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Differentiating Primary and Secondary Hypothermia in a Rat Model: The Role of Biochemical Markers in Postmortem Analysis

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Abstract: Postmortem biochemistry is a valuable tool in forensic investigations, providing insights into the tissue damage and organ dysfunction associated with death. This study aimed to identify biochemical markers that distinguish primary and secondary hypothermia. Twenty-one Wistar rats were allocated into three groups: the Control group (n = 7), which was exposed only to hypothermic conditions, the Alcohol + Hypothermia group (n = 7), and the Benzodiazepines + Hypothermia group (n = 7). The temperature metrics assessed included the normal core temperature, the post-ketamine (0.3 ml injection) core temperature, the immersion temperature, temperature at the onset of hypothermia, and temperature at death. Blood samples were collected from the thoracic aorta in EDTA vacuum tubes for biochemical analysis. The key biochemical parameters measured included the Total Protein (g/L), Albumin (g/L), Globulin (g/L), Albumin to Globulin Ratio, Alanine Aminotransferase (U/L), Alkaline Phosphatase (U/L), Cholesterol (mmol/L), Amylase (U/L), and Lipase (U/L), using an automated IDEXX (Netherlands) cell counter. Significant between-group differences were found for the total protein and globulin levels ($p < 0.001$ and $p = 0.002$, respectively), with post-hoc tests confirming differences between the alcohol and control, and benzodiazepine and control groups. The cholesterol levels were found to be significantly different through an omnibus test ($p = 0.03$), but post hoc tests did not confirm these differences on a statistically significant level. The amylase levels varied significantly across all groups ($p < 0.001$), with post hoc tests confirming significant differences among all pairs: alcohol vs. benzodiazepine ($p = 0.002$), alcohol vs. control ($p = 0.003$), and benzodiazepine vs. control ($p < 0.001$). The lipase levels showed significant differences in the omnibus test ($p = 0.030$), but there was no significance in the post hoc tests. Amylase emerged as the most significant parameter in our study, with reduced levels strongly associated with secondary hypothermia. These findings highlight the potential use of total protein, globulin, and amylase levels as biomarkers to differentiate between primary and secondary hypothermia in forensic contexts.

Keywords: hypothermia; cause of death; primary; secondary

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1. Introduction

Hypothermia, defined as a core body temperature of 35 °C or lower, results from a critical imbalance between heat production and heat loss, disrupting normal physiological function [1–5]. It is categorized by severity: mild hypothermia ranges from 35 °C to 32 °C, moderate from 32 °C to 28 °C, and severe hypothermia occurs when the core temperature drops below 28 °C [6–9]. Hypothermia can be classified into two main types based on its cause: primary hypothermia, which results from direct exposure to cold environments, and secondary hypothermia, which arises due to systemic conditions impairing thermoregulation, such as alcohol intoxication, trauma, or metabolic diseases [9].

In primary hypothermia, the core body temperature drops primarily due to environmental exposure, and metabolic changes may not be pronounced or specific. Conversely, secondary hypothermia often involves additional physiological stressors, leading to more complex biochemical alterations. For instance, secondary hypothermia is frequently associated with underlying conditions that can disrupt normal protein synthesis, immune function, and enzyme activity [10–13]. These factors may manifest as altered total protein (TP), globulins, and amylase (AMY) levels, which are influenced by the body's metabolic state rather than hypothermia itself. Low TP levels could suggest malnutrition or hepatic insufficiency, while changes in globulin levels may indicate chronic inflammatory or immune responses. Elevated AMY might be associated with pancreatic injury or other systemic conditions contributing to secondary hypothermia [2,3,14,15]. However, it is important to recognize that these biomarkers alone are non-specific and can be influenced by a range of factors unrelated to hypothermia.

Postmortem biochemical analysis, while valuable, has limitations in distinguishing primary from secondary hypothermia. Total protein, globulins, and AMY levels can remain within normal ranges or show only minor deviations depending on the underlying condition and time elapsed since death [16–18]. Forensic investigations must therefore rely on an integrated approach, combining clinical history, environmental factors, autopsy findings, and toxicology reports to accurately determine the cause of death [19]. In particular, autopsy findings, such as the presence of cold-induced tissue damage, frostbite, or specific organ injuries, should be considered alongside biochemical data to form a complete picture of the hypothermic process [20–22].

In forensic and clinical contexts, postmortem serum markers like amylase and gamma-glutamyl transferase (GGT) have garnered attention for their potential to indicate specific causes of death. Elevated AMY and lipase levels, typically associated with pancreatic and hepatobiliary conditions, may also signal broader systemic failure in cases of trauma or intoxication [23,24]. GGT has been proposed as a marker for chronic alcohol abuse, which could contribute to secondary hypothermia through impaired thermoregulation and systemic compromise, though its utility in postmortem settings remains debated [25]. However, the postmortem persistence of these markers and their reliability in distinguishing between different causes of death, including hypothermia, requires further investigation.

The objective of this study was to identify biochemical markers that may assist in distinguishing between primary and secondary hypothermia, particularly in cases complicated by acute alcohol and benzodiazepine ingestion. This study seeks to explore the utility of total protein, globulins, and amylase, among other markers, in creating an accurate classification of hypothermia-related deaths.

