010)
Calcium	Vitreous Humor	Not Change Significantly	(Coe, 1969; Dufour, 1982; Farmer et al., 1985; Jashnani et al., 2010; Madea et al., 1989; Madea et al., 1990; Naumann, 1959)
	Synovial Fluid and Vitreous Humor	Not Change Significantly	(Tumram et al., 2011)

	Rat Skeletal Muscle Tissue	Not Change Significantly	(Dogan et al., 2010)
Chloride	Synovial Fluid and Vitreous Humor	Decreases	(Tumram et al., 2011)
	Vitreous Humor	Not Change Significantly	(Coe, 1969; Dufour, 1982; Farmer et al., 1985; Jashnani et al., 2010; Madea et al., 1989; Madea et al., 1990; Naumann, 1959)
	Human Plasma	Decreases	(Jetter, 1959)
	Rat and Human Plasma	Decreases	(Querido, 1990b; Singh et al., 2003)
	Serological	Decreases	(Jetter, 1959; Querido, 1990b; Singh et al., 2003).
	Blood	Decreases	(Coe, 1974)
Sodium	Rat Skeletal Muscle Tissue	Increases	(Dogan et al., 2010)
	Vitreous Humor	Not Change Significantly	(Dufour, 1982; Farmer et al., 1985; Jashnani et al., 2010; Madea et al., 1989; Naumann, 1959)
	Synovial Fluid	Not Change Significantly	(Tumram et al., 2011)
	Vitreous Humor	Decreases	(Balasooriya et al.,

			1984; Jashnani et al., 2010; Singh et al., 2005)
Potassium	Vitreous Humor	Increases	(Adelson et al., 1963; Adjutantis and Coutselinis, 1972; Balasooriya et al., 1984; Blumenfeld et al., 1979; Choo-Kang et al., 1983; Coe, 1969; Farmer et al., 1985; Gregora et al., 1978; Jaffe, 1962; Jashnani et al., 2010; Lange et al., 1994; Naumann, 1959; Prasad et al., 2003; Rognum et al., 1991; Singh et al., 2005; Sturner, 1963)

Enzymes

Enzymes are specialized proteins in the catalysis of biological reactions. Practically all reactions that characterize the cellular metabolism are catalyzed by them. Enzymes are therefore considered functional units of cellular metabolism. Have a high degree of specificity on substrates accelerating specific reactions without being altered or consumed during the process. All

enzymes are synthesized intracellularly (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997).

The *post mortem* enzyme activity has also been investigated by various authors in different specimens for estimation of PMI. The decrease of activity for lactate dehydrogenase enzyme in liver showed good correlation with PMI until 35 days after death (Gos and Raszeja, 1993). The lactate dehydrogenase activity initially increase in activity with a subsequently decline within 100 hours of death (Mann et al., 1978). This increase is explained, not only by the extensive shift to anaerobic glycolysis in anoxic tissues, but also by the autolysis of erythrocytes, since most of this enzyme is found in these blood cells in the serum (Hess et al., 2011; Karlovsek, 2004; Madea et al., 2001; Naumann, 1959; Pla et al., 1986; Schoning and Strafuss, 1980; Vivero et al., 2008).

The creatine kinase activity was studied in rat skeletal muscle up to 40 days after death. The creatine kinase activity declines linearly with time (Mayer and Neufeld, 1980). Gamma glutamyltranspeptidase showed a tendency towards *post mortem* increase time-dependently, although not significantly. The authors also investigated GGT from three sampling sites (left cardiac blood, right cardiac blood, and femoral vein blood). For GGT femoral blood has showed a significantly lower value than the cardiac blood. The GGT showed no significant differences due to the etiology of death (Uemura et al., 2008).

Until this moment, an absence of studies is observed on the kinetics concentrations of most enzymes post *mortem*. Therefore, further studies are needed with better control on the arrays and sample packaging for a conclusion to be taken implying a potential importance of the enzymatic group in the accurate estimation of PMI.

Proteins

In a *post mortem* biochemistry study of 164 consecutive autopsy cases a time-dependent increase of total protein was observed (Uemura et al., 2008). The authors collected cardiac blood and femoral blood to clarify the differences in measured values by sampling site. Total protein and C-reactive protein increased after death and femoral blood has showed a significantly lower value than the cardiac blood. The authors also carried out a comparison by etiology of death and significant differences were found for total protein in the right cardiac blood. For total protein, there were significant differences in fire death-blunt injury, fire death-sharp injury, and fire death-internal death and little differences in fire death-intoxication, fire death-asphyxiation and fire death-drowning. The C-reactive protein showed no significant differences due to the etiology of death (Uemura et al., 2008). Analyses of the mean concentrations of biochemical parameters studied in function of two PMI (up to and including 10 years, and more than 10 years) in medullar and cortical bone zones, show that there was no statistically significant correlation between PMI and total protein (Prieto-Castelló et al., 2007).

Up to date, an absence of studies is observed on the kinetic concentrations of most proteins post *mortem*. Therefore, further studies are needed with better control on the arrays and sample packaging for a conclusion to be taken implying a potential importance of the protein group in the accurate estimation of PMI.

PART II: OBJECTIVES OF THE THESIS

There is a clear need in the discovery of a new reliable and accurate method for estimating the PMI, since all the methods currently used provide a mere approximation. The biochemical complexity of the human organism has been an obstacle for researchers of this topic since there are numerous variables that can influence the rate and form of *post mortem* changes. It is therefore essential to be aware that the human body functions as a whole, and not as a sum of its parts. In other words, no parameter can be studied or analyzed single shape or independently (Madea, 2005b). The major obstacle of the investigations carried so far has been trapped with this riddle. Much of the research focuses in only one set of parameters belonging to the same group (lipids, substrates, electrolytes, enzymes and proteins). Studies should be developed as a whole, and not as the sum of the parts, in the other words, studies should be done by linking parameters of different groups interacting. And not as they have been developed until now, only groups.

A literature review on this topic is not very consistent, since we observe a lack of agreement and consistency in current experiments, especially in the group of electrolytes. In other words, different authors studied the same parameters, the same biological matrices, the same atmospheric conditions and got completely different results. Indeed, while Jashnani *et al.* and Garg *et al.* argue that the environmental temperature has no influence on *post mortem* vitreous potassium concentration (Jashnani et al., 2010) (Garg et al., 2004), others authors showed that potassium concentration were lower in the cold weather (Bray, 1984; Komura and Oshiro, 1977). Moreover, Tumram *et al.* demonstrated that the chloride concentration in synovial fluid and vitreous humor decreases after death and does not show significant correlation with PMI (Tumram et al., 2011), while others authors have found the co-relation of calcium concentration in vitreous humor with increasing PMI (Gregora et al., 1978; Sachdeva et al., 2011).)

Therefore, the aim of this study was to evaluate the kinetic alterations in the different biochemical parameters (lipids, substrates, electrolytes, enzymes and proteins) in human blood. Blood samples were the biological matrix chosen due to its usual collection in *post mortem* situations for other determinations, such as toxicological analysis, and thus can be easily implemented in routine

practice. Moreover, most chemistry analyzers are adapted to blood or serum analysis. We hypothesized that some of them could be valuable for the estimation of PMI and a combined evaluation of some of them could constitute a serious advantage to current methodology since there is no reliable and accurate method to estimate the PMI.

PART III: EXPERIMENTAL WORK

MATERIALS AND METHODS

a. Samples

Human blood samples were collected for tubes without anticoagulant and protected from light. In the initial phase of the study (phase 1), six human blood samples were kept at a temperature of 21 ° C for a period of 10 days. In a second phase (phase 2), eight human blood samples were kept at different temperatures (from 37 at 21 ° C), attempting to mimic the decline in *post mortem* body temperature. After death, the body temperature decreases to room temperature. According to the literature (Siegel et al., 2000), there is an initial maintenance of body temperature which may last for a few hours, followed by a relatively linear rate of *post mortem* cooling, until attainting at room temperature. Thus, during the first hour the samples were maintained at temperature 37°C. Then, the temperature was decreased 0.5˚C/hour up to 12h, 1˚C/hour up to 18h and 0.5˚C/hour to match at room temperature up to 10 days.

b. Kinetic analysis

The 35 biochemical parameters in kinetic changes (electrolytes, proteins, enzymes, substrates, and lipids) were analyzed in human serum samples for each time point, set for a period of 10 days. After the endpoints, blood samples were centrifuged (3000 rpm, 10 minutes) to obtain the serum. The quantification of each biochemical parameter was performed using the PRESTIGE ® 24i automated Clinical Chemistry analyzer (Cormay, Poland). Reagents were obtained from CORMAY (Poland) and from SPINREACT (Spain), accordingly to the data provided in table 1.

