Lasse Pakanen

THROMBOMODULIN AND CATECHOLAMINES AS POST-MORTEM INDICATORS OF HYPOTHERMIA
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THROMBOMODULIN AND CATECHOLAMINES AS POST-MORTEM INDICATORS OF HYPOTHERMIA

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**Abstract**

Hypothermia deaths pose a difficult challenge from the medico-legal point of view because no specific traces are left on the cadaver to be examined post-mortem. The concentrations of urinary catecholamines, adrenaline and noradrenaline increase in various stressful situations including cold stress, and high levels have been considered to be suggestive of lethal hypothermia. There is, however, a need for a better hypothermia indicator. A potential candidate could be thrombomodulin (TM), an endothelially expressed protein whose plasma concentration has been shown to elevate in response to hypothermia.

TM and catecholamine levels were studied in short-term cold exposure (human subjects, n = 7), in mild and severe hypothermia with or without rewarming (rats, n = 96) and in hypothermia deaths compared with deaths from cardiovascular diseases, traumas and other causes (autopsy cases, total n = 552).

Myocardial thrombomodulin transcript expression was increased in severely hypothermic rats, but was lower in hypothermia deaths than in other causes. The circulating TM level was transiently reduced in severe hypothermia. The myocardial and urinary TM protein levels were reduced in lethal hypothermia compared with other causes of death. TM and catecholamine levels correlated significantly in blood and urine both in living subjects and post-mortem examination. In severely hypothermic rats, there was an inverse relationship between plasma adrenaline concentration and myocardial thrombomodulin transcript level.

The results suggest that TM expression and secretion are altered by hypothermia, possibly linked to the actions of catecholamines. Analysing the post-mortem catecholamine and TM levels provides evidence of ante-mortem cold stress in suspected hypothermia deaths. Further studies should be conducted in order to reveal the exact mechanisms behind the regulation of TM on cell level.

**Keywords:** cardiovascular diseases, catecholamines, forensic medicine, hypothermia, thrombomodulin
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Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä

TM- ja katekoliamiinitasoja tutkittiin lyhyessä kylmälääristuksessa (koehenkilöt, n = 7) sekä lievässä ja vaikeassa alilämpöisystilassa joko lämmityksen jälkeen tai ilman lämmitystä (rotat, n = 96). Lisäksi verrattiin paleltumisen, sydän- ja verisuonitautien, vammojen sekä muiden syiden aiheuttamia kuolemi (ruumiinavausaineisto, n = 552).


Asiasanat: hypotermia, katekoliamiinit, oikeuslääketiede, sydän- ja verisuonisairaudet, trombomoduliini
Frigori laecasin dico
(Petronius Arbiter: Satyricon)
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Oulu, April 2015

Lasse Pakanen
Abbreviations

3HB  3-β-hydroxybutyrate
ACE  angiotensin-converting enzyme
ACTH adrenocorticotropic hormone
ADH  antidiuretic hormone
ANR  adrenaline-to-noradrenaline ratio
APC  activated protein C
BZD  benzodiazepine
cAMP cyclic adenosine monophosphate
CB1  cannabinoid receptor type 1
cDNA complementary deoxyribonucleic acid
CgA  chromogranin A
CHF  congestive heart failure
COD  cause of death
CSF  cerebrospinal fluid
CVD  cardiovascular disease
DIC  disseminated intravascular coagulation
EGF  epidermal growth factor
ELISA  enzyme-linked immunosorbent assay
GABA  γ-aminobutyric acid
GAPDH glyceraldehyde 3-phosphate dehydrogenase
GH  growth hormone
HMG-CoA  3-hydroxy-3-methylglutaryl coenzyme A
HSF1  heat shock factor 1
HSP  heat shock protein
ICH  intracerebral haemorrhage
IPA  isopropyl alcohol
KLF2  Krüppel-like factor 2
LR−  negative likelihood ratio
LR+  positive likelihood ratio
MH1–2 mild hypothermia 1–2
NSAID  non-steroidal anti-inflammatory drug
PAI-1  plasminogen activator inhibitor 1
PC  protein C
PCF  pericardial fluid
PMI  post-mortem interval
<table>
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<th>Abbreviation</th>
<th>Full Name</th>
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<tr>
<td>PS</td>
<td>protein S</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RTI</td>
<td>respiratory tract infection</td>
</tr>
<tr>
<td>S100B</td>
<td>S100 calcium binding protein β</td>
</tr>
<tr>
<td>SAH</td>
<td>subarachnoid haemorrhage</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SH1–2</td>
<td>severe hypothermia 1–2</td>
</tr>
<tr>
<td>SHRW1–2</td>
<td>severe hypothermia and rewarming 1–2</td>
</tr>
<tr>
<td>T&lt;sub&gt;b&lt;/sub&gt;</td>
<td>mean body temperature</td>
</tr>
<tr>
<td>T&lt;sub&gt;rect&lt;/sub&gt;</td>
<td>rectal temperature</td>
</tr>
<tr>
<td>T&lt;sub&gt;sk&lt;/sub&gt;</td>
<td>mean skin temperature</td>
</tr>
<tr>
<td>TAFI</td>
<td>thrombin-activatable fibrinolysis inhibitor</td>
</tr>
<tr>
<td>TAFI&lt;sub&gt;a&lt;/sub&gt;</td>
<td>activated TAFI</td>
</tr>
<tr>
<td>THBD</td>
<td>thrombomodulin (gene/transcript)</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TM</td>
<td>thrombomodulin</td>
</tr>
<tr>
<td>t-PA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid-stimulating hormone</td>
</tr>
<tr>
<td>V–XII</td>
<td>factor V–XII</td>
</tr>
<tr>
<td>Va–XII&lt;sub&gt;a&lt;/sub&gt;</td>
<td>activated factor V–XII</td>
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List of original publications

This thesis is based on the following articles which are referred to in the text by their Roman numerals:


Additionally, some unpublished material is presented.
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1 Introduction

A cold environment poses many challenges, and it is a significant factor causing morbidity and mortality. Cold temperature has caused great casualties during many wars, from the campaigns of Hannibal and Napoleon to the battles of the Second World War (Guly 2011, Rodway 2012, Steinman 1987). Even unethical experiments were conducted under cover of finding ways to protect troops from cold injuries (Berger 1990). Extreme weather conditions have claimed many lives in attempts to conquer every corner of the world (Huntford 2000, Krakauer 1998).

In modern everyday society, the threats are somewhat different. Cold affects health and the ability to work (Sormunen 2009), and international guidelines (Hassi et al. 2002, ISO 15743 2008) have been issued to assess and solve cold-related problems at workplaces. Recently, an effort has been made to raise awareness of cold-related health hazards among Finnish health care personnel and the public in order to reduce excess winter morbidity and mortality (Hassi et al. 2011, 2013). Cold-related problems cause a heavy burden on health care, and the majority of excess deaths are presumably preventable.

It is well known that mortality peaks occur yearly during the cold season (Näyhä 2005). The majority of deaths are accounted for by the exacerbation of chronic diseases, but cold exposure also kills directly by causing hypothermia. Though not very frequent among the overall causes, deaths from hypothermia are challenging from the medico-legal point of view. Hypothermia does not leave any specific traces in the cadaver, and the post-mortem diagnosis is, at present, largely based on the outer circumstances and the exclusion of other causes.

Efforts have been made to find an indicator of ante-mortem hypothermia that could be measured as part of the medico-legal examination. Catecholamines, adrenaline and noradrenaline have been suggested to serve as such an indicator (Hirvonen & Huttunen 1982). These are hormones secreted in stressful situations, cold exposure among them. The problem with this approach is that the catecholamine levels are subject to elevate in many other causes of death as well, especially when a long and painful agonal phase is present. In addition to hypothermia, such a situation may for instance often be observed in deaths from cardiovascular diseases (CVD), traumas and hyperthermia. Thus, the value of catecholamines as a tool for post-mortem indication of hypothermia needs to be reassessed.

Thrombomodulin (TM) is a protein expressed in the endothelial cells of most arteries and veins (Maruyama et al. 1985). TM is also found in the circulation and
urine as soluble, functional fragments (Ishii & Majerus 1985). According to current knowledge, the main physiological functions of TM are related to the regulation of haemostasis (Esmon & Owen 1981) and inflammation (Van de Wouwer & Conway 2004) and to the maintenance of pregnancy (Isermann et al. 2003).

The concentrations of TM in plasma have been shown to increase in response to induced hypothermia (Böhrer et al. 1995, Boldt et al. 1996). Hypothermia is known to affect blood coagulation, either impairing it (Rajagopalan et al. 2008, Rohrer & Natale 1992) or enhancing it (Keatinge et al. 1984, Nagelkirk et al. 2012). Little is known, however, about the influence of cold and hypothermia on the expression and secretion of TM. Changes in the TM levels could provide a link between hypothermia and the alterations in the haemostasis and coagulation processes.

Provided that hypothermia affects TM levels, it could be a promising candidate for a novel indicator of lethal hypothermia. Knowledge of the alterations in TM secretion and concentrations due to hypothermia would also shed light on the regulation of critical physiological functions under cold stress.
2 Review of literature

2.1 Influences of cold temperature on humans

Exposure to cold elicits many responses in the human body. Some changes are physiological which initially aim to enhance survival, but may in some aspects be detrimental to health. Cold temperature also produces several beneficial effects which can be exploited in the development of new therapy forms.

2.1.1 Physiological responses to cold

Thermoregulation

Like all mammals, humans are homoeothermic. Temperature is kept constant in order to maintain the essential functions of the body, though there is minor variation depending on the time of the day and state of sleep, among other things. Deviation in either direction from normothermia, around 37 °C, quickly disrupts the delicate balance of, for instance, enzymatic reactions.

Heat is produced in the body by several overlapping systems. The main source of heat is the contraction of skeletal muscles. Basic metabolism, including various endocrine mechanisms and the ingestion of food, are other major heat producers. Heat is lost from the body through four basic mechanisms when the ambient temperature is below that of body temperature. Radiation of heat is usually the most prominent mechanism of heat transfer in cold air. In windy weather conditions or water immersion (Fig. 1a), convection may become the most important way of losing heat. The amount of heat lost through conduction from skin to air or a cold surface is normally not very significant, but if a person is lying on the ground unconscious, it becomes an important factor (Fig. 1b). In cold water immersion, conduction of heat is also increased significantly compared with cold air exposure. Vaporisation of water is not a significant mechanism of heat loss in cold air because sweating is suppressed to a minimum.

The hypothalamus acts as the main thermostat of the body. It gathers information from all the cold receptors and adjusts the temperature regulating responses according to certain threshold temperatures. There is great individual variation in the thermoregulation responses based on factors such as the amount of body fat (Claessens-van Ooijen et al. 2006) and adaptation (Mäkinen et al. ...
Heat preserving mechanisms aim to increase heat production and reduce heat loss. Heat production can be increased by involuntary shivering of muscles as well as voluntary increase in muscle activity. Non-shivering thermogenesis refers to the use of brown adipose tissue to produce heat – a mechanism available in infants and cold-adapted individuals (Leppäluoto et al. 2005, Nedergaard et al. 2007). There is also extensive ongoing research about its role in adult thermogenesis and controlling body weight (Cereijo et al. 2015). Additionally, the secretion of catecholamines, adrenaline and noradrenaline, is increased (Pääkkönen & Leppäluoto 2002). These hormones cause vasoconstriction in the cutaneous vessels which minimises the heat loss from skin surface. Circulation is thus focused on the vital organs, and core cooling is minimised. The role of catecholamines is discussed in more detail later. Behavioural thermoregulation, for instance wearing warm clothing and seeking shelter, is an essential form of protection from low temperatures. It may, however, be compromised because of an illness or the use of medication or intoxicating substances.