2. Materials and Methods

2.1. Experimental Design

This study was conducted as a prospective, randomized experimental study using an albino Wistar rat model of hypothermia. The effects of alcohol and benzodiazepines on hypothermia were investigated. Twenty-one male Wistar rats (3 months old, weighing 200–220 g) were used. Rats were randomly assigned to one of three groups using a computer-generated random order:

1. Control Group: Exposed to hypothermic conditions without any drug administration, serving as a model for primary hypothermia.
2. Alcohol Group: Administered alcohol (red wine, Vranac, 14.0% alc., 10 mL/kg body weight) daily, in the morning, for two weeks prior to hypothermia exposure, modeling secondary hypothermia induced by alcohol ingestion.
3. Benzodiazepines Group: Administered diazepam (5 mg/kg body weight) daily, in the morning, for two weeks prior to hypothermia exposure, modeling secondary hypothermia induced by benzodiazepines use.

The sample size per group was determined based on the power to detect significant differences in effect size (Cohen's d) of 2 between groups with an α of 0.05 and 80% power, and increased by 15% to account for loss to attrition. Further, as this is a pilot experiment, we opted to detect only large effect sizes, in line with 3R principles.

2.2. Ethical Approval and Animal Welfare

The experiment was carried out at the Veterinary Faculty, University of Sarajevo, under ethical approval from the institutional ethics committee (registration number 07-03-161-2/23). All procedures complied with the principles for the care of laboratory animals, as outlined by the Federation of European Laboratory Animal Science Associations (FELASA) [26–28]. The animals were housed in a controlled environment of standard ventilated cages with a sawdust bedding (12 h light/dark cycle, room temperature 20–22 °C, humidity 40–60%) and were acclimatized for 7 days before the experiment. During this period, they had access to standard commercial feed (Pellet, Mixed Nutrition for Laboratory Animals) and water ad libitum. Animal welfare was continuously monitored throughout the study.

2.3. Induction of Hypothermia

After two weeks of treatment with alcohol or diazepam, the rats were intoxicated at the time of hypothermia induction. To induce hypothermia, each rat was anesthetized with intramuscular ketamine (10%, 100 mg/mL, 1.2 mL/kg, Ketaminol®, MSD Animal Health, Unterschleißheim, Germany), and an esophageal temperature probe was inserted 5 cm into the esophagus for continuous core temperature monitoring. The Physitemp Thermalert TH-8 thermometer (Physitemp Instruments, Clifton, NJ, USA) with a precision of $\pm$0.1 °C was used to record internal temperatures at predetermined time points: prior to submersion, after anesthesia, at the onset of hypothermia, and at the time of death.

Rats were secured on wooden boards with their heads above water and immersed in pre-cooled water at 7 °C, verified by a submerged temperature probe to ensure a stable environment. The experiment ended when death occurred, and the survival time was recorded.

2.4. Blood Sample Collection

Blood samples were collected from the thoracic aorta one hour post-mortem in EDTA vacuum tubes. The following biochemical parameters were measured using an IDEXX automated analyzer (Hoofddorp, The Netherlands): Total Protein (g/L), Albumin (g/L), Globulin (g/L), Albumin/Globulin Ratio, Alanine Aminotransferase (U/L), Alkaline Phosphatase (U/L), Cholesterol (mmol/L), Amylase (U/L), and Lipase (U/L). The blood sampling time of 1 h post-mortem was chosen based on previous evidence that this timeframe allows optimal biochemical analysis in post-mortem studies.

2.5. Statistical Analysis

Statistical analysis was performed using R version 4.4.1 (R Foundation for Statistical Computing, Vienna, Austria). Descriptive data are presented as means with standard deviations (SDs) for quantitative variables. Shapiro–Wilk's test and visual assessments (Q-Q plots and histograms) were used to evaluate normality. Levene's test was employed to assess the homogeneity of variances.

Differences in means between groups were assessed using Welch's ANOVA for variables with unequal variances, followed by the Games–Howell post hoc test to explore pairwise differences. Type I error was controlled using the Benjamini–Hochberg correction to adjust for false discovery rates (FDRs). Differences between groups in the post hoc comparisons were shown with standardized mean differences (SMDs).

A significance level of $\alpha = 0.05$ was used for all statistical tests.

3. Results

Our study comprised twenty-one albino Wistar rats, weighing between 200 and 220 g. Core temperature comparisons across the Alcohol, Benzodiazepine, and Control groups revealed significant differences at multiple time points (Table 1). Baseline temperatures varied notably, with the alcohol-treated group displaying the highest baseline mean temperature (38.8 °C ± 0.24), significantly higher than both the benzodiazepine (37.9 °C ± 0.29) and control groups (37.6 °C ± 0.39; $p < 0.001$ for both comparisons). Immediately post-submersion, temperatures remained significantly different among groups ($p = 0.015$), with the alcohol group (37.8 °C ± 0.46) maintaining higher values than the control group (36.9 °C ± 0.52; $p = 0.040$); however, differences between the alcohol and benzodiazepine groups (37.2 °C ± 0.37; $p = 0.073$) and the benzodiazepine and control groups ($p = 0.5$) were not statistically significant. At the onset of hypothermia, the mean temperatures showed no significant differences across groups ($p = 0.074$), suggesting comparable responses to hypothermic conditions. Similarly, terminal temperatures at the time of death did not differ significantly, converging around 28–29 °C across all groups ($p = 0.7$)

Table 1. Comparison of temperature measurements across different time points among groups.