The blood samples were also analyzed for pH, since several mechanisms occurring after death (e.g. autolysis and putrefaction) may modify the pH (Sawyer et al., 1988).

Table 2 – List of parameters and analytic procedures.

Parameter	Purchased From	Analytical Method	Reference
Lipids			
Cholesterol	CORMAY	Enzymatic colorimetric method with cholesterol esterase and cholesterol oxidase	(Siedel et al., 1983)
Triglycerides	CORMAY	Enzymatic colorimetric method with glycerophosphate oxidase	(Jacobs and Vandemark, 1960)
Substrates			
Uric acid	CORMAY	Enzymatic colorimetric method with uricase and peroxidase	(Fossati et al., 1980)
Total bilirubin	CORMAY	Bilirubin react with sulphodiazonium salt and form azobilirubin (Malloy method)	(Malloy and Evelyn, 1937)
Glucose	CORMAY	Enzymatic colorimetric method with glucose oxidase	(Barham and Trinder, 1972)
Lactate	CORMAY	Enzymatic colorimetric method with lactate oxidase	(Wu)
Urea	CORMAY	Enzymatic kinetic method with urease and glutamate dehydrogenase	(Talke and Schubert, 1965)
Creatinine	CORMAY	Jaffe's method without deproteinization	(Fabiny and Ertingshausen,

1971)

Electrolytes			
Phosphorus	CORMAY	Colorimetric method - direct phosphomolybdate reaction without deproteinization	(Munoz et al., 1983)
Iron	CORMAY	Colorimetric method with ferrozine without deproteinization	(Williams et al., 1977)
Potassium	SPINREACT	Enzymatic via potassium-dependant pyruvate linase activity	(Berry et al., 1989)
Magnesium	CORMAY	Xylidyl blue method	(Bohuon, 1962)
Calcium	CORMAY	O-krezoloft method	(Connerty and Briggs, 1966)
Chloride	SPINREACT	Thiocyanate-Hg method	(Schoenfeld and Lewellen, 1964)
Sodium	SPINREACT	Enzymatic colorimetric method with ONPG	(Berry et al., 1988)
Zinc	SPINREACT	Direct colorimetric method with 5 Br-PAPS without deproteinization	(Burtis et al., 1999)
Enzymes			
Alanine Aminotransferase	CORMAY	IFCC method without pyridoxal phosphate	(Bergmeyer et al., 1986b)
Aspartate Aminotransferase	CORMAY	IFCC method without pyridoxal phosphate	(Bergmeyer et al., 1986a)

Lactate Dehydrogenase	CORMAY	DGKC kinetic method	(Berry et al., 1973)
Creatine kinase	CORMAY	IFCC kinetic method	(Lott and Stang, 1980)
Alkaline phosphatase	CORMAY	IFCC kinetic method	(Bowers and McComb, 1966)
γ-glutamyltranspeptidase	CORMAY	Kinetic method with L-γ-glutamyl-3-carboxy-4-nitroanilide	(Persijn and van der Slik, 1976)
Amylase	CORMAY	CNP-G3 method	(Winn-Deen et al., 1988)
Lipase	SPINREACT	Kinetic colorimetric method	(Junge et al., 1983)
Proteins			
Total protein	CORMAY	Biuret reaction	(Gornall et al., 1949)
Albumin	CORMAY	Bromocresol green method	(Doumas et al., 1971)
C-reactive protein	CORMAY	Antigen-antibody reaction	(Burtis and Ashwood, 1994)
α-1-antitrypsin	SPINREACT	Turbidimetry	(Dati et al., 1996)
Complement 3	SPINREACT	Turbidimetry	(Young, 1997)
Complement 4	SPINREACT	Turbidimetry	(Young, 1997)
Ferritin	SPINREACT	Latex turbidimetry	(Cook et al., 1974)
Transferrin	SPINREACT	Turbidimetry	(Dati et al., 1996)

Immunoglobulin M	SPINREACT	Turbidimetry	(Dati et al., 1996)
Immunoglobulin G	SPINREACT	Turbidimetry	(Dati et al., 1996)
Immunoglobulin A	SPINREACT	Turbidimetry	(Dati et al., 1996)
Immunoglobulin E	SPINREACT	Latex turbidimetry	(Plotz and Singer, 1956)
β-2-microglobulin	SPINREACT	Latex turbidimetry	(Chironna et al., 1994)

c. Data Analysis

The quantification and expression data were statistically processed with GraphPad Prism® 6. The determined *P* values of the statistical significance were examined using linear regression and the Pearson correlation (R). We analyze the parameters in linear regressions only. The selection criteria were based on the evaluation of the degree of variability of each parameter and how these correlate with PMI. Level of significance was set for $P \leq 0.0005$. We defined as $R \geq 0.9$ the acceptable Pearson correlation values for posterior analysis. All data were graphically plotted using GraphPad Prism® 6.

PART IV: RESULTS AND DISCUSSION

Lipid Group

We started to evaluate the modification on cholesterol and triglycerides concentration in human serum after collection, since previous studies have proven to be inconsistent and scarce.

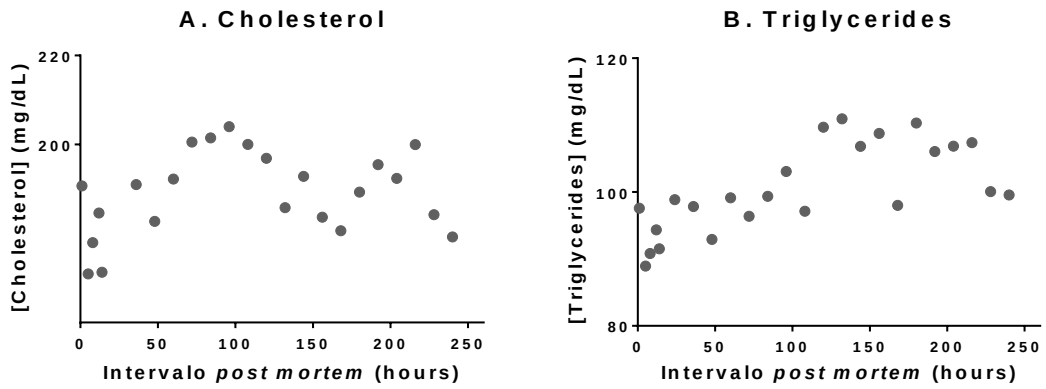


Figure 1 – Blood *post mortem* alterations in lipid concentrations. Kinetic alteration of: A – cholesterol; B – triglycerides.

Our data demonstrate that both the cholesterol concentration (Fig. 1A) and the triglycerides concentration (Fig. 1B) had no kinetic linear. Consequently, this lipids has no relevance to our study.

The concentrations of cholesterol and triglycerides were analyzed and despite previous studies claiming that both tend to decrease over time post collection (Enticknap, 1961; 1962; Glanville, 1960; Sarkioja et al., 1988), our results demonstrated a different outcome. Our data point to a fickle and unstable cholesterol concentration and an increase followed by a slight decrease from 132 hours to the concentration of triglycerides.

