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Biochemical markers of fatal hypothermia

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ABSTRACT

The aim of this study was to investigate the usefulness of postmortem biochemical investigations in the diagnosis of fatal hypothermia. 10 cases of fatal hypothermia and 30 control cases were selected. A series of biochemical parameters, such as glucose, acetone, 3-beta-hydroxybutyrate, isopropyl alcohol, free fatty acids, adrenaline, growth hormone, adrenocorticotropic hormone, thyroid-stimulating hormone, cortisol, calcium, magnesium, C-reactive protein, procalcitonin as well as markers of renal and cardiac functions were measured in blood, postmortem serum from femoral blood, urine, vitreous and pericardial fluid. The results suggested that deaths due to hypothermia, especially in free-ethanol cases, are characterized by increased ketone levels in blood and other biological fluids, increased adrenaline concentrations in urine, increased cortisol levels in postmortem serum from femoral blood and increased free cortisol values in urine. Increased or decreased levels of other biological parameters are either the result of terminal metabolic changes or the expression of preexisting diseases and may provide information to elucidate the death process on a case-by-case basis.

Keywords:
Hypothermia; Postmortem biochemistry; Biochemical markers; Ketone bodies; Blood

1. Introduction

The postmortem diagnosis of hypothermia remains difficult to ascertain today, despite progress made during the past several decades in the realm of forensic pathology [1,2]. The following autopsy findings have been proposed as indicative of death by hypothermia: frost erythema in certain body areas (extensor surfaces and large joints such as the outer hip area, elbows, knees and, less often, on the flanks and face), bright red lividity, hemorrhagic spots of the gastric and, less frequently, duodenal and jejunal mucosa, pancreatic hemorrhages, synovial membrane hemorrhages, bloody discoloration of synovial fluid, signs of acute pancreatitis and hemorrhages into the large muscles of the body, especially the iliopsoas muscle [3–10]. Histological findings, including fatty degeneration of the renal tubular epithelium cells, cardiac myocytes and hepatocytes, as well as vacuolization of pancreatic, hepatic, renal, adrenal and anterior pituitary gland cells, have also been observed in association with hypothermia fatalities [11–19].

Additionally, increases or decreases in the immunopositivity rate for some markers in the hypothalamus, anterior pituitary gland, adrenal medulla, midbrain periaqueductal gray matter, renal tubular epithelial cells and glomerular podocytes have been noted in these cases [20–25].

External and internal observations, both macroscopic and microscopic, can be of diagnostic significance when found concurrently, though they prove non-specific as exclusive findings in themselves. Frost erythema and gastric hemorrhages (Wischnewsky spots), for instance, have been proposed as specific for hypothermia when appearing concomitantly. Furthermore, a strong correlation with such macroscopic findings has been described for the fatty degeneration of the renal tubular epithelium cells [8]. Nonetheless, the diagnosis of death by hypothermia remains a medley of observations including a significant history of exposure to cold, non-specific pathological findings when present and the absence of other causes of death based on all postmortem findings.

Beyond histology and immunohistochemistry, postmortem biochemical investigations in relation to hypothermia fatalities have been carried out over the years. The first reports were published by Mant [26,27] and focused on vitreous magnesium values in a series of hypothermia fatalities and a control group. Thereafter, numerous researchers have shown interest in the postmortem biochemistry related to hypothermia fatalities with

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several, subsequent studies pertaining to blood, vitreous, urine and pericardial fluid to analyze glucose, electrolytes, hormones, ketones, isopropyl alcohol, neurotransmitters as well as renal and cardiac function markers [1,3,22–25,28–58]. Some of these molecules, especially urinary catecholamines and blood ketone bodies, have been targeted as being particularly useful in supporting the diagnosis of fatal hypothermia [1,29–33,37,40,44,50,56–58]. To date, with the exception of the studies performed by Ishikawa et al. [22–24,55] on the human pituitary hormones, there have been no studies proposing a parallel investigation of several biochemical markers simultaneously in hypothermia fatalities. Most studies have associated these deaths with a specific laboratory parameter, often in combination with macroscopic, histological or immunohistochemical findings.

The aim of this study was to investigate the usefulness of postmortem biochemical investigations in the diagnosis of fatal hypothermia. A series of biochemical parameters were analyzed in order to obtain a more integrated and complete perspective pertaining to the metabolic changes that may occur during hypothermia. Most of these parameters were chosen among those that had been described in the medico-legal literature as associated with hypothermia fatalities, including:

- glucose in urine and vitreous humor,
- 3-beta-hydroxybutyrate (3HB) in blood, urine, pericardial fluid and vitreous humor,
- acetone and isopropyl alcohol (IPA) in blood, urine and vitreous humor,
- adrenaline in urine,
- adrenocorticotropic hormone (ACTH) in whole blood,
- thyroid-stimulating hormone (TSH) and growth hormone (GH) in postmortem serum,
- cortisol in postmortem serum and free cortisol in urine,
- troponine I (cTnI), N-terminal pro-brain natriuretic peptide (NT-proBNP) and free fatty acids (FFA) in postmortem serum,
- urea nitrogen, creatinine and uric acid in vitreous humor, pericardial fluid and postmortem serum,
- calcium and magnesium in pericardial fluid,
- procalcitonin (PCT) and C-reactive protein (CRP) in postmortem serum.