	Group				Pairwise Comparisons (p-Value)		
Variable	**Control (n = 7) [1]**	**Alcohol (n = 7) [1]**	**Benzodiazepines (n = 7) [1]**	**p-Value [2]**	**Control vs. Alcohol [3]**	**Control vs. Benzodiazepines [3]**	**Alcohol vs. Benzodiazepines [3]**
Baseline temperature (°C)	37.6 (±0.39)	38.8 (±0.24)	37.9 (±0.29)	**<0.001**	**<0.001**	0.2	**<0.001**
Temperature, immediately after submersion (°C)	36.9 (±0.52)	37.8 (±0.46)	37.2 (±0.37)	**0.015**	**0.040**	0.5	0.073
Temperature, at the start of hypothermia (°C)	35.7 (±0.44)	36.3 (±0.38)	36.2 (±0.40)	0.074	0.2	0.3	0.7
Temperature, at time of death (°C)	28.7 (±0.88)	29.0 (±0.67)	28.4 (±1.84)	0.7	>0.9	>0.9	>0.9

[1] Mean (± SD). [2] One-way analysis of means (not assuming equal variances). [3] Games–Howell post-hoc test, corrected for FDR by Benjamini–Hochberg.

Biochemical analyses were conducted on samples from all three groups, with the results shown in Table 2. This analysis revealed notable differences in protein levels among the groups. The control group exhibited significantly higher total protein and globulin levels compared to the Alcohol and Benzodiazepines groups ($p < 0.001$ and $p = 0.002$, respectively). Albumin levels were also higher in the control group, though the differences were less pronounced ($p = 0.024$). Interestingly, while both treatment groups showed reductions in these markers, the differences between the Alcohol and Benzodiazepines groups were not statistically significant, particularly for total protein and globulin ($p = 0.3$ and $p = 0.6$, respectively). The albumin-to-globulin ratio, while slightly elevated in the Alcohol and Benzodiazepines groups, did not differ significantly across the groups ($p = 0.076$).

Liver enzyme markers exhibited minimal variation between groups. Alanine aminotransferase (ALT) levels were similar across the control, Alcohol, and Benzodiazepines groups, with no significant differences observed ($p = 0.6$). Alkaline phosphatase (ALP) levels showed a trend towards lower values in the Alcohol and Benzodiazepines groups compared to the control group, although this difference did not reach statistical significance ($p = 0.077$).

Table 2. Comparison of biochemical parameters between groups.

	Group							Pairwise Comparisons (*p*-Value)		
Variable	**Control (*n* = 7)** [1]	**95% CI** [2]	**Alcohol (*n* = 7)** [1]	**95% CI** [2]	**Benzo Diazepines (*n* = 7)** [1]	**95% CI** [2]	***p*-Value** [3]	**Control vs. Alcohol** [4]	**Control vs. Benzodiazepines** [4]	**Alcohol vs. Benzodiazepines** [4]
Total Protein (g/L)	77.0 (±1.5)	76, 78	71.3 (±3.5)	68, 75	67.9 (±5.0)	63, 73	**<0.001**	**0.016**	**0.016**	0.3
Albumin (g/L)	29.6 (±1.0)	29, 30	28.4 (±0.8)	28, 29	26.9 (±2.2)	25, 29	**0.024**	0.12	0.12	0.2
Globulin (g/L)	47.4 (±1.5)	46, 49	42.7 (±2.6)	40, 45	40.9 (±4.5)	37, 45	**0.002**	**0.018**	**0.025**	0.6
Albumin to Globulin Ratio	0.6 (±0.0)	0.59, 0.65	0.7 (±0.0)	0.64, 0.69	0.7 (±0.1)	0.59, 0.73	0.076	0.2	0.7	>0.9
Alanine Aminotransferase (U/L)	67.0 (±15.9)	52, 82	67.4 (±10.0)	58, 77	83.9 (±42.1)	45, 123	0.6	>0.9	>0.9	>0.9
Alkaline Phosphatase (U/L)	214.9 (±52.6)	166, 264	164.4 (±29.8)	137, 192	145.9 (±51.0)	99, 193	0.077	0.2	0.2	0.7
Amylase (U/L)	1798.1 (±125.0)	1683, 1914	1549.4 (±74.4)	1481, 1618	1244.4 (±132.6)	1122, 1367	**<0.001**	**0.003**	**<0.001**	**0.002**
Lipase (U/L)	220.1 (±16.9)	205, 236	199.0 (±9.9)	190, 208	191.9 (±20.3)	173, 211	**0.030**	0.064	0.064	0.7

[1] Mean (±SD). [2] CI = Confidence Interval. [3] One-way analysis of means (not assuming equal variances). [4] Games–Howell post-hoc test, corrected for FDR by Benjamini–Hochberg.

Markers of pancreatic function, however, showed more striking differences. The amylase levels were significantly lower in both the Alcohol and Benzodiazepines groups compared to the Control group ($p < 0.001$), and there was also a significant difference between the Alcohol and Benzodiazepines groups themselves ($p = 0.002$). Similarly, lipase levels were lower in the treatment groups compared to the Control group ($p = 0.030$), though no significant difference was found between the Alcohol and Benzodiazepines groups ($p = 0.7$).