Fig. 1. The basic mechanisms of heat loss from the body in wet (a) and dry (b) cold environment.
Changes in blood circulation and diuresis

The vasoconstriction of cutaneous vessels due to cold exposure has notable effects on blood circulation. Up to one litre of blood volume is moved from the periphery to the inner parts of the body. The increased perfusion of kidneys increases diuresis, as excess fluid is excreted into urine. Urine volumes can grow more than twofold in cold stress (Granberg 1991b), and there is a concomitant decrease in urine osmolality (Sun et al. 2003). This is in part also mediated by hormonal mechanisms, particularly by the suppression of renal antidiuretic hormone (ADH) receptors (Sun et al. 2003). The overall circulating ADH seems to be down-regulated in cold as well (Broman et al. 1998), but cold exposure enhances diuresis even with normal or elevated circulating ADH levels (Allen & Gellai 1993, Granberg 1991b, Sun et al. 2003), and ADH deficiency eliminates the cold effect (Sun 2006).

Cold exposure causes haemoconcentration, which increases blood viscosity (Chen & Chien 1978, Keatinge et al. 1984). Plasma volumes have been shown to decrease by 14–15% (Neild et al. 1994, Vogelaere et al. 1992), and there is an increase of up to 12.5% in the erythrocyte count (Keatinge et al. 1984, Mercer et al. 1999, Neild et al. 1994, Vogelaere et al. 1992). An increase in granulocytes but not in lymphocytes has also been demonstrated even after adjustment for haemoconcentration (Mercer et al. 1999), and a direct effect of cold exposure on haematopoiesis has been suggested (Lombardi et al. 2011). The change in the thrombocyte count is, however, equivocal (Keatinge et al. 1984, Mercer et al. 1999, Vogelaere et al. 1992). It was previously thought that enhanced diuresis was mainly responsible for the haemoconcentration during cold stress. However, the changes in blood parameters seem to return to the baseline level fairly quickly after the end of cold stress — plasma volume in one hour (Vogelaere et al. 1992) and erythrocyte count in six hours (Mercer et al. 1999). These observations have led to the assumption that there is also a cold-induced shift of fluid from plasma to interstitial spaces (Heltne et al. 2001, Vogelaere et al. 1990, Vogelaere et al. 1992). According to the study by Vogelaere et al. (1992), almost half of the plasma volume decrease is accounted for by the transient shift between fluid spaces, while the rest is explained by diuresis. Chronic cold exposure of 1–3 weeks, on the other hand, has been shown to increase plasma and extracellular fluid volumes in rats (Sun et al. 1998), suggesting an adaptional response.

Seasonal variation in blood pressure is a well-known phenomenon (Barnett et al. 2007, Brennan et al. 1982, Brook et al. 2011). Both systolic and diastolic
blood pressures are at a higher level in winter time (Brennan et al. 1982), and cold exposure is regarded as the most important cause (Sun 2010). Acute cold exposure increases systolic and diastolic blood pressure by 12–15 mmHg and 16–18 mmHg, respectively (Keatinge et al. 1984, Koehn et al. 2012). Several mechanisms are involved in causing cold-induced hypertension. The vasoconstriction caused by the cold-induced activation of the sympathetic nervous system increases peripheral resistance, which raises arterial pressure. Venous constriction also increases the blood volume returning to the heart, which reflects on the heart rate and cardiac output (Granberg 1991b). It seems that the sympathetic nervous system is also the initiator of complex hormonal and biochemical reactions, including the renin-angiotensin system, nitric oxide and the endothelin system, which together contribute to the development of cold-induced hypertension (Sun 2010). A notable observation is that blood pressure remains at a higher level after the end of a longer cold exposure period (Shechtman et al. 1990). Thus, living in a colder environment may be a remarkable factor in the high prevalence of hypertension in Finland and other northern countries (Lawes et al. 2006, Wolf et al. 1997).

Physical and cognitive performance

Deterioration of physical or cognitive skills may increase the susceptibility to environmental hazards and hinder survival under cold stress. Cold exposure is a significant stress factor, and it impairs both physical and cognitive performance (Rintamäki et al. 2005). Muscular function is hindered even in relatively small decreases in skin temperature, especially when it comes to the control of coordination (Oksa et al. 1997) and dexterity (Chen et al. 2010), leading to progressive ataxia. Strength and the rate of force development of muscular contraction are also reduced in cold exposure (Cornwall 1994, Marrao et al. 2005), but there appear to be compensatory mechanisms at submaximal exercise levels (Ferretti 1992). The cold-induced impairment in muscle performance is due to the slowing of conduction and biochemical reactions in nerves and muscles. A minimum local temperature of 12 °C is needed for the upkeep of manual dexterity, and 10 °C for tactile sensitivity of the skin (Enander 1984).

Cold affects mood, cognition and mental capacity. In numerous studies conducted, cold stress seems to increase errors and the time needed to perform tasks (Palinkas 2001). Palinkas (2001) stated in his review that there is, however, inconsistency in the results of various mild cold exposure studies. Simple task
performance in cold is either impaired (Lieberman et al. 2009, Palinkas et al. 2005, Pääkkönen 2010, Thomas et al. 1989) or not affected (Giesbrecht et al. 1993, Marrao et al. 2005, O'Brien et al. 2007). In complex tasks, even improved performance has been observed (Giesbrecht et al. 1993, Pääkkönen 2010). The improvement is generally thought to be due to the arousing effect of cold (Giesbrecht et al. 1993, Mäkinen et al. 2006). Discomfort and distraction, on the other hand, probably explain the adverse responses reported (Mäkinen et al. 2006, Rintamäki et al. 2005, Spitznagel et al. 2009). Lowered cognitive function may even be sustained during and after recovery from cold exposure (Muller et al. 2012).

**Beneficial impact of cold**

Cold exposure and induced hypothermia are known to exhibit beneficial effects on health in certain respects. The most well-known applications have been the treatment of pain and the prevention of ischaemic damage. Though the scientific evidence is limited, cryotherapy has been shown to relieve the symptoms in some rheumatic diseases (Guillot et al. 2014). The pain-relieving mechanisms are not fully understood, but a systemic response, possibly through the elevation of circulating noradrenaline levels is suspected (Mikkelsson & Leppäluoto 2005). Mild to moderate hypothermia improves the neurological recovery of cardiac arrest patients after resuscitation (Bernard et al. 2002, The Hypothermia after Cardiac Arrest Study Group 2002). Similar neuroprotective impacts have been reported on spinal cord injuries (Ahmad et al. 2014) and many other related conditions. A drastic decrease in the neuronal metabolic rate, accompanied by other complex mechanisms is involved in the hypothermia-induced neuroprotection (Karnatovskaia et al. 2014).

**2.1.2 Morbidity and mortality in cold**

**Cold-related symptoms**

Cold-related respiratory and musculoskeletal symptoms are commonly reported (Näyhä et al. 2011, Raatikka et al. 2007), and the exacerbation of cardiovascular symptoms is also known (Hassi et al. 2005, Mercer 2003, Vuori 1987). Symptoms of peripheral circulation are more prevalent than those arising from the heart.
(Näyhä et al. 2011, Raatikka et al. 2007). In heart-related symptoms, arrhythmias seem to appear in much colder exposure than chest pain (Rytkönen et al. 2005). The overall appearance of cardiopulmonary symptoms is correlated with the extent and severity of cold exposure (Harju et al. 2010, Hassi et al. 2000).

Recurrent cold exposure aggravates respiratory symptoms, especially in people with asthma, chronic obstructive pulmonary disease or other chronic respiratory diseases, but there is no connection between cold exposure and the incidence of these illnesses (Kotaniemi et al. 2003, Kotaniemi & Rintamäki 2005). Breathing cold air increases resistance in the airways through several mechanisms (Giesbrecht 1995), but it does not seem to affect the breathing capacity in healthy people (Ahmed & Danta 1988, Chapman et al. 1990, Larsson et al. 1998). It is possible, however, that living in a cold environment has some effect on pulmonary function, demonstrated by the higher spirometry results in the Finnish population (Kotaniemi & Kataja 2004). An experiment with rabbits showed that chronic cold exposure diminishes lung perfusion even in healthy subjects, which may further contribute to the onset of symptoms (Mustafà et al. 2013).

A major factor affecting health during cold seasons is the rising incidence of respiratory tract infections (RTI) (Eccles 2002b, Silvennoinen-Kassinen et al. 2005). Cold temperature is considered to play a critical role in the appearance of RTIs (Eccles 2002a, 2002b, Mourtzoukou & Falagas 2007, Mäkinen et al. 2009). Mäkinen et al. (2009) reported an increased risk of 4.3% and 2.1% per 1 °C decrease in temperature for upper and lower RTIs, respectively. Eccles (2002a) proposed that cold induces vasoconstriction in the nasal vessels, which facilitates the progress of present subclinical or symptomless infections into manifest disease in the airways. In this respect, humans are poorly adapted to cold environments, compared with other mammals living in the arctic (Ince et al. 2012). The reduced blood flow hinders the supply of nutrients, oxygen, inflammatory cells and mediators as well as the amount of warm blood to the airway epithelium, enhancing the replication of viruses (Eccles 2002a). Thus, a cold-induced predisposition to contract RTIs may exist even though there is no depression of systemic immune functions (Castellani et al. 2002) and the overall amount of acute phase leukocytes may even increase in the circulation (Brenner et al. 1999, Mercer et al. 1999) and lungs (Larsson et al. 1998).
Excess cold-related mortality

Mortality rates are highly correlated with season and environmental temperature (Näyhä 2005). Death rates rise steadily as the mean daily temperature falls. This phenomenon has been widely demonstrated in all those parts of the world where seasonal variation occurs (Anderson & Bell 2009, Cordioli et al. 2000, Davie et al. 2007, Donaldson et al. 1998b, Goldberg et al. 2011, Gouveia et al. 2003, Ha et al. 2009, Healy 2003, Huynen et al. 2001, Keatinge et al. 2000, Kendrovski 2006, McKee 1990, Miron et al. 2012, Moran et al. 2000, Näyhä 2000, The Eurowinter Group 1997). In southern regions, where winters are usually mild, excess winter mortality is relatively higher than in regions with colder winters (Healy 2003, Keatinge et al. 2000, Näyhä 2005, The Eurowinter Group 1997). The threshold temperature, below which mortality starts to rise, is also variable and depends strongly on the climate of each region: 0 °C in Yekaterinburg, Russia (Donaldson et al. 1998b); 14 °C in Finland (Näyhä 2005); 16.5 °C in the Netherlands (Huynen et al. 2001) and 20 °C in São Paulo, Brazil (Gouveia et al. 2003). In Yakutsk, Russia, where the reported mean temperature in winter has been as low as −26.6 °C, mortality is unaffected by low temperature (Donaldson et al. 1998a).

Several factors have been presented so as to explain the paradoxical inverse relationship of mean winter temperature and the number of excess deaths in winter, including poor housing and inequality in socioeconomic factors (Healy 2003). There is also a clear difference in the habits of wearing warm clothing and the heating of dwellings between southern and northern regions (The Eurowinter Group 1997).

The rise in mortality rates during cold seasons is mainly attributed to the rising incidence of cardio- and cerebrovascular events and RTIs (Donaldson & Keatinge 1997, Keatinge et al. 2000, Näyhä 2005, The Eurowinter Group 1997). Up to 70% of excess deaths in winter are considered to be due to CVDs, and around half of the remaining deaths are from respiratory diseases (Mercer 2003). The prevalence of these diseases is highest among elderly people, and thus they are more vulnerable to the effects of cold (Keatinge et al. 2000, Näyhä 2005). A peak in mortality is observed two to five days after the coldest time point regarding cardio- and cerebrovascular events, and after about 12 days regarding respiratory diseases (Keatinge et al. 2000, The Eurowinter Group 1997). The deaths from CVDs are usually regarded as the result of reflex mechanisms such as the rise in blood pressure as well as an increased tendency towards vascular thrombosis, as described above (Rytkönen et al. 2005, The Eurowinter Group 1997).
Respiratory diseases, mainly RTIs, occur later on. People suffering from CVDs are more susceptible to the serious consequences of infections, which at least partially explains the later mortality peak (Bainton et al. 1978, Donaldson & Keatinge 1997). Furthermore, vaccination against influenza has been shown to drastically reduce CVD deaths (Meyers 2003). The link between RTIs and CVDs is not fully understood, but an increase in plasma fibrinogen due to infection probably contributes to the thrombogenic tendency (Woodhouse et al. 1994).