2. Materials and methods

2.1. Study design

10 cases of fatal hypothermia were selected among the medico-legal cases observed at the University Center of Legal Medicine Lausanne–Geneva from 2007 to 2011. The criteria for inclusion in the hypothermia group were as follows:

- circumstantial elements suggesting exposure to cold,
- autopsy findings indicative of hypothermia (frost erythema of the outer hip areas, elbows and knees as well as Wischnewsky spots in the gastric mucosa),
- availability of all biological fluids (femoral blood, postmortem serum from femoral blood, vitreous humor, urine and pericardial fluid) upon autopsy,
- postmortem interval (time between the discovery of the body and the autopsy) within 24 h,
- exclusion of other causes of death based on all postmortem findings.

The selected cases included three females and seven males between 19 and 71 years of age, with a mean age of 44.5 years. According to the medical records, all individuals were non-diabetic. Additionally, the determination of glycated hemoglobin was performed in all cases of suspected hypothermia and revealed normal levels.

Postmortem unenhanced CT-scans, autopsies, histology, toxicology and biochemical analyses were performed in all cases.

Conventional histology (Hematoxylin–Eosin staining) revealed slight, fatty degenerative changes of the renal tubular epithelium cells. Nevertheless, such changes were not systematically observed and therefore not considered as diagnostic.

Biological samples for toxicological and biochemical investigations were collected as soon as possible upon arrival of the bodies at the morgue (from vitreous humor) and upon autopsy itself (from urine, blood and pericardial fluid).

A control group was made up of 30 medico-legal cases (8 females and 22 males), between 20 and 79 years of age, with a medium age of 49.8 years. The criteria for including the cases in the control group were related to the causes of death, postmortem intervals and availability of all biological fluids (femoral blood, postmortem serum from femoral blood, vitreous humor, urine and pericardial fluid) upon autopsy.

The control group included cases with and without injuries as well as sudden and protracted death cases (blunt injuries 5 cases, gunshot wounds 5 cases, sharp instrument injuries 5 cases, coronary thrombosis 5 cases and drug intoxication 10 cases). Investigations at the death scenes were performed in all cases with both rectal and ambient temperatures available. Circumstantial elements did not suggest exposure to cold or hypothermia as a contributing factor to death in any case. Additionally, based on investigative elements and/or medical records, none of the cases had a survival time exceeding 6 h.

All autopsies were performed within 24 h after death. Postmortem unenhanced CT-scans, autopsies, histology, toxicology and biochemical analyses were performed in all cases with biological samples for toxicological and biochemical investigations collected according to the same criteria that had been applied in the hypothermia group.

Lastly, since both study samples originated from forensic practice with deaths occurring outside the hospital in most cases, data on antemortem biochemical results before death were not available.

2.2. Biological samples

Undiluted postmortem vitreous samples were obtained by aspiration using a sterile needle and syringe as soon as possible upon arrival of the bodies at the morgue. Right and left vitreous samples were collected through a scleral puncture at the lateral canthus, aspirated from the center of each eye, pooled in the same syringe and mixed together. After collection, the vitreous samples were immediately centrifuged at 3000 × *g* for 15 min. The separated supernatant was collected and stored in tubes without preservatives.Postmortem urine samples were collected by bladder aspiration during the autopsy and stored in tubes with no preservatives. For the catecholamine determination, between 5 and 10 ml urine were also collected in tubes containing between 30 and 150 μl hydrochloric acid 6 N to adjust pH around 3.

Undiluted pericardial fluid samples (between 5 and 10 ml) were collected immediately after the incision in the pericardium during the autopsy. All the samples were immediately centrifuged at 3000 × *g* for 15 min. After centrifugation, the separated supernatant was collected and stored in tubes without preservatives.

Postmortem blood samples were collected by aspiration with a sterile needle and a syringe from the femoral vein during autopsy. The blood samples were drawn after clamping the vein at the proximal end and lifting the lower limb for several minutes. Blood was stored in tubes containing sodium fluoride (for ethanol, ketone and IPA determination) and in tubes containing ethylenediaminetetraacetic acid (EDTA) for glycated hemoglobin and ACTH determination. Blood samples were also collected in tubes without preservatives and centrifuged immediately after collection at 3000 × *g* for 15 min. After centrifugation, the separated supernatant (postmortem serum) was collected and stored in tubes without preservatives.

All biological samples were transferred to the laboratories immediately after performing the autopsies. When analyses were delayed, the samples (vitreous humor, pericardial fluid, blood, postmortem serum and urine) were stored at −20 °C. Urine samples for the determination of catecholamines were stored at −80 °C.

2.3. Analytical techniques

Glucose was analyzed in vitreous humor and urine on the Roche Modular P clinical chemistry system (Roche glucose hexokinase method calibrated using manufacturer-supplied materials and values).

Acetone and IPA were determined during ethanol analysis in blood, urine and vitreous humor by the use of headspace gas chromatography with flame ionization detection (HS-GC-FID) on an Agilent 1888 headspace and a 6850 GC (Palo Alto, CA, USA). The samples were incubated for 20 min at 80 °C and then expanded to the GC column.

3HB values were determined in blood, vitreous, urine and pericardial fluid samples. All samples were thawed overnight at 4 °C and deproteinized with perchloric acid. Supernatant was used for analysis. 3HB concentrations were determined by an enzymatic photometric method.

Urine catecholamines (adrenaline and noradrenaline) were analyzed using high-performance liquid chromatography (HPLC) with amperometric detection. Urinary catecholamine excretion was related to urinary creatinine.