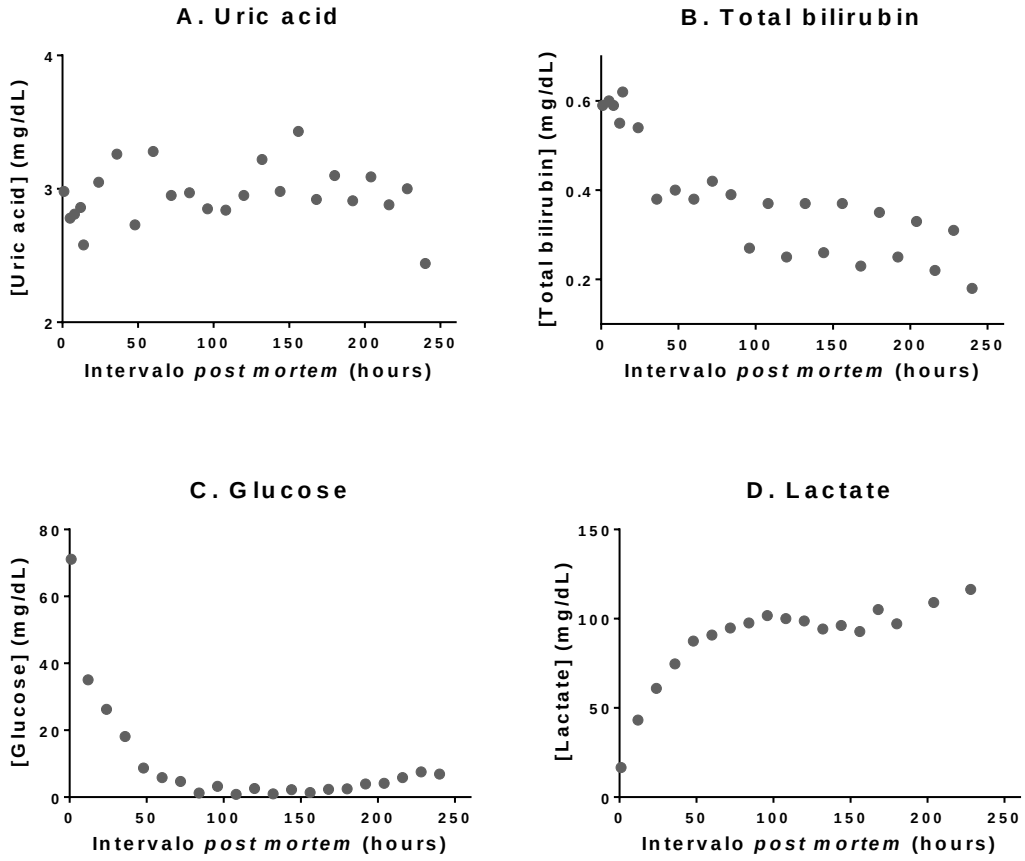
Cholesterol is essential structural component of cell membranes and precursor of bile acids and all steroids hormones. Triglycerides are built of glycerol molecule esterified with three fatty acids molecules. Cholesterol and triglycerides is delivered with food or are synthesized endogenously in liver. Triglycerides stored in adipose tissue constitute a reserve of energy (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). In our case, we analyzed the concentration of cholesterol and triglycerides present in the peripheral blood, so

the concentrations of lipids do not suffer the action of the organs and tissues after death, in other words, lipids cannot be synthesized or consumed. This tendency of lipids to increase can be explained by the unavailability of lipids in the bloodstream.

Thus, in this group, the biochemical parameters cannot be considered as promising targets to estimate the PMI.

Group of Substrates

Further, we evaluated the modification on uric acid, total bilirubin, urea, glucose, lactate and creatinine concentration in human serum after collection. Previous studies were inconsistent in gathering indisputable data. As an exception, the studies evaluating glucose kinetics *post mortem* are abundant and consistent. Several authors reported that glucose concentration decrease rapidly after death (Madea et al., 2001; Schoning and Strafuss, 1980; Tumram et al., 2011).



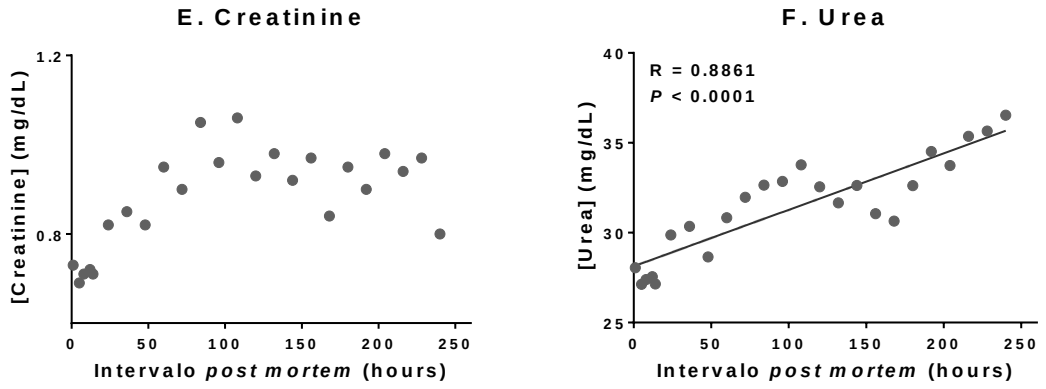


Figure 2 – Blood *post mortem* alterations in substrates concentrations. Kinetic alteration of: A – uric acid; B – total bilirrubin; C – glucose; D – lactate; E – creatinine; F – urea.

Our data demonstrate that the uric acid (Fig. 2A), total bilirubin (Fig. 2B), glucose (Fig. 2C), lactate (Fig. 2D) and creatinine (Fig. 2E) concentrations had no kinetic linear. On the other hand, urea (Fig. 2F), demonstrated a very significant variation during 250 hours post collection. However the urea concentration does not reach the minimum threshold of Pearson correlation predetermined by us.

The concentrations of uric acid, total bilirubin and urea were analyzed. Previous studies claimed that the concentrations of the first two tend to increase and the third to be constant over time post collection (Madea et al., 2001; Naumann, 1959; Uemura et al., 2008). In fact, we found a different set of data. The uric acid concentration remains practically constant over time, the concentration of total bilirubin shows tendency to decrease. The concentration of glucose shows a considerable decrease over the first 78 hours and lactate shows a considerable increase over the first 96 hours post collection. The concentration of creatinine shows a considerable increase over the first 108 hours post collection. Urea shows a considerable increase during 250 hours post collection.

Uric acid is a product of purine catabolism. It is produced in the liver and excreted in the urine. Both, the amount of uric acid production and the efficiency of renal excretion, affect serum urate level (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The slight increase observed in the first 60 hours (Fig. 2A)

can be explained by renal failure post collection. Bilirubin is an product of heme degradation. For clinical purposes, bilirubin is expressed as two fractions (conjugated and unconjugated). In hepatocytes bilirubin is enzymatically conjugated with glucuronic acid residues. This form is called direct or conjugated. Bilirubin without glucuronic acid modification is bound to albumin and is termed unconjugated or indirect (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The decrease observed throughout the 250 hours (Fig. 2B) can be explained by the inaccessibility of the degradation of heme and thus there is no production of bilirubin. In case of glucose (Fig. 2C) and lactate concentrations (Fig. 2D), an inversely proportional phenotype was observed. In the other words, until the first 78-96 hours post collection the glucose decreases while lactate increases. The data of the concentrations of glucose and lactate are consistent with what would be expected, as well as the wide range of previous studies. The product of glycolysis is lactate, so the very significant reduction of glucose and significant rise of lactate are explained by glycolysis anaerobic tissue. (Hess et al., 2011; Karlovsek, 2004; Madea et al., 2001; Naumann, 1959; Schoning and Strafuss, 1980; Vivero et al., 2008). Creatinine is a product of creatine nonenzymatic dehydration in skeletal muscle. The amount of creatinine generated and excreted by kidney is proportional to muscle mass. Daily creatinine generation remains fairly constant, with the exception of crushing injury or degenerative diseases that cause massive damage to muscle (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The increase observed in the first 108 hours (Fig. 2E) can be explained by creatinine be transported in the blood by erythrocytes, so after death or collecting the red cells begin to lose their functions and die and creatinine may be released into the bloodstream.

Urea is a product of amino acids catabolism. It is produced in liver and excreted in urine by the kidneys (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The decrease observed throughout the 250 hours (Fig. 2F) can be explained by dehydration, renal failure and decreased protein catabolism caused by death or in our case caused by collection of peripheral blood.

Thus, in this group, the biochemical parameters cannot be considered as promising targets to estimate the PMI.

The glycemic level is influenced by nutrition, so we decided to make an extra glucose analysis, to see how this is or is not influenced by feeding and pathologies. We analyzed the different concentrations of glucose (fasting true concentration; concentration postprandial (150 to 200 mg/dL); diabetic concentration (350 to 400 mg/dL)) at various times up to 144 hours.

