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Review

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Updated review of *postmortem* biochemical exploration of hypothermia with a presentation of standard strategy of sampling and analyses

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Abstract: Hypothermia is defined as a core body temperature below 35 °C and can be caused by environmental exposure, drug intoxication, metabolic or nervous system dysfunction. This lethal pathology with medico-legal implications is complex to diagnose because macroscopic and microscopic lesions observed at the autopsy and the histological analysis are suggestive but not pathognomonic. *Postmortem* biochemical explorations have been progressively developed through the study of several biomarkers to improve the diagnosis decision cluster. Here, we present an updated review with novel biomarkers (such as catecholamines *O*-methylated metabolites, thrombomodulin and the cardiac oxyhemoglobin ratio) as well as some propositional interpretative *postmortem* thresholds and, to the best of our knowledge, for the first time, we present the most adapted strategy of sampling and analyses to identify biomarkers of hypothermia. For our consideration, the most relevant identified biomarkers are urinary catecholamines and their *O*-methylated metabolites, urinary free cortisol, blood cortisol, as well as blood, vitreous humor and pericardial fluid for ketone bodies and blood free fatty acids. These biomarkers are increased in response either to cold-mediated stress or to bioenergetics ketogenesis crisis and significantly contribute to the diagnosis by exclusion of death by hypothermia.

Keywords: biochemistry; biomarkers; forensic; hypothermia; *postmortem*.

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Introduction

Human beings are homeothermic organisms with a regulating body temperature (T_{body}). Decrease in T_{body} can be poorly tolerated and ultimately lead to death [1]. Mortality studies show that death rate ranges from 38% to 85% from the moderate stage (<32 °C) and up to 100% with associated polytrauma [2–5]. This variability could be related to the studies themselves (from 1987) and the therapeutic approaches of that time. More recently, (between 2003 and 2013), 13,419 deaths were declared in the United-States as being linked to excessive natural cold exposure, with 10% of alcohol or drug poisoning as a contributing cause of death. The annual rates range from 0.3 to 0.5/100,000 persons in the United-States, up to 3.9/100,000 in South Australia and 3.3/100,000 in Sweden [6, 7].

Generalized hypothermia occurs when the T_{body} drops below 35 °C, in relation to exhausting heat production and failure of heat loss prevention [8]. Five degrees of severity are described: mild (32–35 °C), moderate (28–32 °C), severe (24–28 °C), deep (13–24 °C) and irreversible fatal outcome (<13 °C) [1]. As T_{body} drops, the hypothalamus (thermoregulation center) generates a series of reactions aiming to produce heat and prevent its loss. Heat production involves the secretion of stress response hormones (adrenaline [Ad], noradrenaline [Nad] and cortisol), triggering an overall increase in lipidic metabolism, specifically ketogenesis synthesis [8]. The large inter-individual variability in the establishment of these regulatory mechanisms impacts the diversity of hypothermic presentation.

There are two categories of hypothermia, depending on the quality of thermoregulation mechanisms: primary, when thermoregulatory mechanisms are intact but exceeded by the intensity or duration of cold exposure (mountaineers, those shipwrecked, drowned, etc.); secondary, when these mechanisms are altered, and hypothermia occurs despite temperate environmental temperatures (22–24 °C). This may be the case when drug consumption/poisoning (acetaminophen, barbiturate, opioid, tricyclic antidepressant, benzodiazepine, phenothiazine, prazosin, valproic acid,…), ethanol consumption, or in some pathological conditions (thyroid or pituitary insufficiency, neurological disorders, sepsis and extensive burns) [2, 9–11].

The non-specific macroscopic and microscopic findings described in the clinical literature (Table 1) emphasize the major role that *postmortem* biochemical investigations might have in identifying hypothermia-related fatalities and helping pathologists to produce more accurate diagnoses [8, 9, 12–14].

On the other hand, findings obtained from *postmortem* biochemical analyses cannot be considered in isolation, as they may depend on preexisting diseases (which may be responsible for increased or decreased concentrations of specific biochemical markers), and therefore necessitate accurate evaluation of patient conditions, in order to avoid misleading interpretations and mistaking preexisting pathological findings for hypothermia-related results.