NT-proBNP and PCT were measured in postmortem serum from femoral blood with the commercially available immunoassays on the Roche Modular E170 system.

Calcium (o-cresolphthalein complexone), magnesium (xylidyl blue), creatinine (Jaffé method, rate-blanked and compensated), urea nitrogen (kinetic enzymatic UV

assay for urea/urea nitrogen), uric acid (enzymatic uricase colorimetric AU Plus) and CRP (immunoturbidometric Tina-quant CRP) were determined with the Roche standard methods on the Roche Modular P system (Roche Diagnostics GmbH, Mannheim, Germany).

Calcium and magnesium were analyzed in pericardial fluid. Urea nitrogen, creatinine and uric acid were measured in pericardial fluid, vitreous humor and postmortem serum from femoral blood. CRP was determined in postmortem serum from femoral blood.

cTnI was analyzed in postmortem serum from femoral blood with the Access® AccuTnI™ assay on Access II (Beckman Coulter, Fullerton, CA, USA).

FFA were quantified in postmortem serum from femoral blood by the enzymatic colorimetric method "NEFA-HR(2)" (Wako Diagnostics) adapted on a Cobas MIRA Plus (Roche Diagnostics).

ACTH was measured by immunoradiometric assay (IRMA) on whole blood samples obtained from the femoral vein.

TSH was determined by an ultrasensitive chemoluminiscence microparticle immunoassay (CMIA) in postmortem serum from femoral blood (ARCHITECT YOU 8200, Abbott Laboratories).

Postmortem serum GH was measured with use of highly sensitive enzyme immunoassay (EIA) with the commercially available immunoassays on the Immulite analyser.

Postmortem serum cortisol was determined by a fluorescent polarization immunoassay (FPIA) available on the Abbott TDx analyzer.

Determination of free urinary cortisol was performed by radioimmunoassay (RIA).

2.4. Clinical references and statistical analyses

A non-parametric Mann–Whitney *U* test was used to compare the results obtained in hypothermia fatalities with the control cases. The following clinical reference intervals were considered for the analyzed markers:

Blood cortisol:
08:00 h 170–630 nmol/l
17:00 h 40–260 nmol/l
Urinary free cortisol: 100–400 nmol/l
Urine adrenaline: <22 nmol/mmol creatinine
Urine glucose: <0.8 mmol/l
Blood ACTH:
08:00 h 10–60 ng/l
17:00 h 5–35 ng/l
Blood TSH:
06:00 h–18:00 h 0.20–3.50 mU/l
Blood GH:
06:00 h–18:00 h <3 μg/l
Blood troponine I: <0.03 μg/l
Blood NT-pro BNP: <115 ng/l
Blood procalcitonin: <0.06 μg/l
Blood C-reactive protein: <10 mg/l
Blood acetone: <10 mg/l
Blood IPA: 0 mg/l
Blood 3HB: 50–170 μmol/l
Blood FFA: 0.1–0.6 mmol/l
Blood urea nitrogen: 2.9–7.7 mmol/l (male) and 2.9–6.4 (female)
Blood creatinine: 62–106 μmol/l (male) and 44–80 (female)
Blood uric acid: 202–416 μmol/l (male) and 142–339 (female)
Blood calcium: 2.15–2.55 mmol/l
Blood magnesium: 0.6–1.0 mmol/l

2.5. Ethical aspects

All cases collected for this study underwent medico-legal autopsies requested by the public prosecutor. Biochemical analyses were performed as part of the medico-legal investigations. No further ethical permission was required to perform laboratory analyses.

3. Results

The results for each analyzed marker (minimum and maximum values, means, medians, standard deviations and *p*-values) in hypothermia fatalities and control cases are reported in Table 1. In order to maintain the overall Type I error at 5%, differences were considered statistically different for *p*-values of ≤0.147.

Table 2 summarizes macroscopic findings (frost erythema and Wischnewsky spots) and biochemical results (blood ethanol, blood and urine 3HB, blood cortisol and urinary free cortisol levels) observed in hypothermia fatalities.

The highest clinical reference concentrations for blood cortisol, GH, ACTH and TSH levels were considered as reference values.

Statistically significant differences between hypothermia fatalities and control individuals were observed regarding ketones (in all analyzed fluids), urine adrenaline, postmortem serum cortisol and urine free cortisol, which revealed increased values in hypothermia cases. The results concerning these parameters are represented in Figs. 1–5.

Statistically significant results were also noted for serum GH (concentrations within the clinical reference values in hypothermia cases and slightly higher than clinical reference values in control cases), serum FFA (concentrations higher than clinical reference values in both hypothermia and control cases), pericardial fluid calcium and pericardial fluid magnesium (slightly higher values in control cases for both markers).

4. Discussion

Data analysis indicates that deaths by hypothermia, especially in free-ethanol cases, may be characterized by the following biochemical changes:

- increased blood ketones (3HB and acetone) and IPA,
- increased urine adrenaline,
- increased postmortem serum cortisol and urine free cortisol.

Furthermore, increased ketones (3HB and acetone) and IPA levels in ethanol-free cases were observed in all analyzed fluids (vitreous, pericardial fluid and urine).

Considering the biochemical point of view, these findings are not surprising and can easily be interpreted as evidences of the following metabolic pathways:

- enhanced secretion of the counter-regulatory hormones,
- enhanced fat catabolism and increased ketone production,
- inhibition of peripheral utilization of glucose,
- enhanced glycogenolysis and gluconeogenesis.