2.2 Hypothermia

Accidental hypothermia is defined as a drop in core body temperature below 35 °C due to exposure to cold environment. Induced hypothermia refers to the controlled lowering of body temperature for therapeutic purposes, for instance in preventing ischaemic damage during surgical operations. Furthermore, accidental hypothermia can be divided into primary and secondary hypothermia. Primary hypothermia occurs when environmental cold stress exceeds the person’s capacity for heat preservation. Secondary hypothermia is the result of an existing medical condition which subjects the person to core cooling, even without extreme external cold.

The severity of hypothermia is usually classified as mild (35–32 °C), moderate (32–28 °C) and severe (below 28 °C) (Brown et al. 2012). Additionally, staging can be done based on the clinical appearance of the hypothermia victim, ranging from conscious and shivering to absent vital signs (Brugger et al. 2001, Durrer 1991). There are a lot of different rewarming methods, and clinical classification aims to help in determining the optimal approach of therapeutic intervention (Brown et al. 2012, Brugger et al. 2001).

Occurrence of hypothermia

The incidence of accidental hypothermia is difficult to estimate, especially when it comes to secondary hypothermia. Many elderly people with several underlying diseases suffer from chronic, unnoticed hypothermia. However, some reports have been made of accidental hypothermia and hypothermia death incidences. Herity et al. (1991) reported the morbidity caused by hypothermia to be 5.36/100,000 people/year in the Irish population. A similar figure (5.60/100,000/year) has been reported in the United States (Baumgartner et al. 2008), though Noe et al. (2012) reported the annual incidence of emergency department visits due to hypothermia
to be somewhat higher, 8.37/100,000. In a study made in Southern Finland, the annual incidence of severe hypothermia requiring treatment in a university hospital was 0.58/100,000 (Silfvast & Pettilä 2003).

Hypothermia mortality varies according to the environment. Based on unpublished data obtained from Statistics Finland (Fig. 2), there are on average 65 deaths caused by hypothermia yearly and additional 27 deaths in which hypothermia has been a contributory cause of death (COD). This gives an annual mortality of 1.23/100,000. Figures reported from other countries have been 1.81 in Ireland (Herity et al. 1991), 1.35 in northern Sweden (Brändström et al. 2012) and 0.23–0.5/100,000/year in the United States (Centers for Disease Control and Prevention 2007, Noe et al. 2012). Major differences are seen in different states in the USA, and mortality rates up to 4.64/100,000/year have been reported in Alaska (Centers for Disease Control and Prevention 2006). Taylor (1964) estimated the influence of hypothermia on mortality to be much higher: 20,000–100,000 hypothermia-related deaths among the elderly in Great Britain yearly; that is 40–200/100,000/year. A number of hypothermia deaths also occurs in countries with a temperate climate (Lim & Duflou 2008), though no specific statistics are available.

**Progress of symptoms**

The symptoms of hypothermia become gradually more severe as the core temperature decreases. Depending on the circumstances, the rate of cooling has been reported to vary between 0.15–4.1 °C per hour in adults (Lange et al. 2013). In severe conditions, such as in cold water immersion, the cooling rate can supposedly be even higher, as heat loss is thought to occur three to four times faster in water than in dry air (Molnar 1946). Based on estimations by Tikuisis (1995, 1997), an average unclothed person would survive more than 24 hours in calm dry air at +10 °C, but less than five hours exposed to a corresponding water temperature. Cold water causes initially a cold shock response and a so-called diving reflex, which can result in heart arrhythmias, and increase the risk for drowning during the first minutes of immersion (Mantoni et al. 2006). These factors may expedite death even before the development of hypothermia.
In mild hypothermia, the situation is rarely life-threatening, and the symptoms include those discussed in the cold exposure section. Moderate hypothermia is already dangerous, as the risk of arrhythmias rises markedly. Homoeothermia — the ability to spontaneously return the temperature to normal — is lost when the core temperature decreases to 30–26 °C (Barrett et al. 2012, Danzl et al. 1995, Granberg 1991b), and humans become poikilothermic. Survival is still possible, but external rewarming will be necessary. The level of consciousness decreases gradually as hypothermia worsens, and the haemodynamic parameters, such as cardiac output, start to decrease. Oxygen consumption also diminishes greatly, up to 75% (Danzl et al. 1995, Granberg 1991b), which renders tissues much more tolerant of ischaemia. Below 28 °C, reflexes and the sense of pain are lost. Risk of ventricular fibrillation maximises at around 22 °C.

Indeed, cardiac arrhythmia is usually the mechanism of death in hypothermia deaths. Despite circulatory arrest, resuscitation may still be possible. There have been reports of recovery from core body temperatures as low as 13.7 °C (Gilbert et al. 2000) and 9 °C (Niazi & Lewis 1958), for accidental and induced hypothermia, respectively, with no apparent damage to the central nervous system. Nonetheless, despite medical treatment, mortality is high in severe
hypothermia (Turk 2010). In hospital admitted patients, most deaths occur after rather than during rewarming due to various complications, including pulmonary oedema and various infections (van der Ploeg et al. 2010). Perhaps surprisingly, body temperature does not correlate significantly with survival (Vretenar et al. 1994). The most accurate prognostic markers seem to be serum or plasma potassium concentration (Brown et al. 2012, Mair et al. 1994, Muszkat et al. 2002, Silfvast & Pettijä 2003) and partial pressure of carbon dioxide in arterial blood (Silfvast & Pettijä 2003).

Paradoxical undressing

The term paradoxical undressing refers to a peculiar phenomenon sometimes related to accidental hypothermia. The victims remove parts or all of their clothing, and thus expose themselves even more to the cold environment. In the literature there are few references to this phenomenon related to victims who subsequently survived (Sørli 1975), and it is generally regarded to occur immediately before unconsciousness and death (Wedin et al. 1979). There is no definitive explanation for this strange behaviour, but a failure in either the vasomotor activity of the cutaneous vessels or the central temperature regulation is usually thought to be present (Turk 2010, Wedin et al. 1979). A sudden vasodilatation in the peripheral tissues thus leads to a strong sensation of warmth, which, accompanied by the impaired mental status related to hypothermia, causes the victim to undress. Heat loss is then increased dramatically, hastening the death process (Garry 1969).

Most published data about paradoxical undressing are single case reports. However, some study series have also been conducted in order to find common factors behind the phenomenon. The frequency of partial or total undressing in hypothermia deaths is 17–30% (Brändström et al. 2012, Kinzinger et al. 1991, Mizukami et al. 1999). The influence of alcohol, medication and drugs are thought to predispose to the confusion leading to paradoxical undressing (Gormsen 1972, Kinzinger et al. 1991, Wedin et al. 1979). Dementia has also been suggested to contribute to the irrational behaviour (Wedin et al. 1979) although it may be difficult to determine in these situations whether hypothermia has led to undressing or vice versa (Kibayashi & Shojo 2003). These factors may be contributory to paradoxical undressing, but it is clear that it can also occur in healthy people with no signs of medication or intoxication (Sivaloganathan 1985).
Thus, it seems that hypothermia itself is the most significant cause of this phenomenon. Another unique feature, often connected to paradoxical undressing, is terminal burrowing behaviour or hide-and-die syndrome (Rothschild & Schneider 1995, Turk 2010). The terms refer to the finding of the body buried under furniture or other objects in a manner suggesting a final instinctive attempt to seek shelter (Rothschild & Schneider 1995). An undressed cadaver hidden from sight arouses the suspicion of a criminal act. Findings related to paradoxical undressing and terminal burrowing behaviour have been described both in homicidal (Kettner et al. 2012) and suicidal deaths (Hartwig & Tsokos 2007, Müller et al. 2011), emphasising the need for caution in examining the scene and autopsy findings in these cases.

2.2.1 Contributory and predisposing factors

Alcohol

Alcohol consumption is present in 43–75% of hypothermia deaths, making it the most common contributory factor (Brändström et al. 2012, Granberg 1991a, Hirvonen & Huttunen 1982, Kortelainen 1987). Ethanol is the alcohol mainly used, but surrogate alcohols including methanol, ethylene glycol and isopropyl alcohol (IPA) are also used, and they have a similar effect on thermoregulation (Kuitunen 2000, Mohler & Gordon 1990). The availability of ethanol strongly correlates to the rate of hypothermia deaths (Landen et al. 1997). Ethanol concentrations are typically lower in blood, 1.4–2.0 g/l, than in urine, 2.1–2.5 g/l (Brändström et al. 2012, Hirvonen & Huttunen 1982, Kortelainen 1987), suggesting that death occurs during the elimination phase (Kortelainen 1987).

It is commonly thought that alcohol intoxication causes poikilothermia, and the individual becomes more vulnerable to environmental temperatures (Kalant & Lê 1983). Alcohol consumption can result in a drop in core temperature even in neutral ambient temperatures (Risbo et al. 1981), and people unused to alcohol are at a greater risk (Wilson & Waring 2007). Men seem to be more susceptible to alcohol-induced cooling, though the reason behind this is unclear (Graham & Loughheed 1985). Ethanol affects thermoregulation by influencing both heat loss and production. It is probable that the effects are directed both at the central hypothalamic regulation as well as the peripheral thermoregulatory responses.
Examples of such effects are the reduction in shivering directly by muscle relaxation (Kalant & Lê 1983) and the lowering of the central threshold temperature for vasoconstriction of cutaneous vessels (Johnston et al. 1996). Additionally, complex biochemical actions of ethanol have been presented. High ethanol concentration in blood inhibits the production and metabolism of ketone bodies which are essential energy substrates during cold exposure (Teresiński et al. 2002, 2005). The antiketonemic effect of ethanol further impairs muscular shivering and other heat production. Ethanol itself is metabolised to produce energy and heat, but the overall influence seems to be detrimental to the heat balance (Granberg 1991a, Kalant & Lê 1983).

The exact mechanisms through which ethanol affects thermoregulation are not completely understood, and there is controversy in the numerous studies conducted. It has also been suggested that the most important aspect is the disruption of behavioural thermoregulation (Johnston et al. 1996). Ethanol consumption gives a pleasant sensation of warmth and diminishes judgement. Thus, the individual is more likely to go out with light clothing and fail to seek shelter from cold weather.

**Medication and narcotics**

Along with alcohols, certain medications are frequently found in examinations of hypothermia deaths. Common findings in hypothermia-related deaths are neuroleptics, sedatives, antidepressants and antiepileptics (Brändström et al. 2012, Hirvonen & Huttunen 1982, Johansson 2001, Kortelainen 1987, Turk 2010, Wedin et al. 1979). Additionally, illicit drugs such as amphetamine, opioids and cannabis are sometimes associated with hypothermia deaths (Brändström et al. 2012). It has long been known that especially centrally acting pharmaceuticals can affect thermoregulation (Borison & Clark 1967, Johansson 2001, Weihe 1976). The effects can be directed specifically at the thermoregulatory mechanisms, influencing either heat production or loss, or they can be indirect and non-specific (Borison & Clark 1967). Apart from the fact that medication may affect thermoregulation, changes in body temperature may also affect the pharmacokinetics and pharmacodynamics of the substance used (Johansson 2001, Weihe 1973). Lower than normal body temperature slows down the enzymatic reactions responsible for drug metabolism (Hostler et al. 2010) which can even
result in a multifold increase in the concentration of certain medications (Bjørnstad et al. 1990).