The post hoc comparisons of biochemical parameters revealed several significant differences between the groups (Table 3). Total protein showed large standardized mean differences (SMD) between the control and both treatment groups, with SMDs of 2.3 ($p = 0.016$) for Control vs. Alcohol and 2.6 ($p = 0.016$) for Control vs. Benzodiazepines. However, the difference between the Alcohol and Benzodiazepines groups was smaller and not statistically significant (SMD = 0.85, $p = 0.3$). Similarly, globulin levels exhibited significant differences between the control and treatment groups (SMD = 2.4, $p = 0.018$ for Control vs. Alcohol and SMD = 2.1, $p = 0.025$ for Control vs. Benzodiazepines), but no significant difference was observed between the Alcohol and Benzodiazepines groups (SMD = 0.55, $p = 0.6$).

Table 3. Post hoc comparisons of mean differences in biochemical parameters between groups.

	Control vs. Alcohol			Control vs. Benzodiazepines			Alcohol vs. Benzodiazepines		
Variable	**SMD** [1]	**95% CI** [2]	***p*-Value** [3]	**SMD**[1]	**95% CI** [2]	***p*-Value** [3]	**SMD** [1]	**95% CI** [2]	***p*-Value** [3]
Total Protein (g/L)	2.3	0.92–3.6	**0.016**	2.6	1.2–4.1	**0.016**	0.85	−0.24–1.9	0.3
Albumin (g/L)	1.4	0.22–2.6	0.12	1.7	0.50–3.0	0.12	1.0	−0.08–2.1	0.2
Globulin (g/L)	2.4	1.0–3.7	**0.018**	2.1	0.82–3.4	**0.025**	0.55	−0.52–1.6	0.6
Albumin to Globulin Ratio	−1.5	−2.7–−0.30	0.2	−0.73	−1.8–0.35	0.7	0.05	−1.0–1.1	>0.9
Alanine Aminotransferase (U/L)	−0.03	−1.1–1.0	>0.9	−0.57	−1.6–0.50	>0.9	−0.58	−1.6–0.49	>0.9
Alkaline Phosphatase (U/L)	1.3	0.12–2.4	0.2	1.4	0.26–2.6	0.2	0.48	−0.58–1.5	0.7
Amylase (U/L)	2.6	1.2–4.0	**0.003**	4.6	2.6–6.7	**<0.001**	3.1	1.5–4.6	**0.002**
Lipase (U/L)	1.6	0.43–2.9	0.064	1.6	0.43–2.8	0.064	0.48	−0.58–1.5	0.7

[1] SMD = Standardised Mean Difference. [2] CI = Confidence Interval. [3] Games–Howell post hoc test, corrected for FDR by Benjamini–Hochberg.

For albumin, no statistically significant differences were found between the control and treatment groups ($p = 0.12$ for both comparisons), though the SMDs suggested some degree of variation (Figure 1). The albumin-to-globulin ratio did not show significant

differences across the comparisons, with the largest effect size noted in Control vs. Alcohol (SMD = −1.5, $p = 0.2$). Similarly, the alanine aminotransferase (ALT) levels did not exhibit any significant differences between groups ($p > 0.9$ for all comparisons).