Hyperglycaemia may characterize the first phases of hypothermia, resulting from enhanced counter-regulatory hormone release. Thus, hyperglycaemia may theoretically be reflected in increased vitreous and urine glucose concentrations found after death.

In cases where hypothermia developed rapidly, many different processes may contribute to hyperglycemia: insulin release is inhibited, peripheral insulin uptake at the tissue level is impaired, glycogenolysis and gluconeogenesis are actively stimulated.

In cases where hypothermia develops more slowly or lasts longer, muscle and liver glycogen stores may be depleted, increasing the likelihood that hypoglycemia will develop. In the long term, shivering may also contribute to terminal hypoglycemia [59].

In our study, we did not unambiguously observe increased glucose concentrations in urine and vitreous in hypothermia fatalities compared to control subjects. Glucose levels were increased or depressed in both fluids, suggesting that these

Table 1
Results of biochemical investigations carried out in hypothermia fatalities and control group. Clinical reference intervals, measurement units and abbreviations are indicated in the text.

Biochemical marker	Hypothermia group ($n=10$)			Control group ($n=30$)			p-Value
	Min–max	Median [25%;75%]	Mean (SD)	Min–max	Median [25%;75%]	Mean (SD)	
Vitreous glucose	0.2–5.7	1 [0.2;4.7]	2.2 (2.2)	0.2–4.4	1 [0.4;1.8]	1.1 (0.9)	$p=0.4983$
Urine glucose	0–12	0 [1;4]	2.8 (4.1)	0–2	0 [0;0]	0.2 (0.6)	$p=0.0039$ (1)
Blood acetone	0–83	42 [25;56]	44 (25)	0–15	0 [0;8]	4 (5)	$p<0.001$
Urine acetone	0–220	120 [90;136]	120 (58)	0–40	0 [0;15]	9 (11)	$p<0.001$
Vitreous acetone	0–81	42 [24;56]	42 (25)	0–15	0 [0;5]	3 (4)	$p<0.001$
Blood 3HB	300–6700	1850 [400;2900]	2200 (2022)	90–560	110 [90;130]	128 (85)	$p<0.001$
Urine 3HB	400–25,000	2600 [600;3300]	4340 (7367)	120–600	120 [120;120]	143 (87)	$p<0.001$
Vitreous 3HB	100–5800	1700 [390;2800]	1987 (1809)	90–470	100 [100;120]	121 (69)	$p<0.001$
Pericardial fluid 3HB	110–6000	1850 [400–3000]	2130 (1865)	90–500	120 [110;140]	135 (72)	$p<0.001$
Blood IPA	0–15	10.5 [8;13]	9.5 (4.5)	0	0 [0;0]	0 (0)	$p<0.001$
Urine IPA	0–41	21 [16;32]	21.6 (11.8)	0	0 [0;0]	0 (0)	$p<0.001$
Vitreous IPA	0–16	9.5 [7;12]	8.9 (4.7)	0	0 [0;0]	0 (0)	$p<0.001$
Postmortem serum ACTH	10–25	16 [12;20]	16.3 (4.5)	7–20	16 [12;18]	14.7 (4)	$p=0.413$
Postmortem serum TSH	0.73–2.50	1.78 [1.35–2.21]	1.69 (0.61)	0.88–3.43	1.66 [1.43;2.04]	1.74 (0.54)	$p=0.975$
Postmortem serum GH	0.37–2.59	1.65 [1.45–1.69]	1.65 (0.58)	1.73–6.5	2.39 [1.86;2.55]	2.38 (0.84)	$p=0.001$
Postmortem serum cortisol	670–3600	925 [760;1350]	1331 (943)	200–1800	395 [320;500]	476 (309)	$p<0.001$
Urinary free cortisol	250–3530	780 [590;1200]	1140 (977)	120–240	170 [150;210]	179 (38)	$p<0.001$
Postmortem serum cTnI	0.03–1.94	0.75 [0.40;1.01]	0.53 (0.68)	0.03–5.76	0.85 [0.30;1.04]	0.78 (1.25)	$p=0.886$
Postmortem serum NT-proBNP	23–220	148 [136;180]	148 (53)	46–7263	190 [162;225]	1001 (1977)	$p=0.011$ (1)
Postmortem serum PCT	0.06–2.21	0.06 [0.06;0.25]	0.33 (0.67)	0.06–3.01	0.06 [0.06;0.58]	0.46 (0.70)	$p=0.297$
Postmortem serum CRP	2–89	5 [2;20]	16 (27)	2–117	3 [2;24]	22 (33)	$p=0.867$
Postmortem serum FFA	1.6–1.9	1.8 [1.7–1.9]	1.8 (1.1)	1.1–1.7	1.4 [1.2;1.6]	1.4 (0.2)	$p<0.001$
Postmortem serum urea nitrogen	5.6–44	7.4 [6.4;11.1]	11.5 (11.6)	3–46	8.1 [6.4;12.3]	11 (8.5)	$p=0.719$
Vitreous urea nitrogen	3.1–20	5.9 [5.2;6.6]	7.2 (4.7)	3–20	6.3 [5.0;9.3]	8.4 (5.1)	$p=0.482$
Pericardial fluid urea nitrogen	5.4–45	7.5 [6.6;10.5]	11.7 (11.9)	2.9–40	8.9 [6.9;12.9]	11.2 (7.7)	$p=0.522$
Postmortem serum creatinine	56–360	260 [160;360]	246 (122)	60–350	155 [110;190]	154 (60)	$p=0.054$
Vitreous creatinine	40–210	55 [47;120]	85 (56)	58–180	130 [100;150]	121 (36)	$p=0.014$ (1)
Pericardial fluid creatinine	58–290	185 [100;280]	188 (93)	60–300	180 [120;220]	174 (63)	$p=0.661$
Postmortem serum uric acid	160–550	210 [180;310]	264 (123)	140–380	235 [190;290]	244 (64)	$p=0.851$
Vitreous uric acid	110–200	125 [120;150]	136 (28)	90–180	135 [110;160]	134 (26)	$p=0.950$
Pericardial fluid uric acid	170–540	290 [200;340]	303 (121)	150–480	225 [170;300]	248 (90)	$p=0.159$
Urinary adrenaline	160–175	167 [161;175]	167 (6)	5–70	29 [18;41]	31(16)	$p<0.001$
Pericardial fluid calcium	1.10–1.90	1.55 [1.30;1.80]	1.53 (0.26)	1.80–2.30	2.00 [1.90;2.20]	2.0 (0.17)	$p<0.001$
Pericardial fluid magnesium	0.68–0.85	0.77 [0.72;0.80]	0.76 (0.56)	0.81–1.30	1.00 [0.85;1.21]	1.03 (0.18)	$p<0.001$