The thermodyresregulatory function of neuroleptics has been documented in both typical (Borison & Clark 1967, Cranston et al. 1972, Hägg et al. 2001, Hamuro et al. 1999, Maier et al. 1994) and atypical neuroleptics (Al Chekakie et al. 2006, Blass & Chuen 2004, Bookstaver & Miller 2011, Chen et al. 2003, Gibbons et al. 2008, Hägg et al. 2001, Hamuro et al. 1999, Kreuzer et al. 2012, Parris et al. 2001, Rasmayake et al. 2011, Razaq & Samma 2004). The hypothermic effects seem to be mediated through several receptors, including 5-hydroxytryptamine receptor 2A (van Marum et al. 2007), dopamine receptors D1 (Oerther & Ahlenius 2000) and D3 (Millan et al. 1995). Additionally, blocking the α2 adrenergic receptors may aggravate hypothermia by inhibiting shivering and vasoconstriction (van Marum et al. 2007). Thus, the receptor profile of the drug is essential for the risk of hypothermia. This is supported by the finding that atypical neuroleptics, especially risperidone, are more likely to induce hypothermia (van Marum et al. 2007). The risk of hypothermia is dose-dependent (Oerther & Ahlenius 2000) and highest at the beginning of treatment or increase of dosage (van Marum et al. 2007).

Many different substances influence thermoregulation centrally by affecting the γ-aminobutyric acid (GABA) system. A rat study by Zarrindast and Oveissi (1988) demonstrated that activation of GABA<sub>A</sub> receptors induces hypothermia while activating GABA<sub>B</sub> receptors induces hyperthermia. Studies of different GABA receptor agonists show, however, that hypothermia can also be achieved through GABA<sub>B</sub> receptor activation (van Nieuwenhuijzen & McGregor 2009, Rawls et al. 2004). Beside ethanol, common GABAergic drugs include benzodiazepines (BZD), barbiturates, antiepileptics and cannabinoids.

BZDs and their derivatives are commonly used mild sedatives. They have mainly replaced barbiturates, which are potent disrupters of thermoregulation (Hantson et al. 1996, Kortelainen 1987). It is apparent, however, that BZDs also impair cold tolerance (Hostler et al. 2009, Vapaatalo et al. 1984) and cause hypothermia (Echizenya et al. 2004, Hostler et al. 2010, Impallomeni & Ezzat 1976, Irvine 1966, Michaud et al. 2001). Certain interactions with other medication may further aggravate the drop in body temperature (Naylor & McHarg 1977). There is no persistent hypothermic effect in the occasional use of BZDs (Bourdon et al. 1995), suggesting that the thermoregulatory influence is dependent on the effective duration of the substance used. BZDs affect the hypothalamic GABA<sub>A</sub> receptors by binding to a specific site and increasing the
opening frequency of the chloride ion channels (Twyman et al. 1989, Zarrindast & Dibayan 1989). Barbiturates have a similar effect on the receptors, but they bind to a different site (Twyman et al. 1989). Muscle relaxation caused by the sedation further impairs thermoregulation by inhibiting shivering responses (Vapaatalo et al. 1984).

Antiepileptic medications enhance GABA potency by inhibiting GABA transaminase and thus increasing GABA concentration (valproate, vigabatrin) (Nikolov & Yakimova 2011) or by directly binding to the GABA_A receptor (topiramate, carbamazepine, phenytoin) (Granger et al. 1995, White et al. 1997). Reports of hypothermia have been presented related to therapeutic dosages (Longin et al. 2002, Zachariah et al. 2000), as well as intoxications (Alhaj & Alhaj 2001, Robinson & Abbott 2005).

Cannabinoids, most potently Δ^9-tetrahydrocannabinol, induce hypothermia (Haavik 1977, Rawls et al. 2004, Uran et al. 1980). Δ^9-tetrahydrocannabinol binds to central cannabinoid receptor type 1 (CB₁). CB₁ seems to be linked to the GABA_A system which is thought to be the main mediator of cannabinoid-induced hypothermia (Rawls et al. 2004). The effects of cannabinoids on thermoregulation are, however, complex, and many other neurotransmitters are also involved (Malone & Taylor 1998, Rawls et al. 2002).

Interestingly, paracetamol, a widely used mild analgesic, has been reported to cause hypothermia as a side-effect (Rollstin & Seifert 2013, Van Tittelboom & Govaerts-Lepicard 1989). Paracetamol has a CB₁-activating metabolite, but the hypothermic effect is at least partly mediated through other than the cannabinoid and GABA systems (Ayoub et al. 2004, 2011, Massey et al. 1982).

Opioid-induced hypothermia has been demonstrated related to anaesthesia and the treatment of pain (Ryan et al. 2012) as well as illicit drug abuse (Maiulis & Redenbaugh 1972, Platzer et al. 2007). The hypothermic effect of morphine is dose-dependent and comes up with higher dosages, while lower dosages rather produce hyperthermia (Baker & Meert 2002, 2003, Koek et al. 2012). Opioid-induced hypothermia develops mainly through unspecific mechanisms related to anaesthesia (Weihse 1976), but there are probably also several different pathways and mediators involved (Baker & Meert 2003, Milanés et al. 1984, Nemmani et al. 2001, Ulugol et al. 2000). This is further supported by the findings that hypothermia is induced by high doses of naltrexone, an opioid receptor antagonist, without affecting the hypothalamic temperature set point (Ary et al. 1976), and loperamide, a peripherally acting opioid (Baker & Meert 2002).
Age and illnesses

Children are generally more susceptible to cold because their thermoregulatory system is not fully developed. Infants are also dependent on the care of adults in gaining protection against environmental hazards. Compared with adults, the weight-to-surface area ratio of children is higher and skeletal muscle mass is smaller relative to their size (Blatteis 2012, Falk & Dotan 2008). These supposedly compensate for the underdeveloped sweating function to prevent hyperthermia (Falk & Dotan 2008, Inoue et al. 2004), but expose them to greater heat loss and less effective heat production. Heat production in brown adipose tissue is an effective means of protecting neonates from hypothermia (Blackburn 2011, Blatteis 2012). Brown adipose tissue as well as other physiological systems are, however, underdeveloped in preterm infants and neonates with growth defects (Pallotto & Kilbride 2006, Raju 2012), making these groups especially vulnerable to hypothermia. Other developmental disorders affecting the central nervous system may also further predispose small children to severe hypothermia and its complications (Hauer 2008).

There is a debate on whether aging itself has a significant effect on thermoregulation (Blatteis 2012, Kenney & Munce 2003). The elderly are, however, at risk of hypothermia, and most hypothermia-related deaths occur among people over 65 years old (Brändström et al. 2012, Watson 1996). With age usually comes the burden of diseases, medications and lowered nutritional status, which all affect thermoregulation and cold tolerance (Horvath & Rochelle 1977). Hypothermia is a common complication of surgical (Morrison 1988) and pharmacological treatment (Berlinger & Spector 1984) in the elderly. The review of Wilson & Morley (2003) showed that resting metabolic rate declines up to 20% at the age of 80 years compared with young adults. The decline is mainly due to the diminished mass of skeletal muscles (Wilson & Morley 2003). There may also be a decrease in lipid metabolism and other enzymatic reactions, though this is somewhat controversial (Blatteis 2012, Wilson & Morley 2003). Some older people tend to have a lower basal body temperature than younger people (Fox et al. 1973). However, the average change seems to be physiologically insignificant (Blatteis 2012, Kenney & Munce 2003). Body temperature varies with a circadian rhythm. The rhythm is maintained with aging, but its amplitude is slightly reduced and phasing advanced (Kenney & Munce 2003, Van Someren et al. 2002, Weinert 2010, Weinert & Waterhouse 2007). Other functions with a circadian rhythm, such as the sleep-wake pattern, deteriorate with age as well, which is
probably a major factor affecting the body temperature cycle (Weinert 2010). It is, indeed, difficult to distinguish behavioural factors from possible endogenous ones, and there are great differences between and within individuals (Weinert 2010, Weinert & Waterhouse 2007). Aside from the putative changes in resting metabolism, there appears to be a decline in cold tolerance starting from the age of 60–65 years (Smolander 2002). This may partially be due to diminished physiological responses to cold stress, for example attenuated vasoconstrictor reflex (Holowatz & Kenney 2010), but the most important factors are probably related to behaviour (Blatteis 2012, Kenney & Munce 2003). It is evident that the sensitivity to cold temperature is attenuated in the elderly, and that they fail to act properly to repel the chill (Blatteis 2012, Brody 1994, Florez-Duquet & McDonald 1998, Smolander 2002).

Several illnesses increase the risk of developing hypothermia. Basically, all conditions that restrict mobility or cognitive perception may predispose to hypothermia. Such conditions include dementia and neurodegenerative diseases (Kibayashi & Shojo 2003, Li & Lou 2012, Turk 2010). Additionally, some endocrinological diseases, foremost hypothyroidism and diabetes affect thermoregulation. Hypothyroidism slows the overall metabolism including heat production (Horvath & Rochelle 1977, Mizgala et al. 1991, Turk 2010). Deep hypothermia is a typical finding in the most severe form of hypothyroidism, myxoedema coma (Khaleeli 1978, Kogan et al. 2011). Both hypoglycaemia and diabetic ketoacidosis impair thermoregulation and may be complicated by hypothermia (Gale & Tattersall 1978, Nambu et al. 2012, Turk 2010).

**Trauma**

Trauma victims are often at risk of hypothermia. Injuries may prevent mobility, exposing the victim to ambient weather conditions, increased heat loss and decreased heat production. Hypovolaemia, as a result of bleeding, accompanied by shock and hypotension, impairs oxygen transport, which further reduces cold tolerance (Moore 2008). A wet environment, such as in boating accidents (Wanscher et al. 2012), can lead to a rapid cooling of the body with limited possibilities of rescue. Hypothermia is associated with high mortality in severely injured trauma patients (Kobbe et al. 2009, Moore 2008, Rutherford et al. 1998, Sundberg et al. 2011, Trentzsch et al. 2012). A lethal triad is recognised related to severe traumas including hypothermia, acidosis and coagulopathy (Kobbe et al. 2009, Mikhail 1999) which all aggravate bleeding and worsen the prognosis for
the patient. Careless treatment in prehospital setting may also contribute to the cooling of the body and worsen the clinical outcome (Langhelle et al. 2012, Lapostolle et al. 2012). These findings emphasise the need for aggressive rewarming and prevention of hypothermia in all phases of trauma victim management (Kobbe et al. 2009, Moore 2008).

Criminal offences

The majority of hypothermia deaths are accidental, with occasional suicides. Even intentional homicides with hypothermia have, however, been described (Madea & Rittner 2012, Mant 1969). Hypothermia-related deaths can also result from abandonment or failure to rescue.

Abandonment is defined in the criminal code of Finland as abandoning a helpless person to whom there is an obligation of care, and thus endangering his or her life (Rikoslaki 1995/578 21:14 §). The victims are usually infants or small children and elderly people with debilitating illnesses or otherwise people with severe disabilities. Though rare, some hypothermia deaths due to abandonment in nursing homes, and similar circumstances have been described in the literature (Akaza et al. 2003, Corey et al. 1992, Schmidt et al. 2005). Where children are concerned, the family is usually of poor economic and social background, and malnutrition and physical abuse are commonly present (Gustavson & Levitt 1996, Knight & Collins 2005, Trube-Becker 1977, Zumwalt & Hirsch 1980).

Failure to rescue concerns a situation where help is not provided to a person in mortal danger (Rikoslaki 1995/578 21:15 §). Such a situation may, for example, be finding someone lying intoxicated outside in cold weather and not providing help, or arriving at the scene of an accident without acting appropriately. Situations of this kind are rarely mentioned in the literature, and are probably uncommonly the cause of prosecution. The rate of cooling and the timeline of hypothermia are difficult to estimate (Lange et al. 2013), making it hard to prove someone’s involvement in the offence.

2.2.2 Autopsy findings in hypothermia death

Autopsy findings in hypothermia deaths may be scarce and non-specific. The post-mortem diagnosis of hypothermia is based on the exclusion of other notable causes. Thus, knowledge of the circumstances and events before death is vital to assess the influence of cold and possible hypothermia. The on-scene investigation
by the police should include the measurement of ambient temperature and a notion of the weather conditions. The adequacy of clothing and the occurrence of any undressing as well as a description of the place the victim was found should be carefully documented. As always, the victim’s medical history, use of medications, drugs and alcohol are needed in a comprehensive evaluation.