The study herein aims to present the different biomarkers studied over the years and discuss their significance. Location and sampling methods are also discussed in some cases.

Table 1: List of main typical autopsy elements of hypothermia.

	Autopsy findings
Macroscopic elements	Body and external temperature
	Humidity, wind speed
	± thermal insulation of housing
	Hide-and-die syndrome
	Paradoxical undressing
	Wischnewski lesions
	Frost erythema
	Inner knee sign
	Hemorrhage infiltrate in core muscle
Microscopic findings	Lipid vacuolization of renal tubule epithelial cells, pancreatic cells, hepatocytes, cardiac myocytes, adrenal cells and anterior pituitary gland cells
	Expression of adipose differentiation-regulated protein in renal tubule epithelial cells
	Degenerative foci in myocardium

Forensic biomarkers of hypothermia

Generally speaking, *postmortem* biochemical investigation results may be different in different studies based on the clinical standard used. This principle applies also to *postmortem* biochemical investigations pertaining to hypothermia. Indeed, results obtained from biochemical investigations pertaining to catecholamines (and their *O*-methylated metabolites), cortisol and ketone bodies in suspected hypothermia-related fatalities should always consider and evaluate preexisting conditions (such as catecholamine-secreting tumors, diabetes mellitus, chronic alcohol abuse and stress conditions), in order to avoid erroneous interpretations of the measured valued.

Catecholamines and their *O*-methylated metabolites

The three main catecholamines are Ad, Nad and dopamine (DA), synthesized by chromaffin cells of the adrenal medulla and postganglionic neurons from the sympathetic nervous system. These act as neurotransmitters and regulating hormones. In the context of cold mediated stress, their role is to increase heat production and prevent its loss [15–20].

Currently, it is known that cold mediated stress is responsible for increasing urinary catecholamines [9, 12, 14, 17, 21–25]. A sum increase of Ad + Nad in urine higher than 0.1 µg/mL was first described by Hirvonen in 1976, though the difficulty of interpreting urinary catecholamines in relation to cold induced diuresis had already been previously discussed [12]. This latter is thought to be related to an initial increase in renal blood flow resulting from vasoconstriction followed by decreased T_{body}, loss of fluid reabsorption capacity by distal tubules and vasopressin action resistance [14]. It is recommended that urinary catecholamine concentrations be adjusted to creatininuria in order to avoid possible misinterpretations and prevent potential underestimation of the result. This should be disclosed in nmol of catecholamines per mmol of creatinine, and is obtained by adapting the concentration of catecholamines to an estimated renal filtration rate using creatininuria [24].

Recent studies have been conducted on the action of catechol-*O*-methyl transferase on catecholamines, resulting in *O*-methylated metabolites synthesis of which three exist: metanephrine from Ad, normetanephrine from Nad and methoxytyramine from DA [19, 26]. The study of these markers provides a benefit in terms of *in vitro* stability [27, 28].

Table 2: The sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) of urinary catecholamines and their *O*-methylated metabolites determined according to Palmiere et al. [19].

Parameter	Cut-off value	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Adrenaline (Ad)	40	72	87	88	69
Noradrenaline (Nad)	493	48	100	100	58
Dopamine	251	65	90	90	65
Metanephrine	95	87	72	81	80
Normetanephrine	280	81	72	80	73
Methoxytyramine	126	78	75	81	71
Ratio Ad/Nad	0.192	53	73	73	53

Cut-off values are expressed in nmol/mmol creatinine, except for the Ad/Nad ratio (no units).

Urine samples must be taken with a sterile needle. Urine acidification is recommended to prevent *in vitro* degradation, but is not compulsory in the case of immediate freezing. Catecholamines *in vitro* stability is estimated at 3 weeks and up to several months for their *O*-methylated metabolites [19, 27–31]. *Postmortem* stability, is estimated at 2 to "*virtually*" 10 days for catecholamines [19, 32]. Thus, the requirement imposed by the modalities of catecholamines urinary sampling, at the risk of an *in vitro* decay, does not allow a diagnosis of hypothermia to be excluded when normal or slightly increased results are observed.