markers cannot be considered as diagnostic for death by hypothermia.

Our findings concur with those of a former study conducted by Coe [3], who described a significant mean elevation of vitreous glucose levels in a series of hypothermia fatalities and postulated that increased vitreous glucose concentrations could be the result of the enhanced catecholamine secretion in response to stress. However, Coe observed that not all cases of hypothermia showed increased vitreous glucose values, suggesting that exposure to cold did not invariably lead to hyperglycemia and high vitreous glucose levels. Lastly, Coe theorized that a substantial period of time was necessary between cold exposure and death, so that hyperglycemia could be reflected in elevated vitreous glucose concentrations.

Such conclusions are suitable in non-diabetic individuals and concern increased glucose levels in both vitreous and urine.

Indeed, high concentrations of glucose in vitreous and urine may also reflect the presence of preexisting diabetes mellitus and have to be interpreted in the context of a more general metabolic dysfunction, along with glycated hemoglobin and ketone values.

Ishikawa et al. [22–24,55] postulated that the secretion of some pituitary hormones was enhanced in the first phases of hypothermia to generate heat (ACTH and TSH) or in relation to the stress reaction (GH). The conclusions of Ishikawa et al. were partially in contrast with those formerly obtained from animal studies, which had shown increased ACTH secretion in conscious animals exposed to cold in opposition to inhibited adrenal steroid secretion in anesthetized animals and during hibernation hypothermia. These

Table 2
Hypothermia cases.

	Frost erythema	Wischnewsky spots	Blood ethanol	Blood 3HB	Urine 3HB	Postmortem serum cortisol	UFC
Case 1	n.d.	d	n.d.	2200	3800	2350	2050
Case 2	d	d	1.02	300	400	980	880
Case 3	d	Slightly represented	2.08	400	600	760	250
Case 4	d	d	n.d.	6700	25,000	1350	1200
Case 5	d	d	n.d.	4200	3300	3600	3530
Case 6	d	d	1.68	400	500	690	650
Case 7	d	d	n.d.	2400	2600	770	590
Case 8	d	d	1.08	1000	3200	870	680
Case 9	n.d.	d	n.d.	2900	2600	1270	1100
Case 10	d	Slightly represented	n.d.	1500	1400	670	470

d: detected; n.d.: not detected; results for ethanol are expressed in g/kg (‰); 3HB: 3-beta-hydroxybutyrate. Results are expressed in μmol/l; clinical reference interval for 3HB blood: 50–170 μmol/l; Clinical reference interval for blood cortisol; 08:00 170–630 nmol/l; 17:00 40–260 nmol/l; UFC: urinary free cortisol. Results are expressed in nmol/l; clinical reference interval for urinary free cortisol: 100–400 nmol/l.

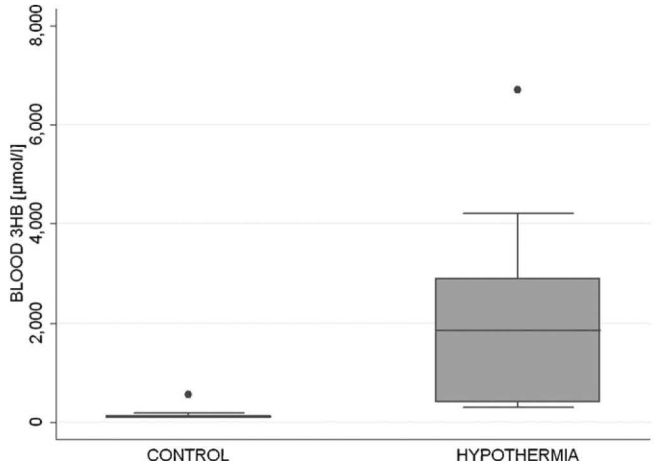

Fig. 1. Shows differences between hypothermia cases and control individuals concerning blood 3HB.