External examination

The most frequent external findings found in hypothermia deaths are frostbites, frost erythema and haematomas or abrasions (Hirvonen 1976, Turk 2010). Frostbites, or congelation, can be observed on the face, extremities and other uncovered skin areas. They are thought to occur most often in severe dry cold exposure (Hirvonen 2000). Frostbites are formed when cold-induced vasoconstriction causes hypoxia, and ice crystal formation damages tissues (Hirvonen 2000, Reddy & Lowenstein 2011). Injured endothelial cells leak plasma, causing oedema. Inflammation, blistering and gangrene are seen only if the frozen areas are rewarmed, and thus they are not usually observed in acute, fatal hypothermia (Hirvonen 2000).

Frost erythema is the reddish colouration of exposed skin areas, commonly seen on the extensor sides of elbows and knees (Turk 2010). The lividity is usually a light red colour, but this is also commonly the case in other deaths that take place in a cold environment. Victims of hypothermia have often crawled on the ground in the last phases before death which leaves variable haematomas and abrasions on the knees, elbows, ankles and hands.

The rectal temperature (T_{rect}) should always be measured when the death has occurred recently. If there is a clear discrepancy between a low T_{rect} and the estimated time of death by other secondary signs of death, hypothermia should be considered (Turk 2010, Türk et al. 2005).

Macroscopic and microscopic findings

Small gastric mucosal lesions, known as Wischnewski’s spots are found in 40–91% of hypothermia deaths (Madea et al. 2004, Takada et al. 1991, Tsokos et al. 2006, Turk 2010) making it the most common internal finding. They may be scattered anywhere on the gastric mucosa and can sometimes be found in the oesophageal and intestinal areas as well (Mant 1969). The lesions are blackish-brown in appearance and vary from 1 mm to more than 10 mm in diameter.
Although no firm conclusions about the exposure can be based on the lesions, they seem to be more prominent when the temperature has been milder, suggesting a longer exposure duration (Takada et al. 1991). The lesions are also more numerous and larger in size in younger than in older people dying of hypothermia (Takada et al. 1991). The exact mechanisms of how and why the lesions develop during cold stress are controversial (Bright et al. 2013a). The absence of Wischnewski’s spots in sedated rats dying of hypothermia suggests that they are related to general stress rather than to hypothermia (Bright et al. 2013b). Originally, they were described as haemorrhagic ulcers or erosions on the mucosa, but the absence of erythrocytes suggests that the lesions are distinct from true ulcerations (Tsokos et al. 2006).

Other hypothermia-related, yet non-specific, manifestations include acute pancreatitis or pancreatic haemorrhages, pulmonary oedema and haemorrhages in large muscles (Hirvonen 2000, Turk 2010). The pancreatic changes due to hypothermia have been described with varying frequency in the literature (Hirvonen 1976, Madea et al. 2004, Mant 1964, 1969), and there is controversy as to whether hypothermia really is a relevant risk factor for pancreatitis (Stiff et al. 2003). Preuß et al. (2007) concluded that only vacuolisation of the pancreatic adenoid cells was present more often in hypothermia deaths than in other deaths. Haemorrhaging of the pancreas was present in all types of deaths, with no significant difference as regards hypothermia (Preuß et al. 2007). Ischaemia due to the failure of microcirculation has been proposed as a possible pathomechanism leading to pancreatic necrosis (Foulis 1982), and it may need a prolonged exposure to develop (Hirvonen 2000). A similar cold-induced reduction in perfusion is thought to cause renal failure (Hirvonen 1976, Yoshitomi et al. 1998), and hypoxia-related haemorrhages in deep muscles, especially in the iliopsoas muscles (Madea et al. 2004). Pulmonary oedema is considered to be a sign of general shock (Turk 2010). Changes in the air and fluid contents of the lungs have also been examined with post-mortem computed tomography (Hyodoh et al. 2013, Kawasumi et al. 2013, Schweitzer et al. 2014). A histological examination of the pituitary gland may reveal non-specific vacuolisation of the cells (Doberentz et al. 2011).

2.3 Biochemical indicators of hypothermia

Because of the non-specific nature of the morphological findings in hypothermia deaths, efforts have been made to find a biochemical indicator of ante-mortem
cold stress. Several different substances have been studied in order to improve post-mortem diagnostics. In living subjects, research on acclimation and acclimatisation has involved quite extensive studies of hormonal responses. The results are of value also from the medico-legal point of view.

### 2.3.1 Catecholamines

Catecholamines — dopamine, noradrenaline and adrenaline — are tyrosine-derivatives which act as stress hormones and neurotransmitters. They are mainly synthesised in the adrenal medulla and nerve cells, noradrenaline in the sympathetic nervous system and dopamine in the central nervous system.

**Living subjects**

It is well documented that cold stress increases the secretion of catecholamines to circulation in healthy subjects (Leppäläluoto et al. 2005, Pääkkönen & Leppäläluoto 2002) and patients undergoing surgical procedures (Frank et al. 1995) as well as in accidental exposure (Hammerle et al. 1980). Noradrenaline levels increase even during mild cold exposure (Leppäläluoto et al. 2005, Pääkkönen & Leppäläluoto 2002) whereas adrenomedullary activation requires a more severe exposure. According to the study by Frank et al. (2002), a drop of at least 1 °C from the base level is needed for the elevation of adrenaline levels in plasma. The excretion of catecholamines to urine is also increased during cold exposure, noradrenaline being more abundant (Budd & Warhaft 1970, Konzett et al. 1971). Repeated cold exposure produces adaptation, and the sympathetic responses are damped down (Huttunen et al. 2001, Radomski & Boutelier 1982).

**Animal experiments**

Animal experiments have enabled the study of severe hypothermia and its effects on the catecholamine levels. Unlike in human studies, catecholamine levels in the plasma of guinea pigs were much lower after severe hypothermia (Hirvonen & Lapinlampi 1989). Urinary catecholamine levels increased, however, greatly — noradrenaline 4–5-fold and adrenaline up to 100-fold (Hirvonen & Lapinlampi 1989, Lapinlampi & Hirvonen 1986). Rats given ethanol showed similar responses in their catecholamine levels (Hirvonen & Huttunen 1995). Interestingly, the serum adrenaline and noradrenaline concentrations of female
rats were increased at the beginning of the cold exposure followed by a significant decrease (Hirvonen & Huttunen 1995). Elevated catecholamine levels have also been measured in the vitreous humour of guinea pigs (Lapinlampi & Hirvonen 1986) and rat adipose tissue (Kvetnansky et al. 2012), and higher concentrations of catecholamine metabolites have been found in the cerebrospinal fluid (CSF) of guinea pigs (Hirvonen & Lapinlampi 1989).

Cadaver studies

Several studies have been conducted on the post-mortem catecholamine levels in different types of death, and the results have been somewhat controversial. Serum adrenaline and noradrenaline concentrations have been reported to be lower in fatal hypothermia than in deaths from injuries, asphyxiation, intoxication and hyperthermia (Zhu et al. 2007a). A high total catecholamine concentration in the cadaveric blood clearly correlated with a long agonal period in the studies of Berg and Bonte (1973) and Hausdörfer et al. (1995). This finding is in contrast to the study of Zhu et al. (2007a) as the agonal period is usually long in hypothermia deaths. Wilke et al. (2007), on the other hand, found no significant differences between long and short agonal period and the catecholamine content of different body fluids. The adrenaline-to-noradrenaline ratio (ANR) in femoral vein blood was, however, significantly lower in short (0.17) than in long agony (0.42) (Wilke et al. 2007).

The use of urinary catecholamine assay in the post-mortem diagnosis of hypothermia was first proposed by Hirvonen and Huttunen (1982). The main reason for this is that the post-mortem catecholamine values are more stable in the urine than in blood (Hirvonen & Huttunen 1996). The adrenaline concentration was found to be more abundant than noradrenaline concentration, and both were at a higher level compared with natural and violent deaths (Hirvonen & Huttunen 1982). In another study, the noradrenaline concentration was more prominent while the total catecholamine content was at a comparable level (Kortelainen 1987). Urinary adrenaline concentration seems, nonetheless, to be a more specific hypothermia marker than noradrenaline (Palmiere et al. 2013, 2014c). To eliminate the impact of varying diuresis, the relation of catecholamine concentration to the concomitant urinary creatinine has been proposed (Sadler & Pounder 1995). No hypothermia death studies have been published using this adaptation, but Tormey et al. (1999) found no differences in the creatinine-
adjusted catecholamine concentrations between myocardial infarction and traumatic deaths.

Studies of post-mortem catecholamine levels measured from other body fluids have been limited. Wilke et al. (2007) concluded that the duration of agony cannot be determined from the catecholamine levels in CSF or vitreous humour. In the studies by Ishikawa et al. (2010, 2013), hypothermia deaths had lower catecholamine concentrations in pericardial fluid (PCF) and CSF compared with intoxication and hyperthermia deaths, among others. Cellular noradrenaline levels in the adrenal medulla follow the pattern of serum levels, being low in hypothermia deaths (Ishikawa et al. 2010).

As a conclusion of the catecholamine studies carried out with animal and human subjects and cadavers, it is evident that cold stress activates both noradrenergic and adrenergic systems. Yet, the activation of adrenal medulla requires severe exposure. The catecholamine levels in circulation rise at the beginning of cold exposure, as do the urinary levels. In severe exposure leading to death, however, the catecholamine concentrations in blood seem to be low, which may be due to the exhaustion of production and waning life force. The concentrations in the blood are also prone to change because of post-mortem autolysis and leaking of catecholamines from the adrenal medulla.

### 2.3.2 Other proposed indicators

**Pituitary hormones and cortisol**

Other hormonal levels are also affected by cold stress, though it is not exactly clear how. Thyroid hormones are known to be essentially involved in thermoregulation. Thyroid hormone levels generally decrease during the cold season and a long-lasting stay in cold environment while the level of thyroid-stimulating hormone (TSH) remains unchanged or increases (Leppäläuo et al. 2005, Pääkkönen & Leppäläuo 2002). In post-mortem examination, serum and CSF TSH levels have been either lower or at the same level in hypothermia deaths than in other deaths (Ishikawa et al. 2009, Palmiere et al. 2013).

The levels of adrenocorticotropic hormone (ACTH) in the hypophysis and CSF are low in hypothermia deaths, although the ACTH levels in serum are at a higher level (Ishikawa et al. 2008). This is in line with the observation that serum cortisol levels rise during cold exposure (Pääkkönen & Leppäläuo 2002) and stay
at a higher level in the post-mortem blood and urine in fatal hypothermia (Palmiere et al. 2013). However, Palmiere et al. (2013) did not find any obvious deviation in the post-mortem ACTH levels in serum. There is a similar equivocality in the growth hormone (GH) levels as well. The GH concentration in serum has been found to be high in hypothermia deaths, but also in deaths caused by injuries and myocardial infarction (Ishikawa et al. 2011). In another study, the GH levels in hypothermia deaths were within the clinical reference values, showing no increase whatsoever (Palmiere et al. 2013). Palmiere et al. (2013) concluded that the pituitary gland hormones are not reliable markers of fatal hypothermia, but high serum and urinary cortisol levels are indicative of cold stress.

**Ketone bodies and isopropyl alcohol**

Acetone and 3-β-hydroxybutyrate (3HB) concentrations in blood have been shown to be increased in hypothermia deaths (Palmiere et al. 2013, Teresiński et al. 2009). This finding is in keeping with the observation that fatty acids are the main energy substrates used during hypothermia, resulting in ketosis (Stoner et al. 1983, Teresiński et al. 2002, 2005). As mentioned earlier, ethanol apparently impairs the use of fatty acids, and thus lower acetone levels are seen if ethanol is present in the blood (Teresiński et al. 2002). The accumulating acetone is partially reduced to IPA (Palmiere et al. 2012b). IPA levels in the blood have also been shown to increase in hypothermia, and this is suggested as an indicator of fatal hypothermia (Palmiere et al. 2013). However, the presence of diabetic and alcoholic ketoacidosis as well as consumption of IPA as surrogate alcohol has to be ruled out (Palmiere et al. 2012a, Palmiere & Mangin 2012, Teresiński et al. 2009).