Historically, urinary catecholamines were interpreted through Ad/Nad ratio analysis [15, 16, 33]. However, the interest in this ratio is put into question as it seems less sensitive and specific than an isolated study of urinary Ad concentrations, for example (Table 1) [18, 19].

According to some authors, Ad injection related to resuscitation does not modify concentrations of urinary catecholamines and their *O*-methylated metabolites, or at least does not increase their concentrations beyond the thresholds usually described for hypothermia. Age and sex would similarly have no influence on the concentrations of these markers [14, 19, 21].

In vivo, urinary catecholamines are explored in 24 h diuresis because of fluctuation in the excretions during a day, i.e. all activation of the sympaticoadrenal system will stimulate the secretion of catecholamines and thus the excretion. This is impossible in *postmortem* where only a sample of urine can be taken. Thus, clinical reference intervals are not applicable and new thresholds have been described adapting the conditions of this *postmortem* sampling and putrefactive phenomena. Palmiere et al. published a multicenter systematic study of urinary catecholamines and their *O*-methylated metabolites in 2014. Eighty-three fatal hypothermia and 144 control deaths were investigated and ROC curves analyzed, putting forward relevant interpretive thresholds for each parameter (Table 2) [19].

Exploring catecholamines and their *O*-methylated metabolites in serum or vitreous humor are not of interest, their *postmortem* concentrations are, respectively, discordant (increased or decreased) from blood, or related to cell autolysis [26].

Two urinary samples must be taken, one in a preservative free tube immediately frozen and/or with acidification and another one in a preservative free tube. The first will allow the study of catecholamines and/or their *O*-methylated metabolites to be carried out and the second creatinine.

Exploring urinary catecholamines and their *O*-methylated metabolites will allow the diagnosis of a perimortem generalized stress, in this situation a cold mediated stress.

Cortisol

The increase of counter-regulating hormones, such as cortisol, stimulates thermal and energy production. Besides contrary results on adrenal cortex hormone evolution in hypothermia, its perimortem elevation might reflect less of an increase in adrenocorticotrophic hormone (ACTH) than a decrease in metabolism and hepatic clearance [14, 34–41]. A study concerning the agonal process and blood cortisol concentrations showed that there are no significant differences between instant death and death with prolonged agony, e.g. subdural hematoma [41]. Some teams recommend the exploration of free urinary cortisol and blood cortisol in suspected hypothermia [14, 21, 35, 38, 42].

In vivo, blood cortisol is explored at a precise time related to cyclic secretion following a nychthemeral rhythm, e.g. the upper secretion is at around 8 a.m. causing awakeness and the lower secretion is at around 12 a.m. to maintain sleep statement. Blood clinical reference intervals are not applicable in *postmortem* because generally the time of death is only suspected or simply

unknown and *postmortem* fluctuation [14]. Bańka et al. [41] proposed a cut-off value of 300 ng/mL for blood cortisol using ultra high performance liquid chromatography. As catecholamines, free urinary cortisol is explored on 24 h diuresis because of the cyclic nychthemeral secretion. Again, in *postmortem* only a sample of urine can be taken. So, forensic pathologists and biochemists must consider a single samples, unknown *postmortem* interval and again inapplicable clinical reference intervals. One study observed a free urinary concentration above 250 nmol/L in all hypothermia fatalities explored and below 240 nmol/L for the control group which included cases with and without injuries as well as sudden and protracted death (analytically performed by radioimmunoassay) [14].

A high urine or blood cortisol is not a typical sign of fatal hypothermia and may reflect the body's reaction to the stress in general [14]. Interpreting cortisol's concentration in *postmortem* can be very difficult and must take into account the circumstances of death, and macroscopic and microscopic findings during and after autopsy exploration.

One peripheral blood sample with one urine sample must be put in preservative free tubes to allow the study of cortisol and free urinary cortisol.

Exploring blood and urine cortisol, as catecholamines and their *O*-methylated metabolites, will allow the diagnosis of a perimortem generalized stress, in this situation a cold mediated stress.