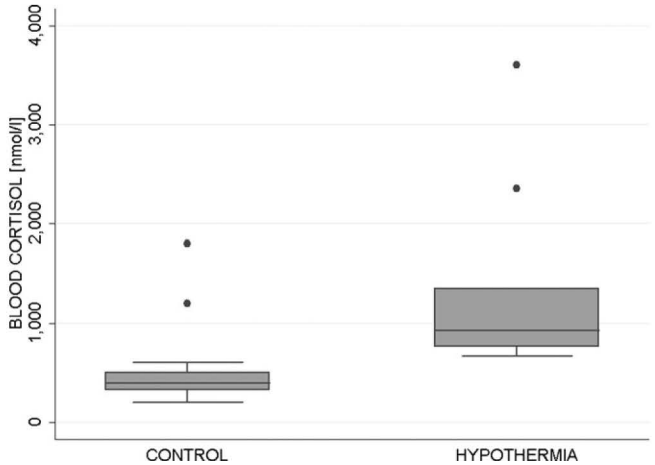

Fig. 3. Shows differences between hypothermia cases and control individuals concerning postmortem serum cortisol.

observations suggested that the increased ACTH and adrenal steroid levels seen after having exposed conscious animals to cold could have been an emotional response to a new, unpleasant environment rather than a response to the cold itself [60].

Pituitary, adrenal and thyroid functions were described as normal in hypothermic patients with physiologic concentrations of TSH and thyroxine, whereas increased cortisol levels were related to reduced hepatic clearance rather than ACTH secretion [59–62].

In our study, ACTH and TSH levels were not univocally increased or decreased in hypothermia fatalities in comparison to the control subjects. Normal ACTH and TSH concentrations may be explained either assuming that the secretion of these hormones is normal in hypothermia, as suggested by the mentioned animal studies, or assuming that their production increases in the early phases of hypothermia to generate heat and subsequently decreases in the advanced phases of hypothermia, as postulated by Ishikawa et al.

In the cases herein selected, GH levels were within clinical reference values in hypothermia fatalities and slightly higher than reference values in control individuals, most likely in relation to stress reactions. These findings suggest that this marker cannot be considered as specific for fatal hypothermia. Furthermore, it should also be noted that increased or decreased pituitary hormone levels can be the expression of preexisting endocrinological disorders.

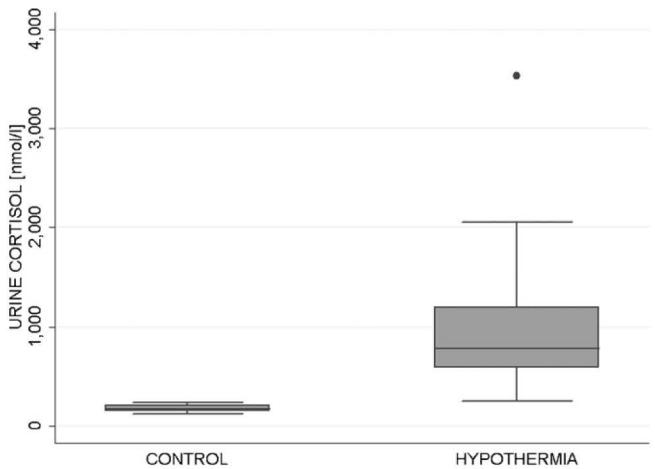

Fig. 4. Shows differences between hypothermia cases and control individuals concerning urine free cortisol.

No extensive studies have been performed on adrenocortical activity and cortisol levels in blood and urine collected upon autopsy, with the exception of the study carried out by Finlayson [63], who observed cortisol levels in postmortem serum from femoral and right atrial blood similar to those obtained in living

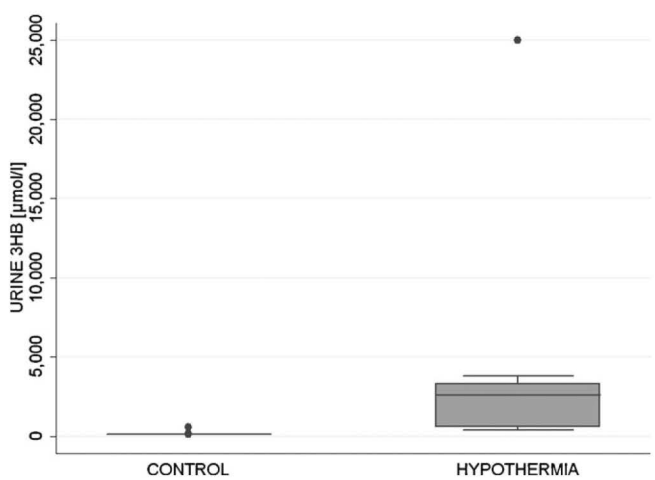

Fig. 2. Shows differences between hypothermia cases and control individuals concerning urine 3HB.

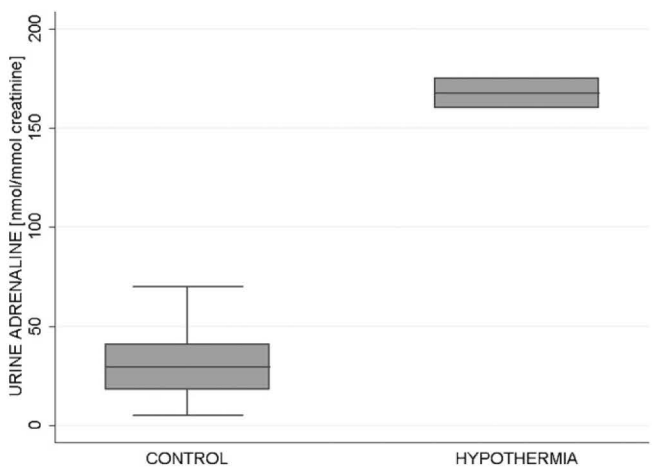

Fig. 5. Shows differences between hypothermia cases and control individuals concerning urine adrenaline.

people. Slight decreases in cortisol blood levels during the first 18 h after death were also noted in this study. On the other hand, clinical studies provided contradictory results concerning the relationship between cortisol levels and cold exposure [64–66].