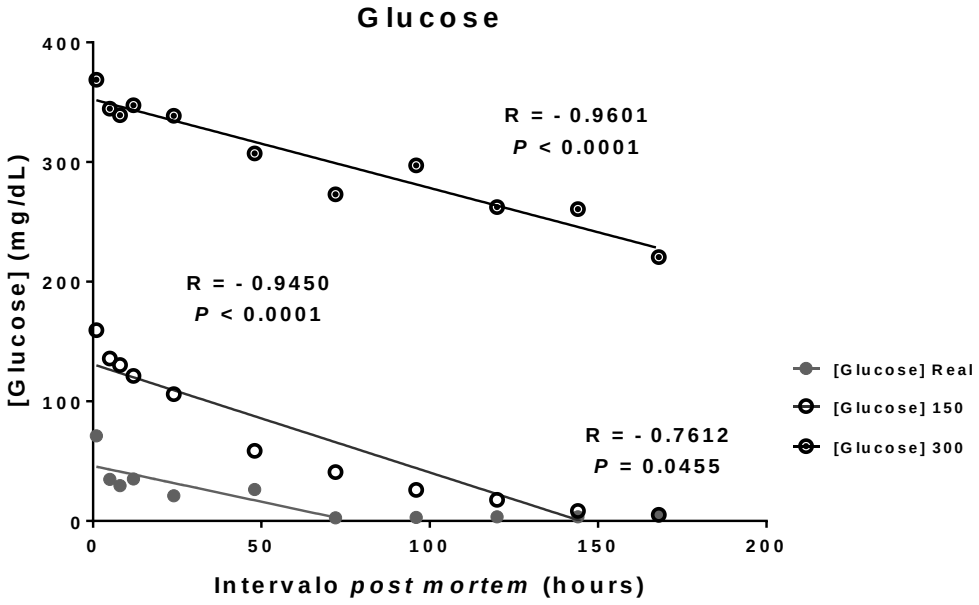


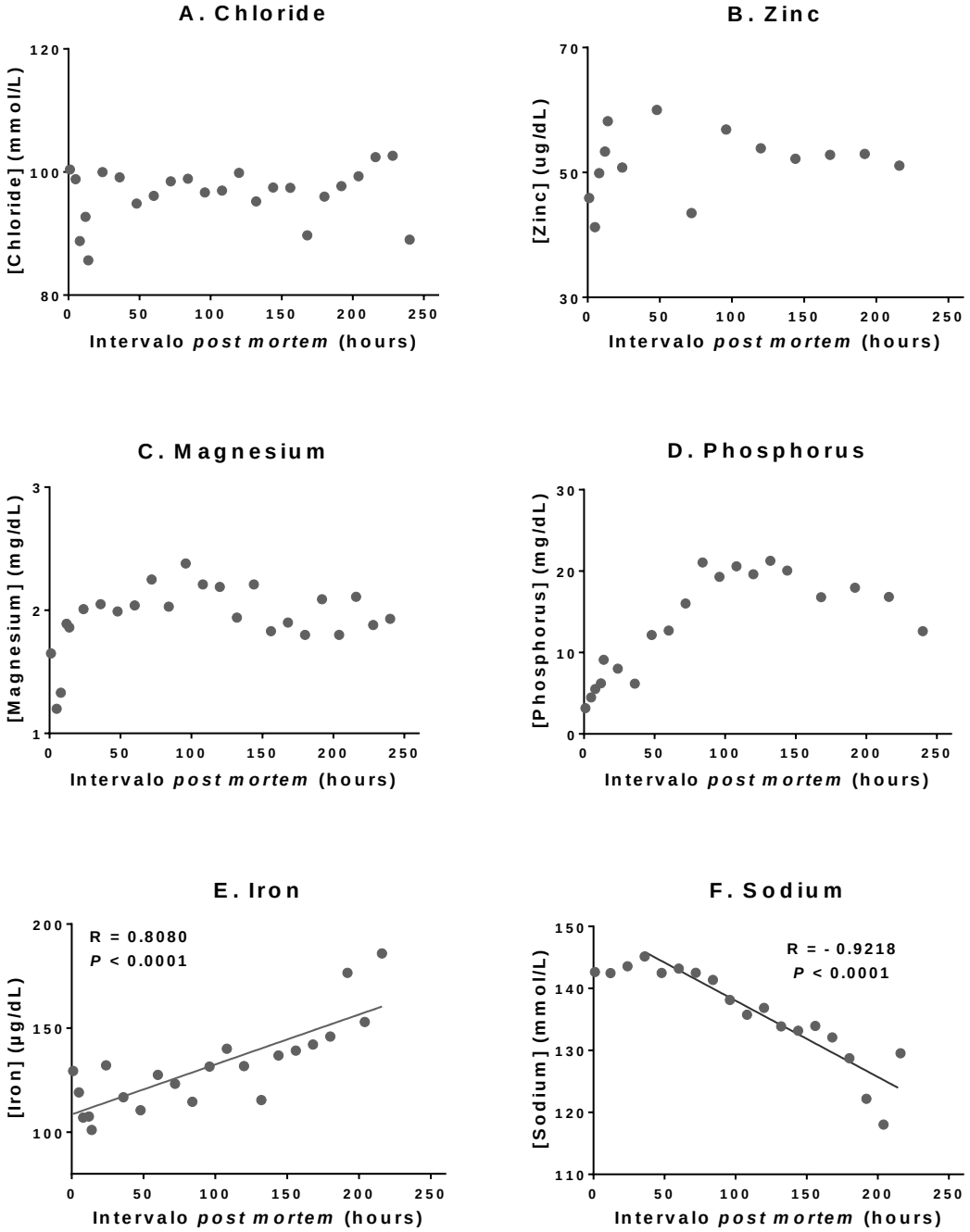
Figure 3 – Blood *post mortem* alterations in glucose at different concentrations.

In testing the hypothesis indeed found that this is not a good parameter. As can be seen in Figure 3, the concentration of glucose is influenced by nutritional status of the individual, as well as a very common disease Diabetes mellitus. Thus, for a parameter that has high degree of correlation with PMI, the parameter cannot be influenced by nutritional status or pathologies that are considered common, affecting as high population number.

Group of Eletrolytes

The electrolytic group is the most studied of this theme. Nevertheless, we decided to evaluate the modification on chloride, zinc, magnesium, iron,

phosphorus, calcium, sodium and potassium concentration in human serum post collection, trying to see if in fact it is the most promising group of this study.



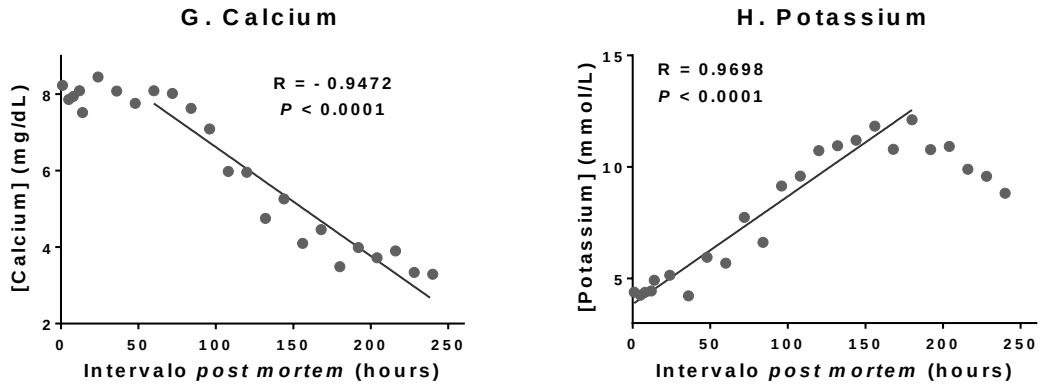


Figure 4 – Blood *post mortem* alterations in electrolytes concentrations. Kinetic alteration of: A – chloride; B – zinc; C – magnesium; D – phosphorus; E –iron; F – sodium; G – calcium; H – potassium.

Our data demonstrate that the chloride (Fig. 4A), zinc (Fig. 4B) and magnesium (Fig. 4C), phosphorus (Fig. 4D) concentrations had no kinetic linear. On the other hand, iron (Fig. 4E), sodium (Fig. 4F), calcium (Fig. 4G) and potassium (Fig. 4H) concentrations demonstrated a very significant variation during 250 hours post collection to iron, from 48 hours to sodium, from 72 hours to calcium and over the first 168 hours post collection to potassium. However the iron concentration does not reach the minimum threshold of Pearson correlation predetermined by us. As a result, only the sodium, calcium and potassium have an acceptable Pearson correlation in our study.