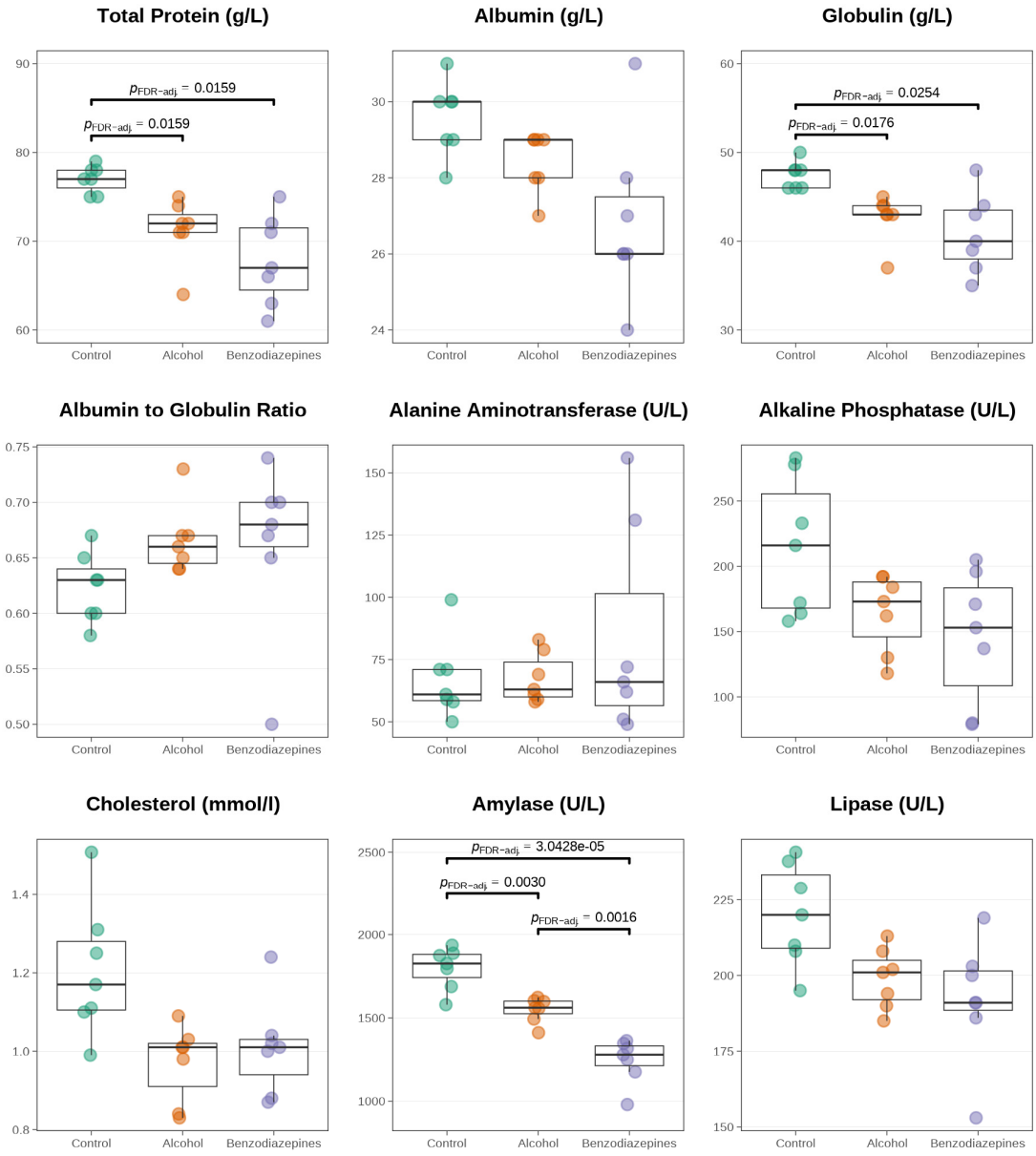

Figure 1. Boxplot of biochemical parameters across groups. Statistically significant values in post hoc tests evaluated with Games–Howell post hoc test, corrected for FDR with Benjamini–Hochberg procedure.

In contrast, pancreatic enzymes showed marked differences (Figure 1). Amylase levels were significantly lower in the Alcohol and Benzodiazepines groups compared to the Control group, with large SMDs of 2.6 ($p = 0.003$) and 4.6 ($p < 0.001$), respectively. Additionally, a significant difference was found between the Alcohol and Benzodiazepines groups (SMD = 3.1, $p = 0.002$). Lipase levels followed a similar pattern, though the differences between the control and treatment groups were not statistically significant (SMD = 1.6, $p = 0.064$ for both comparisons). No significant difference in lipase was observed between Alcohol and Benzodiazepines (SMD = 0.48, $p = 0.7$).

4. Discussion

Postmortem biochemistry has emerged as an essential supplementary technique in forensic investigations, aiding in the identification of the particular tissue injury and overall organ dysfunction that occur during the death process [20,21]. The total protein and globulin levels varied significantly among the groups ($p < 0.001$ vs. $p = 0.002$, respectively). Post hoc tests confirmed these differences for the alcohol vs. control and benzodiazepine vs. control comparisons, but not among the alcohol and benzodiazepine groups. The albumin levels ($p = 0.024$) and cholesterol levels ($p = 0.030$) were initially significantly different, but post hoc tests did not confirm these differences to be highly significant. The lipase levels initially showed significant differences ($p = 0.030$), but post hoc tests did not confirm the significance of these differences in means.

Conditions like cirrhosis or hepatitis can lead to decreased albumin production. In hypothermic patients with liver disease, the combination can exacerbate the effects of low albumin, leading to complications such as edema and impaired drug metabolism. Nephrotic syndrome results in significant protein loss through urine, leading to low serum albumin levels. Hypothermia in these patients can complicate their clinical management, as low albumin further contributes to fluid imbalances and potential cardiovascular issues. In cases of malnutrition, albumin levels may be low due to inadequate protein intake. Hypothermia can worsen the situation as the body's metabolic demands increase, further depleting available proteins. Abnormal albumin levels caused by disease can make differentiation between primary and secondary hypothermia complicated [29].

The clinical importance of determining primary and secondary hypothermia through biochemical analyses is related to power [30].

Biochemical tests help differentiate between primary hypothermia (due to environmental factors) and secondary hypothermia (resulting from medical conditions). This distinction is crucial for appropriate management. Identifying the underlying causes through biochemical markers can inform treatment strategies. For instance, if secondary hypothermia is due to toxication (alcohol consumption or drug), targeted therapy can be initiated promptly [31].

Regular biochemical assessments allow the patient's metabolic state to be monitored, helping clinicians evaluate the effectiveness of interventions and track recovery. Biochemical analyses can reveal complications such as electrolyte imbalances or coagulopathy, enabling early intervention to prevent further morbidity [32].

Understanding biochemical changes can provide insights into prognosis. Specific markers may correlate with outcomes, aiding in risk stratification and decision-making. This shows what is important in clinical medicine, as well as in forensic–legal cases too. In summary, biochemical analyses are vital for diagnosing, managing, and predicting outcomes in patients with hypothermia, leading to improved clinical care and patient safety [30].