Ketones bodies

Exposure to cold is characterized by significant stress reactions that enhance catecholamine and counter-regulatory hormone release. Enhanced fat catabolism and increased ketone body production are the metabolic consequences of hypothermia-induced secretion of insulin antagonist hormones. Acetoacetate and β-hydroxybutyrate (HB) are energy-rich compounds that transport energy from the liver to other tissues. They can be interconverted by the enzyme β-HB dehydrogenase. Acetone is generated through the decarboxylation of acetoacetate (either spontaneously or through the enzyme acetoacetate decarboxylase) and is generally considered of little metabolic significance [9, 43–47].

Acetone production increases due to *postmortem* decompositional changes. On the other hand, these are not associated with HB production, whose concentration does not increase after death but may at most decrease as a result of spontaneous molecule degradation [48–50].

Increased HB can be explored in serum, vitreous humor and pericardial fluid (not recommended in urine) [14, 44, 45]. To these different matrices, Palmiere and Werner [51] have recently added the synovial fluid contained in the knee. This is useful when no other matrix is available due to sampling absence or insufficient volume.

Ketones should be explored in blood because the concentration balance with some other matrix (e.g. vitreous humor or pericardial fluid) takes several hours [14, 44–46]. However, there may be an interest in analyzing all these matrices in order to estimate agonal period duration (e.g. a patient with a long period of agony may have similar HB values in each matrix).

To the different matrices that can be explored in *postmortem* for their HB concentration, "liver homogenates" can be added. Within a long *postmortem* delay and thus taphonomic processes, there is a disappearance of blood, vitreous humor and pericardial fluid though the liver persists. In this context, Palmiere et al. [52] published preliminary but promising results in 2013 regarding the exploration of HB in "liver homogenates". In the future, pathologies with hyperketonemia, in our case hypothermia, could be explored despite long *postmortem* intervals.

Ketones must always be explored in conjunction with ethanol, as the latter is able to inhibit ketogenesis. Hence, a diagnosis of hypothermia cannot be reversed on a lack of increase of the ketone bodies, especially in the presence of ethyl intoxication [44].

Peripheral blood sampling should therefore be carried out in sodium fluoride tubes whereas vitreous humor and/or pericardial fluid should be sampled in preservative free tube as these will allow ketone bodies to be best explored, especially HB and ethanol.

Exploring ketone bodies will allow the diagnosis of a stimulating ketogenesis which occurs during hypothermia.

Free fatty acids (FFAs)

As for Bańka et al. "Elevated levels of FFAs in the blood result from activation of the sympathetic-adrenal system and enhancement of lipolysis as one of the mechanism maintaining heat homeostasis. FFAs play an essential role in counteracting the effects of body cooling as, together with ketone bodies, they are main energy substrate metabolized by the nervous tissue, even before glucose" [20].

In cases of death from hypothermia, Bańka et al. observed an increase in FFA levels in femoral vein blood by 425%. They also pointed out that the most relevant FFAs are: palmitic, stearic and oleic acid. Using a one-step

analytical procedure, gas chromatography coupled to mass spectrometry with a negative chemical ionization, they postulated a cut-off threshold at >0.2 mmol/L for each of these three compounds [20].

Palmiere et al. also observed a significant elevation of FFAs in blood for deaths by hypothermia. For their part, using an enzymatic colorimetric method, they observed concentrations ranging from 1.6 to 1.9 mmol/L (mean 1.8 mmol/L), compared to 1.1–1.7 mmol/L (mean 1.4 mmol/L, control group) which differ largely from clinical reference intervals (0.1–0.6 mmol/L) [14].

Both groups of researchers concluded that elevated blood FFAs cannot be considered a pathognomonic symptom of hypothermia due to a lack of specificity (they may also be increased in starvation, uncontrolled diabetes mellitus, hyperthyroidism and adrenal pheochromocytoma cases) [14, 20].

Peripheral blood sampling should therefore be carried out in preservative free tubes as these will allow FFAs to be best explored, especially palmitic, stearic and oleic acid.