The chloride, zinc and magnesium concentrations tend to remain practically constant over time. The concentration of phosphorus and iron shows a considerable increase over the first 132 hours to phosphorus and during 250 hours post collection to iron. The concentration of calcium and sodium shows a considerable decrease from 36 hours to sodium and from 60 hours to calcium post collection. Lastly, the concentration of potassium shows a considerable increase over the first 180 hours post collection. The concentration of chloride is not consistent with previous studies, previous studies claim that the concentration of chloride tends to decrease (Coe, 1974; Dufour, 1982; Farmer et al., 1985; Jashnani et al., 2010; Jetter, 1959; Madea et al., 1989; Madea et al., 2001; Naumann, 1959; Querido, 1990b; Sachdeva et al., 2011; Schoning and Strafuss, 1980; Tumram et al., 2011). The concentrations of zinc,

magnesium and phosphorus are in agreement with previous studies, the first two parameters tend to remain unchanging over time *post mortem* and the third parameter tends to increase during the first hours after death (Dogan et al., 2010; Farmer et al., 1985). The concentrations of iron and potassium are in agreement with previous studies both have a tendency to increase after death (Dogan et al., 2010; Balasooriya et al., 1984; Jashnani et al., 2010; Singh et al., 2005; Farmer et al., 1985). The concentration of sodium and calcium are not consistent with previous studies, previous studies claim that the concentration of sodium tends to decrease (Dogan et al., 2010) and remain constant (Dufour, 1982; Farmer et al., 1985; Jashnani et al., 2010; Madea et al., 1989; Naumann, 1959; Tumram et al., 2011), and the concentration of calcium tends to remain constant (Dufour, 1982; Farmer et al., 1985; Jashnani et al., 2010; Madea et al., 1989; Naumann, 1959; Madea et al., 1990; Tumram et al., 2011).

The chloride is an element that is mainly involved in the regulation of osmotic pressure of extra cellular fluid and to its significant role in acid-base balance of the human body (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significant variation observed during 250 hours (Fig. 4A) can be explained because the collection of peripheral blood does not influence or intervene in the regulation of osmotic pressure of extra cellular fluid or in the regulation of acid-base balance. Zinc has a very important function in the immune system, both in the synthesis of immune cells used in the processes of defense against infectious agents (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significant variation observed during 250 hours (Fig. 4B) can be explained because the collection of peripheral blood does not influence or intervene in the immune system. Magnesium in human organism occurs mainly in bone (about 50%) but is present also intracellularly in other tissues. Magnesium serves as a cofactor for multiple enzymatic reactions involved in nucleic acids synthesis, transport and production of energy (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significant variation observed during 250 hours (Fig. 4C) can be explained because the collection of peripheral blood inhibits the occurrence of enzymatic reactions, as well as the synthesis and transport of nucleic acids and essentially energy production. Phosphorus is present in all body cells as a component of nucleic acids, phospholipids and phosphoproteins. Phosphorus is essential for

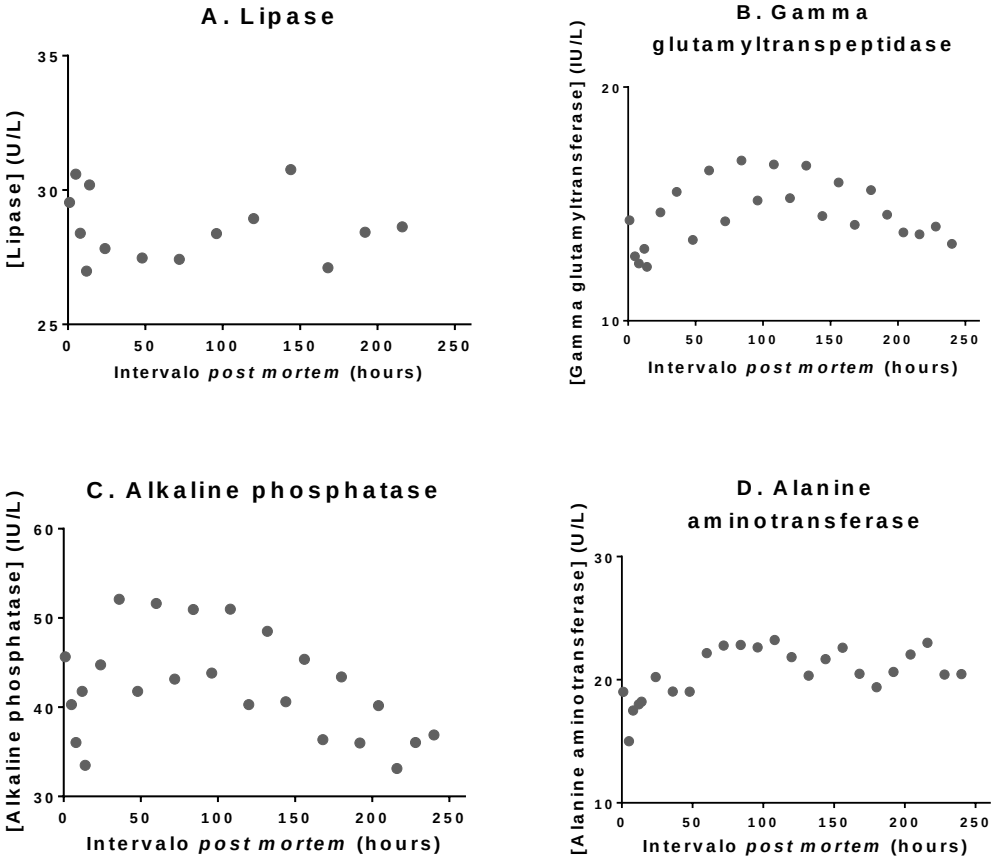
intracellular storage and conversion of energy (adenosine triphosphate (ATP), creatine phosphate) and participates in carbohydrates metabolism (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The increase observed in the first 132 hours (Fig. 34D) can be explained because the collection of peripheral blood inhibits the occurrence of intracellular stores as well as energy conversion.

Iron is the most abundant trace element in the organism. Most of the iron in humans is located within heme molecule which is incorporated into hemoglobin, myoglobin, catalase, peroxidase and cytochromes. Iron is stored bound to ferritin and is transported by transferrin (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The increase observed during 250 hours (Fig. 4E) can be explained by cell death that occurs after collection of peripheral blood consequently the degradation of heme group is releasing iron into the extracellular space. Calcium occurs as divalent cations (free or bound with negatively charged proteins) which participate in blood coagulation, neuromuscular excitability, skeletal and cardiac muscle contractility and in multiple cellular functions (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The decrease observed from 60 hours (Fig. 4G) can be explained because the collection of peripheral blood stimulates the blood coagulation and the formation of chelates and carbonates. The sodium and potassium play an important role in maintaining osmotic balance, the so-called sodium-potassium pump. Sodium is the extracellular space and tends to enter the cell, while the potassium is in the intracellular space and tends to leave the cell. But in reality happens that sodium is forced out and potassium is forced to enter cells, through active transport (requires energy in the form of ATP) (Balasooriya et al., 1984; Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The decrease observed from 60 hours to sodium (Fig. 4F) and the increase observed in the first 180 hours to potassium (Fig. 4H) can be explain by inactivating sodium-potassium pump, due to an energy shortage. Eletrolytes usually transport themselves by a passive movement per concentration gradient (requires no energy) so when there is no more energy, sodium enters cells and potassium comes out equaling their concentrations.

Therefore, in this group of biochemical parameter, only sodium, calcium and potassium can be considered as promising targets to estimate the PMI.

Enzymatic Group

Next, we evaluated the modification of lipase, gamma glutamyltranspeptidase, alkaline phosphatase, alanine aminotransferase, amylase, aspartate aminotransferase, creatine kinase and lactate dehydrogenase concentration in human serum post collection, since the few existing studies on this group showed that only a few enzymes are studied.