The results of tests measuring total proteins, globulins, and amylase can provide valuable insights in distinguishing between primary and secondary hypothermia as causes of death, though they are not definitive on their own [23,33]. Total protein levels can provide an indication of overall nutritional status and systemic health [33]. In cases of severe hypothermia, total protein levels might be affected by underlying acute stress responses. However, significant deviations from normal levels are not exclusively diagnostic for hypothermia but can suggest broader issues. Globulins are a group of proteins in the blood that play roles in immune response and inflammation [34]. Elevated or decreased levels of globulins might indicate chronic disease or an inflammatory response. In hypothermia cases, changes in globulin levels can reflect the body's response to the cold stress and possible secondary infections or complications. Our findings suggest that, despite reductions in protein levels, liver function as assessed by ALT and ALP may not have been significantly impacted by either alcohol or benzodiazepine exposure in this model.

A challenge when investigating the globulin concentration in hypothermia is also represented by certain conditions that can affect protein values, such as trauma. Trauma

in combination with hypothermia can cause co-agulopathies that can affect the globulin concentration. Traumatic injuries, especially those involving significant blood loss, environmental exposure, or severe pain, can precipitate hypothermia by disrupting thermoregulation and increasing metabolic demands. Trauma triggers an inflammatory response, which can lead to the increased production of globulins, particularly immuno-globulins. This response is aimed at combating potential infections and facilitating healing. In cases of significant blood loss due to trauma, the concentration of globulins can be affected by hemodilution if fluid resuscitation is employed. This may lead to falsely low measurements of globulin levels despite ongoing inflammatory responses. In traumatic cases, the combination of injury and hypothermia can significantly impact globulin levels. Elevated globulins may indicate an inflammatory response to trauma. Such conditions make it difficult to determine whether it is primary hypothermia that has resulted in a change in the globulin concentration or secondary hypothermia after trauma, which also affects the globulin concentration [35].

In our study, the protein levels varied significantly among the groups ($p < 0.001$ for total protein and $p = 0.002$ for globulin, respectively). Post hoc tests confirmed these differences for the comparisons of alcohol vs. control and benzodiazepine vs. control comparisons, but not between the alcohol and benzodiazepine groups. This indicates the potential of globulin to be used as a parameter for distinguishing primary from secondary hypothermia.

The amylase levels were significantly different among the groups ($p < 0.001$), with post hoc tests confirming significant differences between all pairs: alcohol vs. benzodiazepine ($p = 0.002$), alcohol vs. control ($p = 0.003$), and benzodiazepine vs. control ($p < 0.001$). Elevated amylase levels can indicate pancreatic stress or damage. In the context of hypothermia, elevated amylase might be associated with secondary complications like pancreatitis or stress from severe cold exposure [36,37].

Hypothermia can reduce the metabolic rate and slow down enzymatic reactions. As temperatures drop, the activity of amylase may decrease, potentially leading to lower serum amylase concentrations. In conditions leading to secondary hypothermia, such as pancreatitis or sepsis, elevated amylase levels may occur due to tissue damage or inflammation. In these cases, hypothermia may coexist with elevated amylase, complicating the clinical picture; for example, acute pancreatitis occur as an underlying disease or be caused by some chemical agent [38].

The context in which hypothermia occurs is crucial, and while this study did not investigate the possible interaction of alcohol and benzodiazepines, this is worth exploring in future research using animal models, as CNS depressants are shown to have multiplicative effects on the thermoregulatory response when taken in combination [39].

5. Conclusions

The assessment of total proteins, globulins, and amylase levels can provide valuable insights into differentiating between primary and secondary hypothermia as causes of death in the studied rat model. Notably, amylase emerged as the most significant indicator, with reduced values strongly correlating with secondary hypothermia. These biochemical markers can enhance our understanding of the physiological changes associated with different types of hypothermia and may serve as crucial tools in the clinical evaluation and management of hypothermic conditions. A further investigation of these parameters could improve diagnostic accuracy in both experimental and forensic clinical settings.

Postmortem laboratory analysis presents several complexities due to the limitations of studies in forensic and clinical biochemistry, especially when interpreting the results. Some of the limitations are the small sample, the range of biochemical markers, the number of groups, which should be larger, and the number of measurements in which each animal would be a control itself, not only in relation to the control group. Considering that this was a pilot study, we will try to correct these limitations in the continuation of the research.