Other biomarkers

Thrombomodulin (TM) is a transmembrane protein expressed in the endothelial cells of most blood vessels. TM functions essentially as anticoagulant and anti-inflammatory regulator by acting as a cofactor for thrombin. Severe cold stress has been shown to cause changes in the expression and secretion of TM [53]. Pakanen et al. have described that lethal hypothermia is associated with low myocardial TM transcript and TM protein levels as well as low urinary TM level compared with other causes of death. They also demonstrate a *postmortem* stability of TM during at least 24 h. Using urinary TM as a biomarker, with a cut-off at 15.5 ng/mL, they found sensitivity and specificity at 70.8% and 70.3%, respectively [54].

In fatal hypothermia, right cardiac blood is darker than left cardiac blood. This color difference could result from an increased oxyhemoglobin ratio (%O_2-Hb) in left cardiac blood. %O_2-Hb can be measured using CO-oximetry, spectrophotometry or gas chromatography. Kanto-Nishimaki et al., using co-oximetry, identified that %O_2-Hb in left cardiac blood is significantly higher than in right cardiac blood (63.39% average for left cardiac blood versus 18.97% for right). Their study concluded that the difference between left and right cardiac blood could be a significant biological finding in death due to hypothermia [55].

Thyrotropin (TSH) and ACTH increase to produce heat should there be a loss. After this initial increase, there is a secondary decrease due to lipid disorders and/or depletion of reserves and central production capacities. Though concentrations vary according to the *postmortem* period, they are often lower [56, 57].

Although vitreous magnesium was widely used in the early 1960s during biochemical investigations for hypothermia, neither it nor vitreous calcium are recommended today due to inconsistent result reliability [14, 21, 58, 59].

Chromogranin A is a glycoprotein present in the secretory granules of endocrine and neuroendocrine cells. Immunohistochemical staining of the hypothalamus shows a decrease of its staining in the case of hypothermia while its concentration rises in cerebrospinal fluid. These findings would indicate a terminal stage of dysfunction involving the secretion of chromogranin A for cases of death by hypothermia with prolonged agony [60].

Information regarding biochemical markers that have already been studied but have not demonstrated their significance in the exploration of hypothermic deaths include: urinary histamine, urinary serotonin, blood, vitreous and urinary glucose and blood, vitreous and endocan [12, 14, 61].

Sampling and analyses strategy

This updated review of the literature allows proposing that the most adapted strategy to identify biomarkers of hypothermia would currently retain the following sampling and analyses (Table 3).

Discussion

Postmortem exploration of biochemical markers, also called thanatobiochemistry refer to the study of biomarkers that contribute to the understanding of the origin of a death. This discipline, which appeared in the middle of the 20th century, has been gaining interest over the last several years due to the diversifications of its implications [62–65]. The *postmortem* biochemical exploration of hypothermia, presented here, is an example of these implications.

The increase of catecholamines and their *O*-methylated metabolites is related to cold mediated stress but can also be identified in pheochromocytoma. Catecholamines and their *O*-methylated metabolites must be explored in the urine because of discordant conclusions in blood and insufficient exchange in vitreous humor. Related to a cold induced diuresis with dilution-effect some authors recommend expressing the results by a ratio Adr/Nad, which is increased, with different thresholds between the studies (1 or 0.19) [22, 33]. As with Sadler and Pounder [24], we

Table 3: Strategy of sampling and analyses to identify biomarkers of hypothermia.