In our study, the determination of cortisol levels in postmortem serum from femoral blood and free cortisol in urine revealed increased values of both markers in hypothermia fatalities compared to those in control cases. Based on the results of the mentioned animal studies and assuming that ACTH production and adrenal function are depressed or normal during hypothermia [59–62], increases in both serum and urine cortisol may be explained by physiological cortisol secretion, hepatic metabolism inhibition and reduced hepatic clearance. Furthermore, stress-induced ACTH and cortisol productions, as postulated by some clinical investigations pertaining to cold exposure, may also explain such results.

All hypothermia fatalities observed in our study presented increased postmortem serum cortisol levels compared to clinical reference intervals, and 9 cases out of 10 also showed increased urinary free cortisol values.

However, among blunt traumas, we observed one case presenting markedly increased serum cortisol and GH concentrations, suggesting that such findings may also reflect the body's reaction to the stress in general.

Lastly, no relationship was observed between ethanolemia and postmortem serum cortisol or urinary free cortisol levels.

Postmortem serum free fatty acid levels, though statistically different in the studied groups, were higher than the reference values in both hypothermia and control cases, which could be due to postmortem cellular autolysis and the release of these molecules in the bloodstream.

Blood acetone, 3HB and IPA levels were significantly increased in hypothermia fatalities, with the highest levels found in free-ethanol cases. Increased levels of acetone, 3HB and IPA were also observed in vitreous and urine, as well as higher pericardial fluid 3HB levels, in comparison to the control subjects. Urine, pericardial fluid and vitreous levels of these markers displayed the same metabolic relationship with ethanolemia as they did in blood, with the highest increases observed in cases with no ethanol in blood at whatsoever.

Our findings concur with those of the studies performed by Teresiński et al. [1,37,50], who investigated ketones as biochemical markers of hypothermia and the usefulness of determining ketone levels in blood and other biological fluids to support the diagnosis of death by hypothermia. Based on the results of their investigations, Teresiński et al. concluded that increased blood ketone levels could be considered laboratory hallmarks of fatal hypothermia. Nevertheless, normal ketone levels in suspected hypothermia fatalities did not allow this diagnosis to be excluded, particularly in cases with increased ethanol blood levels.

Furthermore, the same authors also highlighted that the severity of ketosis could not be evaluated based on vitreous ketone levels alone. Indeed, the equilibrium between blood and vitreous requires some time to be established and rapid increases of blood ketone levels could not be reflected in simultaneous increases in vitreous ketone concentrations.

3HB concentrations have been shown not to increase with postmortem changes, hence postmortem concentrations in blood are considered reliable markers of 3HB concentrations at the time of death, even in decomposed bodies [67]. Kadiš et al. [68] also observed that 3HB does not increase after death but, at most, may decrease due to the spontaneous degradation of the molecule.

The results of our investigations also indicate that IPA blood levels may be increased in hypothermia fatalities in comparison to the control subjects. From the metabolic point of view, it is known that IPA can be observed in blood and other biological fluids in conditions, other than IPA exposure, that are characterized by ketosis and an elevated NADH/NAD+ ratio. In these situations, the presence of IPA is related to the metabolism of acetone and its conversion by alcohol dehydrogenase [58].

Thus, the presence of increased blood IPA levels in hypothermia cases, in comparison to the control individuals, is not surprising and confirms the usefulness of IPA determination to better characterize the metabolic profile of hypothermia fatalities.

As with ketone levels, the interpretation of IPA levels in other biological fluids requires cautiousness. However, in the cases selected for our study, IPA levels were concordantly increased in all analyzed biological fluids, with modest differences among the tested fluids. This may suggest a substantial period of time that elapsed between the exposure to cold and death.

Urine adrenaline concentrations were higher than clinical reference intervals in both studied groups. However, as already observed by several authors [28–33,44], urine adrenaline levels were proportionally higher in hypothermia fatalities than in control individuals and proportionally increased more than noradrenaline levels.

As proposed by Sadler and Pounder [33], urine adrenaline levels were interpreted after having measured creatininuria.

It should be highlighted that preservation measures during sample collection and time after death are factors significantly influencing catecholamine stability in urine [69]. Thus, normal urine adrenaline levels in suspected hypothermia fatalities did not allow this diagnosis to be excluded, particularly in cases with prolonged postmortem intervals.

In mild hypothermia, there is cold-induced diuresis, which occurs before any fall in body temperature. This is initially due to an increase in renal blood flow following vasoconstriction then, with a decrease in temperature, a loss of distal tubular ability to reabsorb water and resistance to the action of vasopressin. This cold-induced diuresis is accompanied by an increase in urinary electrolyte excretion, which is likely the result of reduced tubular sodium reabsorption. In moderate hypothermia, the glomerular filtration rate falls as cardiac output and hence renal blood fall, the last of these being reduced by half at 27–30 °C. There is also a further reduction in tubular function as well as renal clearance of glucose. At lower temperatures still, tubular capacity for H^+ ion secretion is reduced with the consequential renal contribution to acidosis. Clinically, acute renal failure is seen in over 40% of patients with accidental hypothermia who require admission to an intensive care unit [59].