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References

1. Li, J.; Silverberg, M.A.; Decker, W.; Edelstein, J.A.; Talavera, F.; Walker, J.S.; Alcock, J.; Danzl, D. Hypothermia: Background, Pathophysiology, Etiology. Available online: https://emedicine.medscape.com/article/770542-overview?form=fpf (accessed on 15 August 2023).
2. Kempainen, R.R.; Brunette, D.D. The evaluation and management of accidental hypothermia. *Respir. Care* **2004**, *49*, 192–205. [PubMed]
3. Brychta, R.J.; Chen, K.Y. Cold-induced thermogenesis in humans. *Eur. J. Clin. Nutr.* **2017**, *71*, 345–352. [CrossRef] [PubMed]
4. Bohl, M.A.; Martirosyan, N.L.; Killeen, Z.W.; Belykh, E.; Zabramski, J.M.; Spetzler, R.F.; Preul, M.C. The history of therapeutic hypothermia and its use in neurosurgery. *J. Neurosurg.* **2018**, *130*, 1006–1020. [CrossRef] [PubMed]
5. Wang, F.S.; Fan, J.G.; Zhang, Z.; Gao, B.; Wang, H.Y. The global burden of liver disease: The major impact of China. *Hepatology* **2014**, *60*, 2099–2108. [CrossRef]
6. Jeican, I.I. The pathophysiological mechanisms of the onset of death through accidental hypothermia and the presentation of "The little match girl" case. *Clujul Med.* **2014**, *87*, 54–60. [CrossRef]
7. Zhang, C.; Pulligheddu, M.; Zhang, L.; Car, R.; Galli, G. Thermal Conductivity of Water at Extreme Conditions. *J. Phys. Chem. B* **2023**, *127*, 7011–7017. [CrossRef]
8. Yang, C.; Sugimoto, K.; Murata, Y.; Hirata, Y.; Kamakura, Y.; Koyama, Y.; Miyashita, Y.; Nakama, K.; Higashisaka, K.; Harada, K.; et al. Molecular mechanisms of Wischnewski spot development on gastric mucosa in fatal hypothermia: An experimental study in rats. *Sci. Rep.* **2020**, *10*, 1877. [CrossRef]
9. Cappaert, T.A.; Stone, J.A.; Castellani, J.W.; Krause, B.A.; Smith, D.; Stephens, B.A. National Athletic Trainers' Association Position Statement: Environmental Cold Injuries. *J. Athl. Train.* **2008**, *43*, 640–658. [CrossRef]
10. Palmiere, C.; Teresiński, G.; Hejna, P. Postmortem diagnosis of hypothermia. *Int. J. Leg. Med.* **2014**, *128*, 607–614. [CrossRef]
11. Režić-Mužinić, N.; Mastelić, A.; Benzon, B.; Markotić, A.; Mudnić, I.; Grković, I.; Grga, M.; Milat, A.M.; Ključević, N.; Boban, M. Expression of adhesion molecules on granulocytes and monocytes following myocardial infarction in rats drinking white wine. *PLoS ONE* **2018**, *13*, 196. [CrossRef]
12. Dervišević, E.; Hasić, S.; Katica, M.; Salihbegović, A.; Ajanović, Z.; Sarajlić, N. Forensic significance of cTnI serum for the detection of terminal myocardial damage in rats (*Rattus norvegicus*) caused by hyperthermia. *J. King Saud Univ. Sci.* **2022**, *34*, 1018–3647. [CrossRef]
13. Dervišević, E.; Jesenković, D.Ž.A.; Avdić, E.; Bešić, A.; Šuta, N.; Selmanagić, A. The forensic significance of core temperature in identifying primary and secondary hypothermia as a cause of death: A pilot study on Wistar rats. *Leg. Med.* **2024**, *30*, 102523. [CrossRef] [PubMed]
14. Teległów, A.; Romanovski, V.; Skowron, B.; Mucha, D.; Tota, Ł.; Rosińczuk, J.; Mucha, D. The Effect of Extreme Cold on Complete Blood Count and Biochemical Indicators: A Case Study. *Int. J. Environ. Res. Public Health* **2021**, *19*, 424. [CrossRef] [PubMed]
15. Paal, P.; Gordon, L.; Strapazzon, G.; Maeder, M.B.; Putzer, G.; Walpoth, B.; Wanscher, M.; Brown, D.; Holzer, M.; Broessner, G.; et al. Accidental hypothermia—An update. *Scand. J. Trauma Resusc. Emerg. Med.* **2016**, *24*, 111. [CrossRef]
16. Durrer, B.; Brugger, H.; Syme, D. International Commission for Mountain Emergency Medicine. The medical on-site treatment of hypothermia: ICAR-MEDCOM recommendation. *High Alt. Med. Biol.* **2003**, *4*, 99–103. [CrossRef]
17. Petrone, P.; Asensio, J.A.; Marini, C.P. In brief: Hypothermia. *Curr. Probl. Surg.* **2014**, *51*, 414–415. [CrossRef]
18. Paal, P.; Pasquier, M.; Darocha, T.; Lechner, R.; Kosinski, S.; Wallner, B.; Zafren, K.; Brugger, H. Accidental Hypothermia: 2021 Update. *Int. J. Environ. Res. Public Health* **2022**, *19*, 501. [CrossRef]