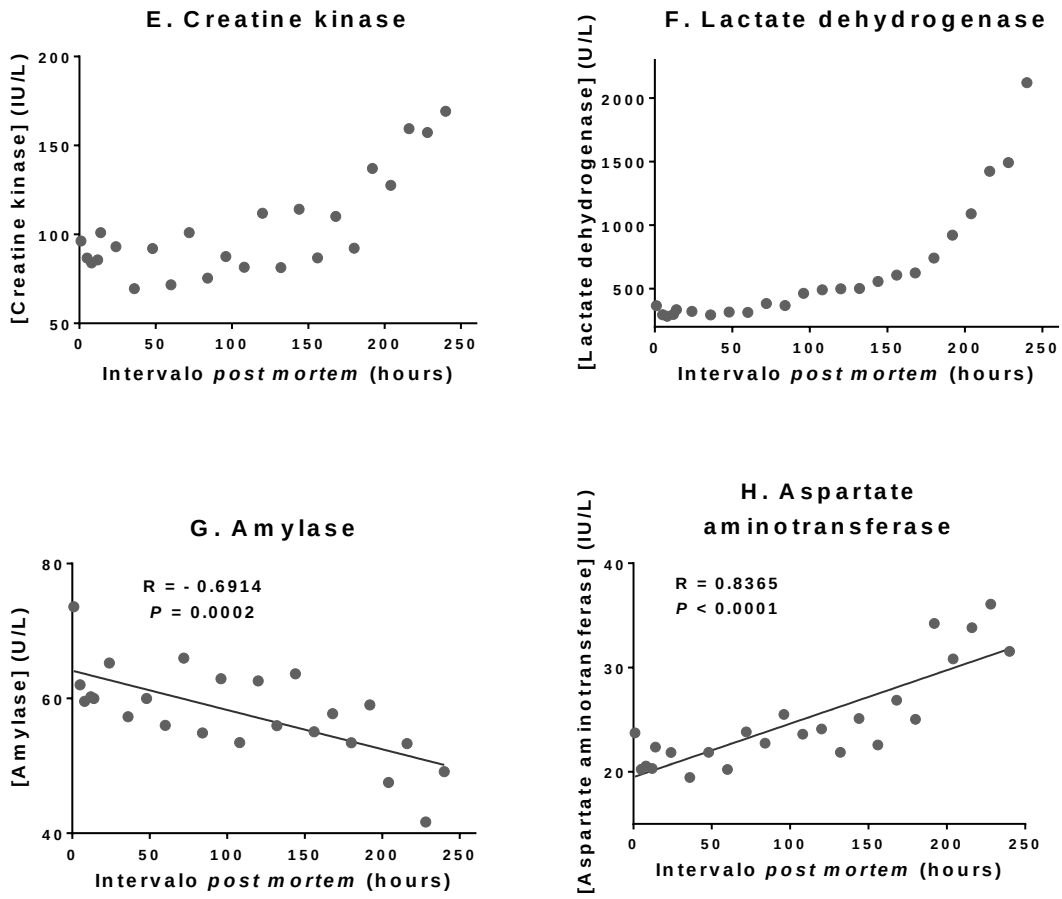


Figure 5 – Blood *post mortem* alterations in enzymes concentrations. Kinetic alteration of: A – lipase; B – γ-glutamyltranspeptidase; C – alkaline phosphatase; D – alanine aminotransferase; E – creatine kinase; F – lactate dehydrogenase; G – amylase; H – aspartate aminotransferase.

Our data demonstrate that the lipase (Fig. 5A), γ-glutamyltranspeptidase (Fig. 5B), alkaline phosphatase (Fig. 5C), alanine aminotransferase (Fig. 5D), creatine kinase (Fig. 5E) and lactate dehydrogenase (Fig. 5F) concentrations had no kinetic linear. On the other hand, amylase (Fig. 5G) and aspartate aminotransferase (Fig. 5H) concentrations demonstrated a very significant variation during 250 hours post collection. However the amylase and aspartate aminotransferase concentrations do not reach the minimum threshold of Pearson correlation predetermined by us.

The lipase, γ-glutamyltranspeptidase, alkaline phosphatase and alanine aminotransferase concentrations tend to remain practically constant over time. The concentration of creatine kinase and lactate dehydrogenase shows a considerable increase from 84 hours to creatine kinase and from 60 hours post

collection to lactate dehydrogenase. The concentration of amylase shows a considerable decrease during 250 hours post collection. Lastly, the concentration of aspartate aminotransferase shows a considerable increase during 250 hours post collection. The concentration of γ-glutamyltranspeptidase is not consistent with previous studies, previous studies claim that the concentration tends to increase (Uemura et al., 2008). The concentration of creatine kinase is not consistent with previous studies, previous studies claim that the concentration tends to decrease (Mayer and Neufeld, 1980). The concentration of lactate dehydrogenase is in agreement with previous studies, previous studies claim that the concentration of lactate dehydrogenase tends to increase (Coe, 1993; Hess et al., 2011; Karlovsek, 2004; Mann et al., 1978; Petsatodis et al., 2013) and tends to decrease (Gos and Raszeja, 1993).

Lipase is a pancreatic enzyme necessary for the absorption and digestion of nutrients that catalyzes the hydrolysis of glycerol esters of fatty acids functions (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significant variation observed during 250 hours (Fig. 5A) can be explained because the collection of peripheral blood does not influence or intervene in the absorption and digestion of nutrients. γ-glutamyltranspeptidase (GGT) is a membrane localized enzyme that catalyzes the transfer of glutamyl groups from glutathione to amino acids or peptides. Large GGT amounts are present in secretory organs like kidney, liver, bile duct and pancreas functions (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significant variation observed during 250 hours (Fig. 5B) can be explained because the collection of peripheral blood inhibits the occurrence of enzymatic reactions, as well as the synthesis and transport of nucleic acids. Alkaline phosphatase (ALP) is actually a group of isoenzymes that hydrolyse monophosphate esters in alkaline medium functions (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significant variation observed during 250 hours (Fig. 5C) can be explained because the collection of peripheral blood does not influence or intervene in the regulation of acid-base balance. Alanine aminotransferase (ALAT) is an enzyme participated in aminoacids metabolism. ALAT is present in all tissues but particularly high level is found in liver and kidney cells functions (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significant variation observed during 250 hours (Fig. 5D) can be explained by

inhibition of the metabolism of amino acids caused by collection of peripheral blood. Creatine kinase (CK) is a dimmer, composed of two subunits called M and B. Three different isoenzymes formed from these subunits are found in brain and smooth muscle, skeletal muscle and cardiac muscle. CK catalyzes the transfer of phosphate group between creatine phosphate and adenosine diphosphate. The product of this reaction is ATP (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The increase observed from 84 hours (Fig. 5E) can be explained because the collection of peripheral blood inhibits the energy production. Lactate dehydrogenase (LDH) is intracellular enzyme occurred in all tissues, especially in the myocardium, liver, skeletal muscle, kidney and erythrocytes. LDH catalyzes the reversible conversion of lactate to pyruvate using NAD^+ as a cofactor (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The increase observed from 60 hours (Fig. 5F) can be explained by cell death that occurs after collection of peripheral blood consequently the degradation of all cells is releasing LDH into the extracellular space.

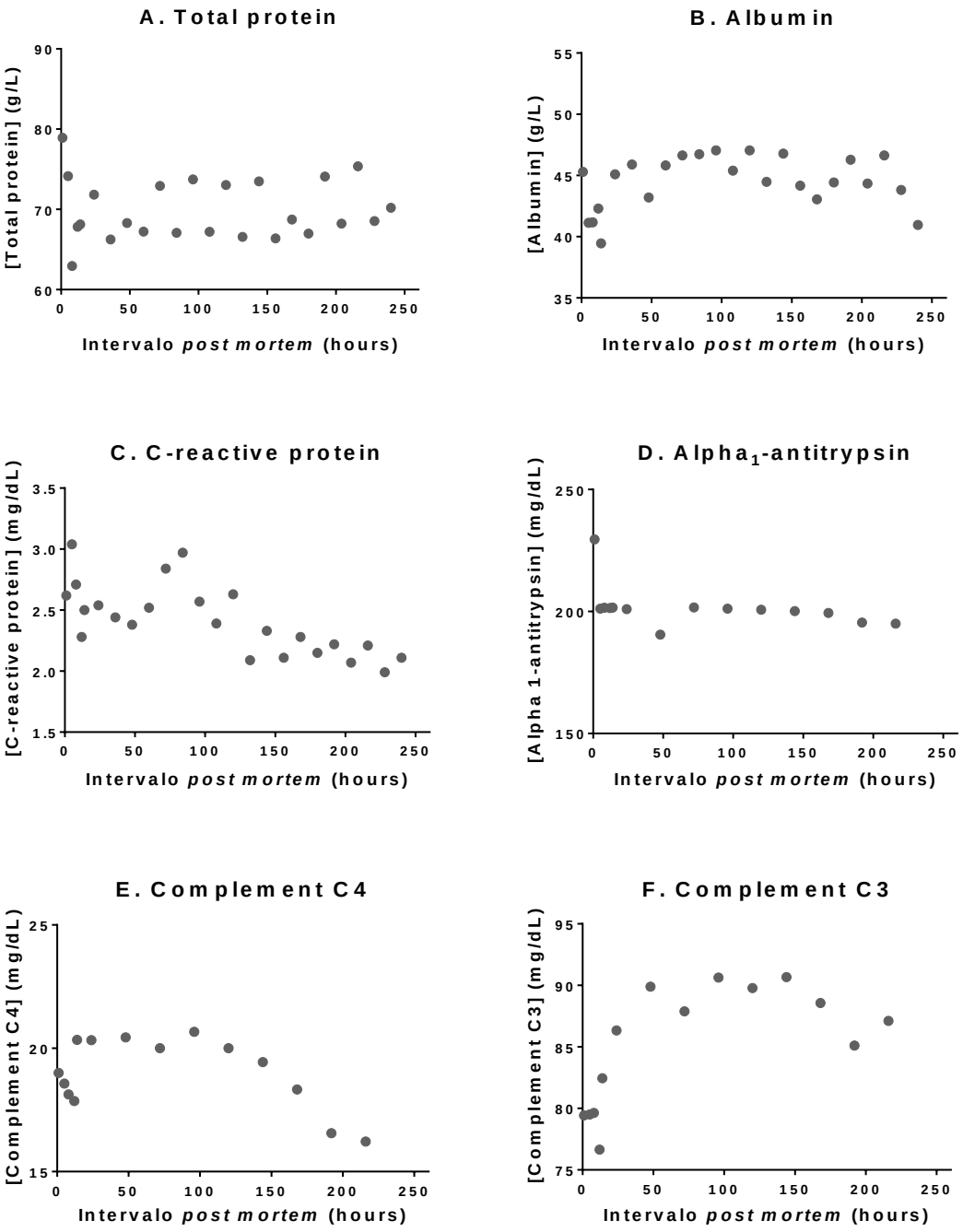
Amylase is a digestive enzyme secreted by salivary glands and pancreas (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The decrease observed during 250 hours (Fig. 5G) can be explained because the collection of peripheral blood inhibits the salivary activity. Aspartate aminotransferase (ASAT) is an enzyme participated in aminoacids metabolism. ASAT is found in all tissues but particularly high level of ASAT is observed in heart muscle, skeletal muscle, liver and kidney (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The increase observed during 250 hours (Fig. 5H) can be explained by inhibition of the metabolism of amino acids caused by collection of peripheral blood, leading to the accumulation of ASAT in extracellular space.