**Proteins**

Chromogranin A (CgA) is a neuroendocrine secretory protein found, for example, in the chromaffin cells of the adrenal medulla and beta cells of the pancreas. It is clinically used as a prognostic marker for carcinomas (Wu et al. 2000). The CgA level was found to be lower in serum and higher in CSF in hypothermia deaths compared with other causes (Yoshida et al. 2011).

S100 calcium binding protein β (S100B) is another protein studied as a possible hypothermia indicator. It is expressed by the astrocytes in the central
nervous system, and the serum levels of S100B elevate in cerebral injuries and neurodegenerative diseases (Zongo et al. 2012). Hypothermia seems to reduce the S100B concentration in the CSF (Li et al. 2009a, Wang et al. 2012). This may in part reflect the neuroprotective attribute of hypothermia (Roka et al. 2012, Sun et al. 2012).

Heat shock proteins (HSP) are a group of proteins which activate in response to heat and other stress factors. Cold stress has also been shown to induce the production of HSPs in cellular (Rada et al. 2005, Sonna et al. 2002) and animal studies (Tveita et al. 2012) as well as post-mortem examination (Preuß et al. 2008). Contradictory results showing decreases in the HSP levels have also been presented (Hashiguchi et al. 2003, Sonna et al. 2006). The severity of the exposure and the possible rewarming probably play a significant role (Sonna et al. 2006). Freezing of tissues may also influence the protein levels, which has to be considered in post-mortem examination (Edgerton et al. 2000).

**Electrolytes and markers of renal function**

Electrolyte disturbances are common in severely stressful situations. Changes in electrolyte concentrations in blood and other body fluids have been studied as possible hypothermia markers. Mant (1969) found elevated magnesium levels in the vitreous humour of hypothermia death victims, but concluded that it cannot be used as an indicator. On the other hand, markedly lower levels of magnesium have been found in the PCF in hypothermia deaths compared with deaths from drowning and injuries (Li et al. 2009b). There were no significant differences in the calcium levels in different tissues (Jakubeniene et al. 2009a, 2009b) or PCF (Li et al. 2009b) between hypothermia and other deaths. The sodium content in skeletal muscles was, however, much higher in hypothermia deaths than in control cases consisting of asphyxiation, injuries, intoxication and cardiac deaths (Jakubeniene et al. 2009a).

Urea nitrogen, creatinine and uric acid levels reflect renal function. Their post-mortem levels have been shown to be quite stable in blood (Zhu et al. 2002). Lethal hypothermia seems to increase the concentrations of urea nitrogen, creatinine and uric acid in blood and PCF (Maeda et al. 2008, Zhu et al. 2002, 2005, 2007b). As these compounds are mainly derived from muscle proteins, a catabolic process due to prolonged death has been proposed as an explanation (Palmiere & Mangin 2013). The changes in PCF take place later than in the
blood, and thus high concentrations of renal function markers in PCF may suggest longer survival times in the cold (Zhu et al. 2007b).

2.4 Thrombomodulin

TM is a protein originally discovered from vascular endothelium and proven to be essentially involved in blood coagulation (Esmon & Owen 1981, Esmon et al. 1982). Further studies have shown that TM also plays a critical role in inflammatory pathways and the maintenance of pregnancy (Weiler & Isermann 2003). The discovery of the vital functions of TM has spawned vigorous research trying to discover whether there is a link between TM and different illnesses (Takano et al. 1990, Weiler & Isermann 2003).

2.4.1 Structure, expression and physiological functions

The detailed structure of TM is quite well established (Fig. 3). It is expressed as a transmembrane protein and consists of several different domains, of which epidermal growth factor (EGF) -like domains are essential for cofactor activity and binding to thrombin (Sadler 1997). TM is expressed in the endothelium of both arteries and veins as well as some lymphatic vessels and placental cells (Maruyama et al. 1985). Additionally, TM is found in the circulation and urine as soluble fragments of variable sizes (Ishii & Majerus 1985, Ishii et al. 1990) which retain their functional properties (Bajzar et al. 1998, Öhlin et al. 2005). The regulation and exact mechanisms of TM release into circulation remain unclear. Damage to endothelial cells results in an increased release of TM from cells (Boehme et al. 2002, Ishii et al. 1991), but the cleavage of soluble fragments from the cell surface occurs most likely also in physiological conditions (Menschikowski et al. 2010).

Regulation of blood coagulation and fibrinolysis

Blood coagulation is a complex system involving cascades of enzymatic activations and feedback regulation. This ensures the delicate balance between clotting and lysis. The formation of a stable blood clot requires fibrin which is formed from its precursor fibrinogen by one of the central serine proteases, thrombin. Thrombin, in turn, is activated enzymatically from prothrombin after several similar enzymatic activations (Fig. 4). The coagulation cascade can be
started by two distinct pathways, the contact activation pathway (intrinsic system) and the tissue factor pathway (extrinsic system).

Fig. 3. The structure and functional domains of the thrombomodulin molecule. Epidermal growth factor (EGF) -like domains 5–6 bind thrombin. EGF module 4 is involved in protein C activation and module 3 in the activation of thrombin-activatable fibrinolysis inhibitor. The lectin-like domain has anti-inflammatory properties. The serine (Ser) and threonine (Thr) -rich domain is important for the binding of thrombin and activation of protein C. The cytoplasmic domain is involved in the cleavage of the transmembrane part, and its removal does not affect the functional properties of the molecule. Modified from Sadler (1997) and Weiler & Isermann (2003).
Fig. 4. Thrombomodulin (TM) as part of the coagulation and fibrinolysis cascades, and the effects of certain anticoagulant drugs (in blue). Arrows indicate inhibition or inactivation (red); and promotion of action or activation (green). APC, activated protein C; PAI-1, plasminogen activator inhibitor 1; PC, protein C; PS, protein S; TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated TAFI; t-PA, tissue plasminogen activator; V–XII, factor V–XII; Va–XIIa, activated factor V–XII.

TM acts as cofactor for thrombin and changes its function from procoagulant to anticoagulant (Esmon 2000). The TM-thrombin complex activates protein C (PC) much more efficiently than thrombin by itself (Esmon & Owen 1981). Activated protein C (APC) and its cofactor, protein S (PS), inactivate the active forms of factors V (Va) and VIII (VIIIa), which are parts of the thrombin activating chain reaction, as well as the plasminogen activator inhibitor 1 (PAI-1). The inactivated PAI-1 is unable to inhibit the tissue plasminogen activator (t-PA) which then catalyzes the formation of plasmin from plasminogen together with thrombin. Fibrin degradation products formed by fibrinolysis, in turn, inhibit thrombin.

The TM-thrombin complex has been demonstrated also to activate another plasma protein — thrombin-activatable fibrinolysis inhibitor (TAFI) (Nesheim et al. 1997). The active form of TAFI (TAFIa) is a potent inhibitor of fibrinolysis (Bajzar et al. 1996, Nesheim et al. 1997). In vitro studies have shown that the
TM-thrombin complex is able to activate both PC and TAFI simultaneously without significant competition (Bajzar et al. 1998). Thus, TM not only promotes anticoagulation, but also prevents the lysis of formed clots (Bajzar et al. 1998). The delicate balance is sustained in a normal situation, but it is unclear how different disease states may affect the balance.

Anticoagulation seems to be the prevailing function of TM in vivo. There are, indeed, ongoing studies about using recombinant TM or a TM analogue as anticoagulant therapy in thrombotic states (van Iersel et al. 2011, Moll et al. 2004, Vincent et al. 2013).

Inflammation

The regulation of inflammation is perhaps even more complicated than that of coagulation. The role of TM in inflammation has been quite extensively reviewed, and the TM-thrombin complex has been established as one of the essential links between coagulation and inflammation (Conway 2012, Weiler 2010, Weiler & Isermann 2003, Van de Wouwer et al. 2004, Van de Wouwer & Conway 2004). TM regulates inflammatory pathways through three main mechanisms: binding to thrombin, activating PC, and activating TAFI. In addition to being central in the coagulation cascade, thrombin has numerous proinflammatory effects such as regulating leucocyte proliferation and cytokine production (Naldini et al. 2002, 2005). The binding of TM to thrombin hinders these activities effectively (Conway 2012, Van de Wouwer & Conway 2004). APC modulates inflammation mainly by binding to the endothelial PC receptor and then activating the protease-activated receptor 1 (Weiler 2010, Weiler & Isermann 2003). Finally, TAFIa has anti-inflammatory properties by inactivating parts of the complement and other inflammatory mediators (Van de Wouwer & Conway 2004).

Embryogenesis and pregnancy

TM is expressed in placental cells (Maruyama et al. 1985, Uszyński et al. 2006a) and it is also found in other gestational tissues and amniotic fluid (Uszyński et al. 2006a, 2006b). Studies on mice have shown that TM is needed for normal foetal development (Healy et al. 1995, Weiler & Isermann 2003). Lack of TM leads to the end of proliferation and the death of placental trophoblast cells (Isermann et al. 2003). These changes are caused by the effects of tissue factor expressed by
trophoblasts and the subsequent fibrin deposition, both of which TM is able to counteract (Weiler & Isermann 2003).

Based on these findings and the fact that pregnancy greatly increases the risk of thrombus formation (Greer 1999), malfunctions in the coagulation system have been proposed as a cause for recurrent miscarriages. Several procoagulant factors increase in the circulation during normal pregnancy (Greer 1999, Joly et al. 2013) and there is a parallel change in the circulating TM concentration during early pregnancy (Van Dreden et al. 2012, Hui et al. 2012). In a study of recurrent miscarriages, the placental TM levels were lower (Stortoni et al. 2010), but there were no differences in the circulating TM levels between women with and without recurrent miscarriages (de Larrañaga et al. 2005). A state of TM resistance has been described related to early miscarriage, especially between gestational weeks 9–12 (de Saint Martin et al. 2011), although the ability of TM to activate PC with thrombin seems to be higher in early and late miscarriages than in uncomplicated pregnancy (Van Dreden et al. 2012). The influence of different variations in the thrombomodulin gene (THBD) on the prognosis of pregnancy has been studied, but no conclusive findings have been presented (Cao et al. 2013, Kaare et al. 2007, Said et al. 2012).

### 2.4.2 Thrombomodulin and diseases

As TM is an essential factor in many critical functions, its role in various diseases has been under extensive research. Most studies have concerned the changes in TM concentrations in different diseases compared with healthy subjects in order to find a diagnostic tool or a prognostic marker. Other studies have attempted to discover a relation between variations of the THBD and an increased susceptibility to contract a disease. Given the fundamental role of TM in coagulation and inflammation, most interest has focused on atherosclerosis and other CVDs, disorders in coagulation and malignancies.

**Normal values**

There are no known reference values for TM in blood, urine or other fluids. TM concentrations are analysed immunologically, and there appears to be great variability in the values depending on the kit in use (Boehme & Stremmel 2004). Published values of TM in the blood of healthy adults have ranged between 2.4–35.9 ng/ml (Hoshi et al. 1995, Huang et al. 2008, Iwashima et al. 1990, Matsuda 1991).
et al. 1992, Oida et al. 1990, Schumacher et al. 2002, Seigneur et al. 1993, Sosothikul et al. 2007, Takano et al. 1990, Yin et al. 2009). Variation according to sex and age is negligible, but postmenopausal women appear to have higher values than younger women (Nilsson et al. 1993). There are not many studies concerning urinary TM levels. Hanyu et al. (1999) reported mean values of 48.0 and 51.1 ng/mg creatinine for men and women, respectively.