Unlike Zhu et al. [34,36,41], who observed higher urea nitrogen and uric acid levels in pericardial fluid and postmortem serum from cardiac blood in hypothermia fatalities, we did not observe increased urea nitrogen, creatinine or uric acid levels in these deaths compared to the control cases in any of the tested fluids. Postmortem serum from femoral blood and pericardial fluid presented more similar and comparable values for these parameters than postmortem serum/vitreous or pericardial fluid/vitreous.

Urea nitrogen and creatinine levels in postmortem serum have been shown to be close to those of antemortem serum levels, even after moderate decomposition [57,70].

Increased serum creatinine concentrations or plasma urea concentrations, or both, are considered laboratory features of acute kidney injury. Unfortunately, these analytes are insensitive markers of the glomerular filtration rate and are modified by nutrition, presence of gastrointestinal blood, muscle mass, age, sex and muscle injury. Furthermore, they become abnormal only when the glomerular filtration rate decreases by more than 50% and do not show dynamic changes in filtration rates [71].

In rapidly developed hypothermia fatalities, increased urea nitrogen and creatinine levels may reflect the existence of

preexisting renal dysfunctions rather than changes in glomerular filtration rate and tubular function, which generally occur in advanced phases of hypothermia. However, since other situations, including nutrition, dehydration and muscle injury, may contribute to the increase of both markers in advanced hypothermia, they cannot be considered as specific of death by hypothermia.

Similar to renal function markers, increased CRP or procalcitonin concentrations may indicate the presence of preexisting diseases of inflammatory or infectious origin (pneumonia) rather than the onset of the inflammation processes due to cold exposure. In our study, CRP and PCT values presented similar distributions in both groups.

cTnI levels in postmortem serum from femoral blood were significantly increased in the control subjects with coronary thrombosis accompanied by histologically documented myocardial necrosis, whereas hypothermia cases and control individuals presenting blunt, sharp or gunshot injuries revealed normal or mildly increased postmortem serum cTnI levels, suggesting the existence of terminal myocardial hypoxia in the absence of extensive myocardial damage. Analogously, increased NT-proBNP levels in postmortem serum from femoral blood were more prominent in control individuals than in hypothermia fatalities, where increased values may indicate the severity and duration of heart failure before death, even without substantial myocardial damage [72].

Lastly, calcium and magnesium levels in pericardial fluid were higher in control cases, in contrast with the findings of Li et al. [47] who described higher calcium concentrations and lower magnesium levels in hypothermia fatalities compared to other causes of death. Since the pathophysiological mechanisms responsible for these changes are not known at present, it is still arduous to consider increased or decreased calcium or magnesium levels in pericardial fluid as pathognomonic of fatal hypothermia. The usefulness of such measurements in confirming the diagnosis of fatal hypothermia therefore remains indeterminate.

5. Conclusions

Based on the results of our investigations, we can formulate the following conclusions:

- blood ketones and urine adrenaline should systematically be determined in all suspected hypothermia cases,
- ketone levels in blood and other biological fluids, such as vitreous, pericardial fluid and urine, show an inverse relationship with the concentration of ethanol in blood,
- since the equilibrium between blood, vitreous and pericardial fluid requires some time to be established, vitreous and pericardial ketone concentrations may provide additional information concerning the duration of the hypothermia process,
- IPA levels may also be increased in blood in hypothermia fatalities, as a consequence of acetone metabolism. IPA levels show the same relationship with ethanolemia as acetone;
- cortisol levels in postmortem serum and urine are increased in hypothermia fatalities. Their determinations may provide further information in order to confirm the diagnosis of death by hypothermia,
- increased concentrations of certain molecules (such as cTnI and NT-proBNP) are the consequence of terminal heart failure and cannot be related to the hypothermia process itself,
- increased levels of other parameters (e.g., urea nitrogen, creatinine, CRP and procalcitonin) can be the expression of preexisting diseases or complications (e.g., pneumonia) and their changes may be useful to elucidate the death process on a case-by-case basis. Furthermore, malnutrition, dehydration, presence of gastrointestinal blood, muscle mass, age, sex and muscle injury may contribute to increasing the concentrations of some of these markers,
- urine and vitreous glucose do not appear to be of any particular usefulness in the postmortem diagnosis of death by hypothermia. Increased levels of glucose in vitreous and urine may reflect the existence of diabetes mellitus and must be interpreted in the context of a more general metabolic dysfunction, along with glycated hemoglobin and ketone concentrations,
- increased or decreased pituitary hormone levels are not systematic and can also be the consequence of stress reactions or preexisting endocrinological disorders,
- antemortem organ damage as well as postmortem changes related either to the environment or to the interval after death must be considered as potentially contributing factors in increasing or decreasing the concentrations of certain molecules,
- some biochemical findings can be the result of metabolic changes involved in the death process. However, these findings must be discriminated from similar results that can characterize other causes of death,
- postmortem changes may influence the concentration of specific molecules. Hence, the combined use of biochemical markers should be recommended in order to formulate appropriate conclusions of death by hypothermia.

Conflict of interest

The authors have no conflict of interest to declare.

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