Matrices	Sampling	Analyses	Interfering conditions/disturbing factors
Peripheral blood	Sodium fluoride tube	Hydroxybutyrate and/or acetone	Ketoacidosis (diabetic, alcoholic, starvation and septic)
		Ethanol	Inhibit ketogenesis
	Preservative free tube	Cortisol	All generalized stress reaction
		Free fatty acids	Starvation, uncontrolled diabetes mellitus, hyperthyroidism, adrenal pheochromocytoma
Urines	Preservative free tube immediately frozen and/or with acidification	Catecholamines and/or their *O*-methylated metabolites	Adrenal pheochromocytoma
	Preservative free tube	Free cortisol	All generalized stress reaction
		Creatinine	(for proper interpretation of urinary catecholamines)
Vitreous humor	Sodium fluoride tube	Ethanol	Inhibit ketogenesis
		Hydroxybutyrate and/or acetone	Retinal cells and ketoacidosis (diabetic, alcoholic, starvation and septic)
Pericardial fluid	Preservative free tube	Hydroxybutyrate and/or acetone	Ketoacidosis (diabetic, alcoholic, starvation and septic)

recommend adjusting the catecholamines' results to creatininuria as is done *in vivo*, in order to correlate levels to an estimated renal filtration rate thus, preventing the dilution effect and because of less specificity and sensitivity of this ratio [19].

Cortisol increases during hypothermia related to cold mediated stress. The specificity of this biomarker seems lower than for catecholamines as it increases in all stress situations. Anyway, its exploration is important to obtain an additional argument for a stress-related death, such as hypothermia. Cortisol can be explored in blood or urine with a higher statistical significance in urine [14]. Notably, no study focused on the correlation between urinary free cortisol and creatininuria.

Ketogenesis is stimulated during hypothermia to allow energy and thermal production as ketone bodies are easily metabolized. This finding is not a specificity of hypothermia and is observed in all situations of ketoacidosis and some metabolic dysfunctions [43]. Nevertheless, ketone bodies must be explored to identify stimulating ketogenesis as occurs during hypothermia.

FFA are increased in death from hypothermia because of the activation of the sympathetic-adrenal system and the enhancement of lipolysis as one of the mechanism maintaining heat homeostasis. FFA play an essential role in counteracting the effects of body cooling as, together with ketone bodies, they are the main energy substrate metabolized by the nervous tissue, even before glucose [20].

The parameters described in the section "Other biomarkers" have not been yet confirmed by different research teams or found in consistent result reliability or simply have no statistical significance. These are the reasons why they did not figure in our strategy of sampling and analyses.

Sample collection is crucial in thanatobiochemistry as *postmortem* redistribution can quantitatively affect some parameters, e.g. catecholamines' levels are much higher in cardiac than peripheral blood [66]. That is why we recommend the exploration of peripheral blood as many studies have focused on this matrix instead of cardiac blood. Blood, urine and vitreous humor must be punctured gently with a sterile syringe to avoid contamination and the dilution effect.

Exploring ethanol consumption is also crucial when a diagnosis of lethal hypothermia is suspected. First, chronic or acute alcohol consumption are risk factors of hypothermia for many reasons: acceleration of heat loss, general vasodilatation, sedation stimulation, inhibition of shuddering abilities, denutrition and hypothalamic dysfunction. Secondly, ethanol has a antiketonemic action which mainly influences acetone levels. Thus, some authors postulated that a normal or subnormal result of ketone bodies cannot exclude hypothermia, particularly in cases of alcohol consumption [9]. However, it seems that ethanol intake does not influence the levels of urinary catecholamines, blood FFAs, blood cortisol or urinary free cortisol [14, 20, 41].

The last point is the most important: these different biological markers (just like the macroscopic and microscopic elements) make it possible to carry out a diagnosis of hypothermia but not a specific death by hypothermia.

Only the absence of arguments for another etiology, after a thorough per- and post-autopsy exploration (histological, toxicological and imaging), leads to the conclusion of death by hypothermia. Several diagnostic findings (macroscopic, microscopic and biological) and an absence of arguments for any other lethal origin are required to carry out this diagnosis [67].

Conclusions

A death by hypothermia remains a diagnosis by exclusion according to a cluster of arguments. Biochemical markers take an increasingly important place in such decision clusters. The studies reviewed here have considerably increased the knowledge of such biomarkers, by demonstrating the relevance or not of each of them. For the most pertinent biomarkers, their *postmortem* stability, the better biological fluid in which to dose them, as well as their specificity and limits, have been progressively affirmed by these studies. Despite the complexity to develop clinical biochemistry adapted to forensic medicine, the potential of such approach remains considerable.

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