**Cardiovascular diseases**

Atherosclerosis is a condition where fatty materials accumulate in the arterial walls, forming plaques. The plaques disrupt the normal functions of the vessels and are susceptible to rupture and thrombus formation. Additionally, inflammatory processes are known to be involved in the formation of atherosclerotic plaques. Thus, it is plausible that atherosclerosis influences the expression and secretion of TM. Changes in TM levels could also affect the development of vascular lesions.

Indeed, the expression of TM has been shown to inversely correlate with the level of atherosclerosis in the coronary arteries (Laszik et al. 2001). The ability of TM to bind thrombin seems to be a key factor in preventing atherosclerosis (Wei et al. 2011). However, the changes in circulating TM levels in CVD patients have been equivocal (Weiler & Isermann 2003). Elevated plasma TM concentrations have been found in both coronary artery disease and peripheral arterial disease (Seigneur et al. 1993), but there seems to be no correlation between the severity of the disease and the measured TM levels (Blann et al. 2000, Nasser et al. 2006, Peter et al. 1997, Seigneur et al. 1993). Also, the plasma TM level is not connected to ischaemic signs of the heart (Nilsson et al. 1993). An inverse relationship was revealed between TM concentrations in plasma and the risk of developing coronary artery disease in the Atherosclerosis Risk In Communities study (Salomaa et al. 1999, Wu 2003), though another large prospective study did not find any support for this (Karakas et al. 2011). Somewhat paradoxically, there was a positive relationship between the circulating TM level and the risk of atherosclerosis of the carotid artery (Salomaa et al. 1999). It was suggested that this might be because of the different mechanisms of TM cleavage from the cell surface, whether physiological or due to endothelial cell damage (Wu 2003).

Numerous studies have been conducted on TM levels in arterial hypertension, cardiac hypertrophy and congestive heart failure (CHF) with inconsistent results. Concerning essential arterial hypertension, the circulating TM levels have been
lower (Makris 1997, Sawada et al. 2003), higher (Dohi et al. 2003, Papadopoulos et al. 2012) or inconclusive (Trifiletti et al. 1995, Wang et al. 2006) compared with normotensive controls. Cardiac hypertrophy and CHF may be related to a number of different conditions, including coronary artery disease and arterial hypertension. The circulating TM levels related to these conditions have been mostly higher than in healthy controls (Chong et al. 2006, Cugno et al. 2004, Dimitrow et al. 2007), but some studies have found no differences (Nonaka-Sarukawa et al. 2003). Chong et al. (2006) reported higher TM levels in acute CHF compared with chronic CHF. In another study, there was no correlation between TM levels and the severity of CHF (Nonaka-Sarukawa et al. 2007). Animal studies have shown a decrease in TM expression in the left atrium (Kapur et al. 2007), but a progressive increase in the left ventricle (Li et al. 2010) after induced CHF or cardiac hypertrophy, respectively.

It is clear that the pathological conditions of the heart and vasculature influence the expression of TM. Because of the variable and overlapping nature of these conditions, however, it is difficult to draw firm conclusions about the correlations between different illnesses and the expression and secretion levels of TM.

Coagulopathies

Due to the essential role of TM in coagulation, it is understandable that disturbances in its production and metabolism cause serious defects in haemostasis. Heterozygous point mutations have been found behind hereditary coagulopathies (Öhlin & Marlar 1999, Öhlin et al. 1997). There is, however, discrepancy in the relationship between the risk of thromboembolism and plasma TM level. The circulating TM level has been higher regarding deep venous thromboembolism in some studies (Takano et al. 1990), but others have found no significant differences between patients and controls (Aleksic et al. 2003, Sakamaki et al. 2003, Smith et al. 1999, Trifiletti et al. 1997). Yin et al. (2009) concluded that low plasma TM level is associated with a higher pulmonary thromboembolism risk in women but not in men.

Disseminated intravascular coagulation (DIC) is a disorder where blood clotting is activated inside the blood vessels. Small clots are formed and they consume circulating coagulation factors, which leads to haemorrhaging. DIC may result from a number of underlying causes, for example septic infections, severe trauma, cancer and intoxications (Levi 2007). Higher TM levels are associated
with DIC in sepsis (Gando et al. 2005, Lin et al. 2008) and trauma (Zhu & Huang 2009) compared with non-DIC patients. There was also an evident correlation between the circulating TM concentration and mortality, while the resolution of DIC reflected as lowered concentration (Lin et al. 2008). The release of TM in DIC has been speculated to come from the abnormal activation of the coagulation-fibrinolysis system (Takano et al. 1990) and the DIC-related vascular endothelial damage (Gando et al. 2005). Because DIC is a complex and multifaceted disorder, there are probably also numerous other factors involved, such as inflammatory processes and the underlying cause itself.

* Cancer *

Disorders in blood coagulation, inflammation as well as other important physiological functions are commonly related to malignancies. Thus, it is no wonder that TM expression and secretion are affected by various types of cancer. It has become clear that TM is expressed in different cancer cell types and is a crucial factor in tumour growth and regulation (Hanly et al. 2005). The expression of TM is closely linked to the growth behaviour and prognosis of many malignant tumours — a low expression level reflects a more advanced stage (Hanly et al. 2005). Measuring the TM expression level might also be useful in distinguishing some tumour types from each other (Boffa et al. 1994, Collins et al. 1992). The plasma levels seem to be of opposite significance, and high concentrations have been related to advanced stage and metastasising (Lindahl et al. 1993, Xu et al. 2004).

* Renal failure and diabetes *

Although the exact excretion routes of TM are unclear, renal filtration probably plays a major part in it. This is supported by the pharmacokinetic studies of soluble recombinant TM (Moll et al. 2004). Renal function is clearly associated with the concentration of TM in plasma (Hergesell et al. 1993). Higher plasma TM levels are found in patients with renal failure, from early nephropathic changes to dialysis (Ishii et al. 1996, Jacobson et al. 2002, Mezzano et al. 1997, Segarra et al. 2001), irrespective of the cause (Borawski et al. 2001). A strong correlation has also been shown between the rate of proteinuria in diabetic nephropathy and plasma TM level (Iwashima et al. 1990, Oida et al. 1990). However, the TM concentrations in plasma and urine seem to be higher in
diabetic patients even without nephropathy (Aso et al. 1998). Diabetes and renal function are closely related to CVDs, which probably is a confounding factor. Somewhat controversially, though in line with the Atherosclerosis Risk In Communities study (Salomaa et al. 1999), low plasma TM level predicted the onset of type 2 diabetes while subjects with higher levels became diabetic less frequently (Thorand et al. 2007). The interpretation of these findings is difficult and emphasises the multifactorial and complex nature of the expression and release of TM.

Medication

Some pharmacological agents may influence the production and release of TM. These substances include 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), angiotensin-converting enzyme (ACE) inhibitors and drugs affecting blood coagulation.

Statins are the most studied substance group in this context. In addition to effectively lowering blood lipid levels, they upregulate TM expression in endothelial cells (Undas et al. 2005) with a dose-dependent increase (Góralczyk et al. 2009, Rossi et al. 2010). Some interesting observations have been made about the pathways behind the up-regulation, and the effects seem to be independent of the lipid-lowering functions. Mevalonate is an essential product of HMG-CoA reductase, and it provides geranylgeranylpyrophosphate which is needed in the activation of small G proteins (Masamura et al. 2003, Shi et al. 2003). The Rho family of G proteins negatively regulate Krüppel-like factor 2 (KLF2), which appears to be a direct upregulator of the THBD gene (Sen-Banerjee et al. 2005). Statins inhibit HMG-CoA reductase, thus promoting the actions of KLF2 and ultimately increasing TM expression. Statins also induce the endothelial nitric oxide synthase by downregulating the G proteins (Laufs & Liao 1998). Nitric oxide, in turn, enhances the dissociation of heat shock factor 1 (HSF1) from the HSP90 complex. HSF1 binds to the THBD promoter, enhancing its expression (Fu et al. 2008), thus providing another pathway of statin-induced increase in TM expression. Furthermore, it has been demonstrated that statins induce TM expression despite attenuation by tumour necrosis factor α (Lin et al. 2007, Shi et al. 2003), which is most likely closely connected to these pathways.

Considering plasma TM levels, a decrease is usually seen in response to statins (Krysiak et al. 2003), though inconclusive results have been obtained (Seljeflot et al. 2002). As Masamura et al. (2003) pointed out, this is probably not
in contradiction with the up-regulation of expression, since the plasma TM is at least partially derived from cellular damage. It is likely, however, that physiological changes are involved in the statin-induced effects.

ACE inhibitor enalaprilat was shown to reduce the circulating TM concentration in patients with septicaemia (Boldt et al. 1998). In renal failure patients, the use of ACE inhibitors was rather associated with a higher TM level (Borawski et al. 2001) and perindopril did not have any effect on TM concentration in hypertensive patients (Okrucká et al. 1998). ACE inhibitors are commonly used in the treatment of hypertension in diabetic nephropathy patients and are known to reduce proteinuria. The renoprotective effects may partially explain the effects on TM levels in these patient groups and the lack of effects in patients without such conditions.

The anticoagulants used for preventing blood clotting affect different phases in the coagulation cascade (Fig. 4), and, thus, some influence in the TM production could be possible. Unfortunately, there are no comprehensive studies involving TM and anticoagulants.

Warfarin prevents the reduction of vitamin K and thus impairs the synthesis of prothrombin, factors VII, IX and X as well as PC and PS (Freedman 1992). High TM level in plasma during warfarin treatment is a risk factor for bleeding complications (Jansson et al. 1997, Lind et al. 2009), but it is unknown how the medication itself affects TM levels.

Low molecular weight heparins, such as enoxaparin and dalteparin, potentiate antithrombin III which inhibits thrombin and several other activated factors in the cascade. The administration of unfractionated heparin was associated with reduced TM levels in circulation (Cella et al. 1997), though a positive correlation has also been presented regarding unfractioned heparin but not enoxaparin (Borawski et al. 2001).

Recently discovered anticoagulant agents, such as dabigatran and rivaroxaban, directly inhibit either thrombin or activated factor X (Xa) (Schulman 2014). The activation of TAFI and generation of thrombin are reduced during dabigatran administration (Ammollo et al. 2010) which could shift TM production in either way.

Additionally, several pharmacological agents act as anticoagulants by preventing thrombocyte reactions including clopidogrel, dipyridamole, acetylsalicylic acid and other non-selective non-steroidal anti-inflammatory drugs (NSAID). However, there are no studies regarding their influence on TM production or metabolism.
2.4.3 Thrombomodulin in cold exposure and hypothermia

Little is known about the effects of cold exposure and hypothermia on the expression and shedding of TM. Elevation of the plasma TM level was observed in patients undergoing a cardiopulmonary bypass under hypothermia (Böhrrer et al. 1995). Based on the given circumstances, it is probable that the underlying illness as well as the procedure itself may have caused the changes. In another study, however, it was shown that hypothermia was a significant factor behind the increase when hypothermic (T$_{rect}$ 27–28 °C) and normothermic bypass procedures were compared (Boldt et al. 1996). On the other hand, a mild cold exposure (10 minutes at 4 °C, no hypothermia) did not have any effect on the measured TM levels (Matsuda et al. 1992). An immunohistochemical study revealed an increase in the TM expression in the small muscular and subcutaneous arterioles and venules in a rat hind leg after a 30-minute period of cooling down to 4 °C (Rücker et al. 2009). It is notable that the changes in the TM levels in the previous studies were observed 2–24 hours after the cold stress period (Böhrrer et al. 1995, Boldt et al. 1996, Rücker et al. 2009) while the differences measured initially after cold exposure were insignificant (Matsuda et al. 1992).
3 Aims of the study

The objectives of this study were to find a tool to help the post-mortem diagnosis of hypothermia deaths and to acquire knowledge of the pathophysiological effects of cold. The specific aims were:

1. to evaluate the accuracy and usefulness of urinary catecholamine assays as a post-mortem indicator of hypothermia (I);
2. to discover how cold exposure, hypothermia and rewarming affect TM expression and secretion in living subjects (II, III);
3. to study the relationship between catecholamines and TM (II–IV);
4. to assess the usefulness of TM as a post-mortem indicator of hypothermia (IV).
4 Materials and methods

4.1 Study permits

The experimental protocols were approved by the University of Oulu and Northern Ostrobothnia Hospital research committee (statement number 31/2007). A permit to take tissue samples in autopsies was acquired from the National Supervisory Authority for Welfare and Health (2932/06.01.03.01/2013). Permits to collect data from the autopsy reports, death certificates and other material related to the medico-legal investigations were acquired from the State Provincial Office of Oulu (currently the Regional State Administrative Agency of Northern Finland) (OLH-2009-02008/SO-41), the Regional State Administrative Agency of Lapland (LAAVI-2010-01-01294/SO-41) and the National Institute for Health and Welfare (THL/479/5.05.01/2013). A permit to use experimental animals was acquired from the National Animal Experiment Board (ESAVI/974/04.10.03/2012).