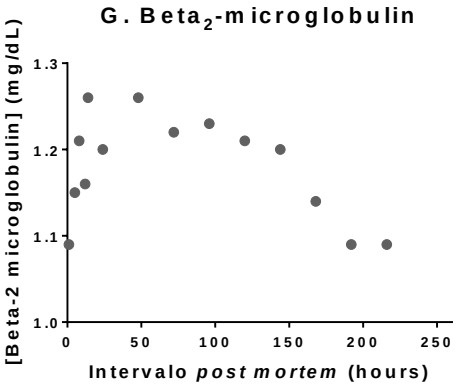
Thus, in this group, the biochemical parameters cannot be considered as promising targets to estimate the PMI.

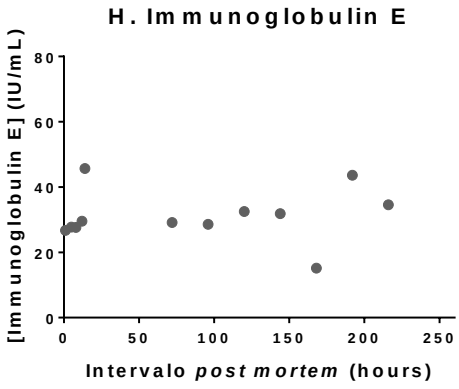
Protein Group

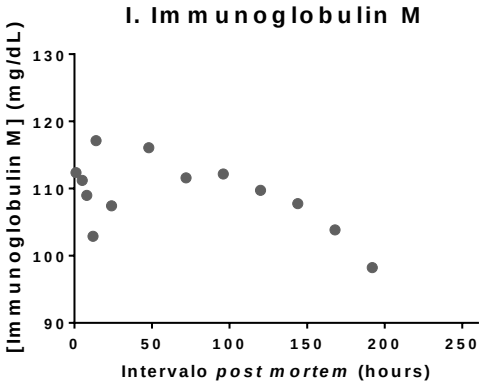
Finally we evaluated the modification on ferritin, total protein, immunoglobulin E, albumin, alpha-1-antitrypsin, complement 4, immunoglobulin

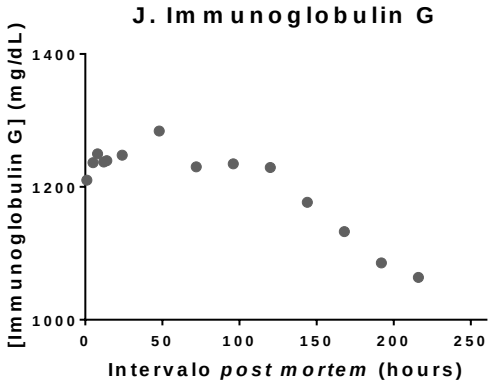
M, complement 3, C-reactive protein, immunoglobulin A, immunoglobulin G, transferrin and beta-2-microglobulin concentration in human serum after collection, since the few existing studies on this group showed that only a few proteins are studied.

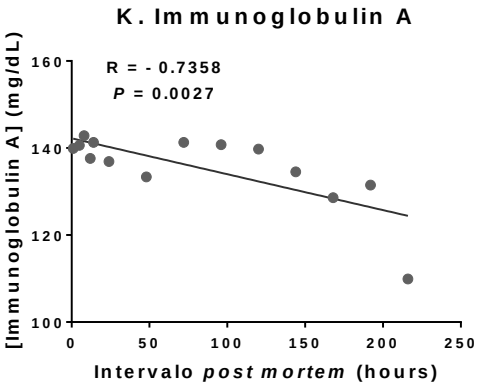


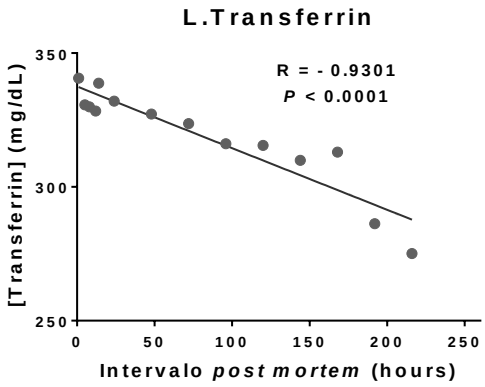

G. Beta2-microglobulin
[Beta-2 microglobulin] (mg/dL)
1.3
1.2
1.1
1.0
0
50
100
150
200
250
Intervalo post mortem (hours)


H. Immunoglobulin E
[Immunoglobulin E] (IU/mL)
80
60
40
20
0
0
50
100
150
200
250
Intervalo post mortem (hours)


I. Immunoglobulin M
[Immunoglobulin M] (mg/dL)
130
120
110
100
90
0
50
100
150
200
250
Intervalo post mortem (hours)


J. Immunoglobulin G
[Immunoglobulin G] (mg/dL)
1400
1200
1000
0
50
100
150
200
250
Intervalo post mortem (hours)


K. Immunoglobulin A
[Immunoglobulin A] (mg/dL)
R = - 0.7358
P = 0.0027
160
140
120
100
0
50
100
150
200
250
Intervalo post mortem (hours)


L.Transferrin
[Transferrin] (mg/dL)
R = - 0.9301
P < 0.0001
350
300
250
0
50
100
150
200
250
Intervalo post mortem (hours)

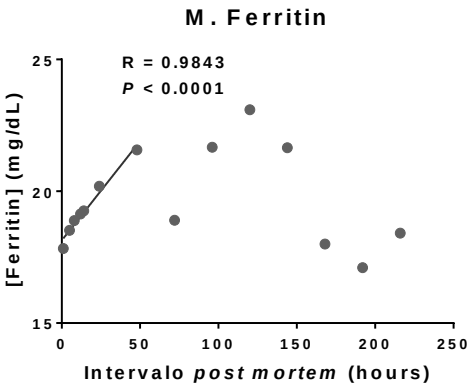

M. Ferritin
[Ferritin] (mg/dL)
R = 0.9843
P < 0.0001
25
20
15
0
50
100
150
200
250
Intervalo post mortem (hours)

Figure 6 – Blood *post mortem* alterations in proteins concentrations. Kinetic alteration of: A – total proteins; B – albumin; C – C-reactive protein; D – α_1-antitrypsin; E – complement C4; F – complement C3; G – β_2-microglobulin; H – immunoglobulin E; I – immunoglobulin M; J – immunoglobulin G; K – immunoglobulin A; L – transferrin; M – ferritin.