19. Drummer, O.H.; Kennedy, B.; Bugeja, L.; Ibrahim, J.E.; Ozanne-Smith, J. Interpretation of postmortem forensic toxicology results for injury prevention research. *Inj. Prev.* **2013**, *19*, 284–289. [CrossRef]
20. Muniraj, T.; Dang, S.; Pitchumoni, C.S. Pancreatitis or not?—Elevated lipase and amylase in ICU patients. *J. Crit. Care* **2015**, *30*, 1370–1375. [CrossRef]
21. Frossard, J.L.; Steer, M.L.; Pastor, C.M. Acute pancreatitis. *Lancet* **2008**, *371*, 143–152. [CrossRef]
22. Fallat, R.W.; Vester, J.W.; Glueck, C.J. Suppression of amylase activity by hypertriglyceridemia. *JAMA* **1973**, *225*, 1331–1334. [CrossRef] [PubMed]
23. Rompianesi, G.; Hann, A.; Komolafe, O.; Pereira, S.P.; Davidson, B.R.; Gurusamy, K.S. Serum amylase and lipase and urinary trypsinogen and amylase for diagnosis of acute pancreatitis. *Cochrane Database Syst. Rev.* **2017**, *4*, CD012010. [CrossRef] [PubMed]
24. Banks, P.A.; Bollen, T.L.; Dervenis, C.; Gooszen, H.G.; Johnson, C.D.; Sarr, M.G.; Tsiotos, G.G.; Vege, S.S.; Acute Pancreatitis Classification Working Group. Classification of acute pancreatitis—2012: Revision of the Atlanta classification and definitions by international consensus. *Gut* **2013**, *62*, 102–111. [CrossRef] [PubMed]
25. Yadav, D.; Agarwal, N.; Pitchumoni, C.S. A critical evaluation of laboratory tests in acute pancreatitis. *Off. J. Am. Coll. Gastroenterol. AGG* **2002**, *97*, 1309–1318. [CrossRef]
26. Al-Qirim, T.; Al Bawab, A.Q.; Al-Hiari, Y.; Ahmad, N.H.; Alwahsh, M.; Al-Kouz, S.; Shattat, G. Synthesis and lipid-lowering properties of novel N-(4-benzoylphenyl) pyrrole-2-carboxamide derivatives in Triton WR-1339-induced hyperlipidemic rats. Indonesia. *J. Appl. Pharm. Sci.* **2024**, *14*, 64–70. [CrossRef]
27. Kukoyi, A.J.; Coker, T.A.; Arowora, K.; Ukperoro, J.E.; Alabi, M.A.; Ishola, A. Comparative analysis of the effect of white and red table wines on the brain of wister rats. *Open J. Med. Res. (OJMR)* **2020**, *1*, 28–35. [CrossRef]
28. Wadee, S.A. Effect of Diazepam on Salivary Gland (Parotid Gland) in Rats. *South Asian Res. J. Pharm. Sci.* **2023**, *5*, 61–71.
29. Kim, S.H.; Youn, C.S.; Kim, H.J.; Choi, S.P. Prognostic Value of Serum Albumin at Admission for Neurologic Outcome with Targeted Temperature Management after Cardiac Arrest. *Emerg. Med. Int.* **2019**, *2019*, 6132542. [CrossRef]
30. Elmsjö, A.; Ward, L.J.; Horioka, K.; Watanabe, S.; Kugelberg, F.C.; Druid, H.; Green, H. Biomarker patterns and mechanistic insights into hypothermia from a postmortem metabolomics investigation. *Sci. Rep.* **2024**, *14*, 18972. [CrossRef]
31. Elmsjö, A.; Vikingsson, S.; Söderberg, C.; Kugelberg, F.C.; Green, H. Post-mortem metabolomics: A novel approach in clinical biomarker discovery and a potential tool in death investigations. *Chem. Res. Toxicol.* **2021**, *34*, 1496–1502. [CrossRef]
32. Urso, C.; Brucculeri, S.; Caimi, G. Acid-base and electrolyte abnormalities in heart failure: Pathophysiology and implications. *Heart Fail. Rev.* **2015**, *20*, 493–503. [CrossRef] [PubMed]
33. Lin, H.; Deng, K.; Zhang, J.; Wang, L.; Zhang, Z.; Luo, Y.; Sun, Q.; Li, Z.; Chen, Y.; Wang, Z.; et al. Biochemical detection of fatal hypothermia and hyperthermia in affected rat hypothalamus tissues by Fourier transform infrared spectroscopy. *Biosci. Rep.* **2019**, *39*, BSR20181633. [CrossRef] [PubMed]
34. Hashash, J.G.; Koutroumpakis, F.; Anderson, A.M.; Rivers, C.R.; Hosni, M.; Koutroubakis, I.E.; Ahsan, M.; Gkiaouraki, E.; Dunn, M.A.; Schwartz, M.; et al. Elevated serum globulin fraction as a biomarker of multiyear disease severity in inflammatory bowel disease. *Ann. Gastroenterol.* **2022**, *35*, 609–617. [CrossRef] [PubMed]
35. Tsuei, B.J.; Kearney, P.A. Hypothermia in the trauma patient. *Injury* **2004**, *35*, 7–15. [CrossRef] [PubMed]
36. Mallet, M.L. Pathophysiology of accidental hypothermia. *QJM* **2002**, *95*, 775–785. [CrossRef]
37. Lam, R.; Muniraj, T. Hyperamylasemia. In *StatPearls [Internet]*; StatPearls Publishing: Treasure Island, FL, USA, 2024. Available online: https://www.ncbi.nlm.nih.gov/books/NBK559273/ (accessed on 4 November 2024).
38. Khan, M.Z.; Yousaf, H.; Jamil, A.; Shah Zaib, M.; Haddad, N. Acute Pancreatitis Associated with Hypothermia: An Uncommon Presentation. *Cureus* **2020**, *12*, e8859. [CrossRef]
39. Kortelainen, M.L. Drugs and alcohol in hypothermia and hyperthermia related deaths: A retrospective study. *J. Forensic Sci.* **1987**, *32*, 1704–1712. [CrossRef] [PubMed]