Our data demonstrate that the total proteins (Fig. 6A), albumin (Fig. 6B), C-reactive protein (Fig. 6C), α_1-antitrypsin (Fig. 6D), complement C4 (Fig. 6E), complement C3 (Fig. 6F), β_2-microglobulin (Fig. 6G), immunoglobulin E (Fig. 6H), immunoglobulin M (Fig. 6I) and immunoglobulin G (Fig. 6J) concentrations had no kinetic linear. On the other hand, immunoglobulin A (Fig. 6K), transferrin (Fig. 6L) and ferritin (Fig. 6M) concentrations demonstrated a very significant variation during 250 hours to the first two parameters and over the first 48 hours post collection to ferritin. However the immunoglobulin A concentration does not reach the minimum threshold of Pearson correlation predetermined by us. As a result, only the transferrin and ferritin have an acceptable Pearson correlation in our study.

The total proteins, albumin, C-reactive protein, α_1-antitrypsin, complement C4, complement C3, immunoglobulin E concentrations tend to remain practically constant over time. The concentration of β_2-microglobulin, immunoglobulin M and immunoglobulin G shows a considerable decrease from 48 hours to β_2-microglobulin and immunoglobulin M and from 120 hours post collection to immunoglobulin G. The concentration of immunoglobulin A and transferrin shows a considerable decrease during 250 hours post collection. Lastly, the concentration of ferritin shows a considerable increase over to the first 48 hours post collection. The concentration of total proteins and C-reactive are not consistent with previous studies, previous studies claim that the concentrations tends to increase (Uemura et al., 2008).

Most serum proteins except gamma globulins and hemoglobin are synthesized in the liver. Proteins participate in transport, catalysis and coagulation, act as hormones and receptors, antigens and antibodies, regulate osmotic pressure and play structural functions. Correct serum level of total protein depends mainly on balance between synthesis and degradation of albumin and immunoglobulins (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significant variation observed during 250 hours

(Fig. 6A) can be explained because the collection of peripheral blood does not influence or intervene in the transport and catalysis of proteins. Albumin is the major serum protein, but is present also in other body fluids: cerebrospinal, pleural and peritoneal. Albumin regulates blood oncotic pressure and serves as amino acids reservoir. Beyond of these functions albumin is very important transport protein (binds and keeps dispersed bilirubin, hormones, vitamins, calcium, magnesium, fatty acids and medicines) (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significant variation observed during 250 hours (Fig. 6B) can be explained because the collection of peripheral blood does not influence or intervene in the regulation of blood oncotic pressure. C-reactive protein (CRP) is an acute phase protein whose concentration is seen to increase as a result of the inflammatory process, most notably in response to pneumococcal (bacterial) infectious, histolytic disease and a variety of disease states. CRP to be used as a marker or general diagnostic indicator of infections and inflammation, in addition to serving as a monitor of patient response to therapy and surgery (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significant variation observed during 250 hours (Fig. 6C) can be explained because the collection of peripheral blood does not influence or intervene in the inflammatory process or histolytic disease or infections and inflammation. The α_1-antitrypsin is a glycoprotein synthesized in the hepatic parenchyma cells that circulates in the bloodstream. It is the second highest proteinase inhibitor in plasma (after α_2-macroglobulin). α_1-antitrypsin is a strong reactor with elastase, skin collagenase, chemiotrypsin, plasmin and thrombin, and also shows inhibitory activity against fungal and leukocytic proteases (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significant variation observed during 250 hours (Fig. 6D) can be explained because the collection of peripheral blood does not influence or intervene in the activity against fungal and leukocytic proteases. The complement C4 (C4) is the second component reacting in the classical pathway cascade. Most synthesis occurs in the hepatic parenchymal cells, although some may be synthesized by monocytes or others tissues. And the complement C3 (C3) is the functional link between classical and alternative pathways of activation and it is the most concentrate component of the complement system in human plasma. Hepatic cells synthesize C3, although bacterial endotoxins induce synthesis by monocytes and fibroblasts

(Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significants variations observed during 250 hours (Fig. 6E and Fig. 6F) can be explained because the collection of peripheral blood does not influence or intervene in the classical and alternative pathway cascade. β_2-microglobulin is a protein located on the surface human lymphocytes and other nucleated cells. Free β_2-m is filtered by glomerulus and subsequently reabsorbed in the proximal tubular cells (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The decrease observed from 48 hours (Fig. 6G) can be explained by cell death that occurs after collection of peripheral blood consequently lymphocytes and other nucleated cells are destroyed. The immunoglobulin E (IgE) is an immunoglobulin normally present in trace amounts. Continuous production of IgE antibodies in response to common naturally occurring allergens (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The absence of significant variation observed during 250 hours (Fig. 6H) can be explained because the collection of peripheral blood does not influence or intervene in the production of IgE antibodies.

Immunoglobulin M (IgM) is an immunoglobulin with a structure pentamer of five IgG molecules and its high molecular weight prevents its passage into extravascular spaces. The immunoglobulin G (IgG) is the most important immunoglobulin produced by plasma cells, and represents about 75% of total immunglobulins. Its main function is to neutralize toxins in tisular spaces. The immunoglobulin A (IgA) represents approximately 10 to 15% of total serum immunglobulins. Its structure is monomeric, but 10 to 15% of IgA in serum is polymeric (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The decrease observed from 48 hours to IgM (Fig. 5I), from 120 hours to IgG (Fig. 6J) and during 250 hours to IgA (Fig. 6K) can be explained by cell death that occurs after collection of peripheral blood. Transferrin is a plasma protein that contains a single polypeptide chain with approximately 6% carbohydrate. It is synthesized in the liver and transfers iron trough the serum (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The decrease observed during 250 hours (Fig. 6L) can be explained because the collection of peripheral blood means that transferrin is not produced, consequently reducing its concentration levels after the death.Ferritin is an iron-containing protein who is found in the human liver and spleen, where its function is to eliminate and store iron in body,

and is also found in small amounts in human serum (Baynes, 2005; Glew and Ninoyama, 1997; Laker, 1997). The increase observed over the first 48 hours (Fig. 6M) can be explained because the collection of peripheral blood means that ferritin does not eliminated or stored iron consequently accumulates into the extracellular space.

Thus, in this group of biochemical parameter only transferrin and ferritin can be considered as promising targets to estimate the PMI.

Table 3 – Equation, Pearson correlation (R) and *P* values of linear regression were calculated for each parameter.

Parameters	**Results**		**Statistics**
	Equation	Pearson Correlation (R)	P value (*P*)
	Substrates		
Urea	Y = 0.03141*X + 28.13	0.8861	< 0.0001
	Electrolytes		
Iron	Y = 0.2400*X + 108.6	0.8080	< 0.0001
Sodium	**Y = - 0.1230*X + 150.3**	**- 0.9218**	**< 0.0001**
Calcium	**Y = - 0.02849*X + 9.466**	**- 0.9472**	**< 0.0001**
Potassium	**Y = 0.04836*X + 3.848**	**0.9698**	**< 0.0001**
	Enzymes		
Amylase	Y = - 0.05827*X + 64.11	- 0.6914	0.0002

Aspartate Aminotransferase	Y = 0.05126*X + 19.49	0.8365	< 0.0001
	Proteins		
Immunoglobulin A	Y = - 0.08248*X + 142.2	- 0.7358	0.0027
Transferrin	**Y = - 0.2304*X + 337.5**	**- 0.9301**	**< 0.0001**
Ferritin	**Y = 0.07536*X + 18.14**	**0.9843**	**< 0.0001**

The other parameters did not show linear regressions

PART V: CONCLUSIONS AND FUTURE PERSPECTIVES

Our data demonstrated that some of the biochemical parameters studied have the potential to estimate the PMI. The conditions developed in our work showed the existence of a positive or negative correlation between some biochemical parameters and the time elapsed after collection. Sodium, calcium, potassium, transferrin and ferritin showed to be promising. Thus, these parameters showed a high potential for estimating the PMI and could in future play a key role in assessing and estimating accurate and correct PMI.

The results will be used to develop a mathematical model with predictive value in order to estimate the PMI. This study may provide a new paradigm to estimate the PMI, in the way that it could be considered as a complementary procedure that can be used along with the methods that are already used. Confirmatory studies will be conducted in *post mortem* blood samples.

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