4.2 Medico-legal autopsies (I, IV)

The medico-legal autopsies were performed in the University of Oulu, Department of Forensic Medicine during 1990–2014. Prior to 2012, medico-legal autopsies were also partly carried out in the pathology units of Lapland Central Hospital, Rovaniemi and Länsi-Pohja Central Hospital, Kemi.

4.2.1 Study groups

The autopsy cases were divided into five groups (I and IV combined):

- Hypothermia as the main COD (n = 195)
- Hypothermia as a contributory COD (n = 79)
- CVD as main COD (n = 182)
- Trauma as main COD (n = 52)
- Other non-traumatic, non-CVD main cause (n = 44)

The distribution of autopsy cases was done based on the main and contributory causes of death recorded in the death certificate by a medical examiner. The measurement of urinary catecholamines has been included in many of the autopsy cases as part of the medico-legal investigation. This may have influenced some of
the diagnoses given by the medical examiner, especially concerning the hypothermia death groups.

The traumatic deaths group included sudden deaths from sharp and blunt force trauma, namely injuries to the head, neck, thorax and lower extremities, as well as combinations of severe injuries. The other deaths group consisted of deaths from accidental and suicidal causes and diseases not included in the other groups. A detailed description of the causes of death is shown in Tables 1 and 2.

**Table 1. The frequencies of diagnoses as main cause of death in different groups.**

<table>
<thead>
<tr>
<th>Main cause of death</th>
<th>Hypothermia</th>
<th>Hypothermia</th>
<th>CVD</th>
<th>Trauma</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>main cause of death</td>
<td>contributory cause of death</td>
<td>deaths</td>
<td>main cause of death</td>
<td>causes</td>
</tr>
<tr>
<td>Hypothermia CVDs</td>
<td>195</td>
<td></td>
<td></td>
<td>195</td>
<td></td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>30</td>
<td>165</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertensive cardiomyopathy</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Other cardiomyopathies</td>
<td>5</td>
<td>9</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Valvular diseases</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Rupture of aorta</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>SAH/ICH</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Traumas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head or neck</td>
<td>4</td>
<td>34</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thorax</td>
<td>2</td>
<td>10</td>
<td>12</td>
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<tr>
<td>Extremities</td>
<td>1</td>
<td>2</td>
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<tr>
<td>Combinations</td>
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<td>Intoxications</td>
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<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>7</td>
<td>11</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medication/drugs</td>
<td>4</td>
<td>3</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combination</td>
<td>8</td>
<td>3</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>4</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drowning</td>
<td>9</td>
<td>4</td>
<td>13</td>
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<td></td>
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<tr>
<td>Hanging/asphyxia</td>
<td></td>
<td>8</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>3</td>
<td>11</td>
<td>14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CVD, cardiovascular disease; ICH, intracerebral haemorrhage; SAH, subarachnoid haemorrhage
Table 2. The frequencies of diagnoses as contributory causes of death in different groups.

<table>
<thead>
<tr>
<th>Contributory cause of death</th>
<th>Hypothermia main cause of death</th>
<th>Hypothermia contributory cause of death</th>
<th>CVD deaths</th>
<th>Trauma main cause of death</th>
<th>Other causes</th>
<th>All groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Use of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>126</td>
<td>29</td>
<td>25</td>
<td>17</td>
<td>16</td>
<td>213</td>
</tr>
<tr>
<td>Medication/drugs</td>
<td>34</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>CVDs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Coronary artery disease</td>
<td>65</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td>12</td>
<td>93</td>
</tr>
<tr>
<td>Hypertensive cardiomyopathy</td>
<td>6</td>
<td>7</td>
<td>55</td>
<td></td>
<td></td>
<td>68</td>
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<td>Other cardiomyopathies</td>
<td>25</td>
<td>5</td>
<td>11</td>
<td>1</td>
<td>6</td>
<td>48</td>
</tr>
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<td>Valvular diseases</td>
<td>3</td>
<td>6</td>
<td>12</td>
<td>1</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>SAH/ICH</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cerebral infarction</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Other CVDs</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
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<td>79</td>
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<td>79</td>
</tr>
<tr>
<td>Traumas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head and neck</td>
<td>10</td>
<td>6</td>
<td>1</td>
<td>4</td>
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<td>22</td>
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<tr>
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<td>1</td>
<td>1</td>
<td>10</td>
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<td>16</td>
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<tr>
<td>Abdomen</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Extremities</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Combinations</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
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<td>2</td>
<td>26</td>
<td>1</td>
<td>1</td>
<td>34</td>
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<tr>
<td>Dementia</td>
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<td>1</td>
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<td>Psychiatric disorders</td>
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<td>3</td>
<td>1</td>
<td>9</td>
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<td>Pulmonary diseases</td>
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<td>2</td>
<td>14</td>
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<td>24</td>
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<td>Neurological diseases</td>
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<td>2</td>
<td>1</td>
<td></td>
<td></td>
<td>13</td>
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<tr>
<td>Hyperlipidaemia</td>
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<td>11</td>
<td></td>
<td></td>
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<td>13</td>
</tr>
<tr>
<td>Obesity</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignancies</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Acute infections</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Drowning</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Other</td>
<td>12</td>
<td>4</td>
<td>9</td>
<td>2</td>
<td>1</td>
<td>28</td>
</tr>
</tbody>
</table>

CVD, cardiovascular disease; ICH, intracerebral haemorrhage; SAH, subarachnoid haemorrhage
4.2.2 Data collection

Basic demographic data was collected from death certificates and autopsy reports. This data included sex, age, manner of death as well as main and contributory causes of death. Post-mortem interval (PMI) was determined as the time period between the time of death, either certain or estimated, and sample taking in autopsy.

4.2.3 Sample collection

All samples were collected as part of the routine medico-legal autopsy. Blood was taken from the common iliac veins, the post-mortem serum was separated and stored at \(-80^\circ\text{C}\) before analysis. Urine was taken directly from the bladder and stored at \(-80^\circ\text{C}\). Myocardial samples were taken from the anterior wall of the left ventricle and either fixed in formalin and embedded in paraffin or deep frozen with liquid nitrogen. If sufficient amounts of blood or urine were not available, vitreous humour and liver tissue were collected for toxicological analysis.

4.3 Live subjects (II–III)

Live subjects were used to determine the baseline values of TM and to assess the effects of short-term cold exposure as well as mild and severe hypothermia on the TM levels.

4.3.1 Human experiment protocol and measurements

Seven male subjects volunteered for the study, and gave their informed consent after learning of the nature and risks of the experiment. The mean (± standard deviation) characteristics were: age, 26 ± 2 years; height, 178 ± 4 cm; weight, 75 ± 7 kg; body mass index 23.7 ± 1.6 kg/m². Mean maximal oxygen consumption during maximal exercise test was 52.3 ± 5.2 ml/kg/min.

The experiment was carried out at the Finnish Institute of Occupational Health, Oulu, Finland, in late spring and early summer. Two immersions of different exposure temperatures were conducted in random order for each subject. The immersions were accomplished with head-out immersion in upright posture in a pool of water with a temperature of either +10 °C or +30 °C. The duration of the +10 °C exposure was 10 minutes followed by an immersion in a pool with
water temperature of +28 °C for 20 minutes. This was done so as to limit the severity of the exposure, and also to allow further heat loss in a slightly below thermoneutral temperature. In the +30 °C exposure, the same water temperature was used for the corresponding time (10 + 20 minutes). The total immersion time was thus 30 minutes. Blood and urine samples were taken 1 hour before the immersion, and 1, 4, 6 and 23 hours after the start of immersion. Additional blood samples were taken at the start and in the end of immersion and 2 hours after the start of immersion.

Skin temperatures were measured from 10 sites: forehead, upper back, chest, abdomen, upper arm, lower arm, back of the hand, anterior thigh, dorsal side of the foot and calf (NTC DC 95, Digi Key, USA). The thermistors were attached to the skin with adhesive material. T_rect was measured 10 cm beyond the anal sphincter with a YSI 401 probe (Yellow Springs Instrument Co., Yellow Springs, USA). Skin and rectal temperature values were recorded at 30-s intervals with a datalogger (SmartReader 8+, ACR Systems, Canada) throughout the cold and warm exposure. Mean skin temperature (T_sk) was calculated as an area-weighted average according to the following formula: 0.07 • (T_forehead) + 0.35 • mean(T_chest, T_scapula, T_abdomen) + 0.14 • mean(T_upper_arm, T_lower_arm) + 0.05 • (T_dorsal_hand) + 0.19 • (T_thigh) + 0.13 • (T_calf) + 0.07 • (T_dorsal_side_of_foot) (Hardy & Du Bois 1938). Mean body temperature (T_b) was calculated by the equation: T_b = 0.65 • T_rect + 0.35 • T_sk (Burton 1935).

4.3.2 Experimental animals

96 male Sprague-Dawley rats (Rattus norvegicus) aged between 8 and 9 weeks and with an average weight of 329 g were divided into 7 groups: control, mild hypothermia 1 (MH1), mild hypothermia 2 (MH2), severe hypothermia 1 (SH1), severe hypothermia 2 (SH2), severe hypothermia followed by rewarming 1 (SHRW1) and severe hypothermia followed by rewarming 2 (SHRW2). The experimental parameters for each group are presented in Table 3.

The rats were anaesthetised with a mixture of fentanyl/fluanisone, midazolam and aqua 1:1:2 (0.5 ml) injected subcutaneously into the back. The control group rats were killed immediately after the administration of the anaesthetic. In SHRW2 group, no anaesthetic was given during the last three hours to ensure the recovery of normal temperature. However, no observable arousal from sleep was present. After inducement of anaesthesia, the rats were weighed and their body temperature was measured with a rectal thermometer. The rats were monitored
continuously, and an intramuscular injection (0.12 ml) of the anaesthetic was administered into the thigh if a toe pinch reflex appeared. The temperature data was collected at 5-minute intervals except for the rats in SHRW1 and SHRW2 groups, where the temperature was recorded only in the beginning of the test, at the 2-hour interval and at the end of the test. After completion of the experiment, the rats were killed by decapitation, dissected and the myocardium, blood and urine samples were collected. One half of the heart was fixed with formalin (24 h) and embedded in paraffin. The other half was frozen with liquid nitrogen and stored in −80 °C. EDTA-plasma, separated from blood, and urine were stored at −80 °C.

Table 3. The description and mean study parameters of the rat groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description of exposure conditions</th>
<th>n</th>
<th>Weight (g)</th>
<th>Cold exposure</th>
<th>Rewarming</th>
<th>Endpoint Trect (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Temperature (°C)</td>
<td>Time (min)</td>
<td>Temperature (°C)</td>
</tr>
<tr>
<td>Control</td>
<td>Killed immediately</td>
<td>15</td>
<td>334</td>
<td>22.0</td>
<td></td>
<td>37.2</td>
</tr>
<tr>
<td>MH1</td>
<td>2 h at RT</td>
<td>6</td>
<td>326</td>
<td>21.5</td>
<td>120</td>
<td>30.3</td>
</tr>
<tr>
<td>MH2</td>
<td>4.5 h at RT</td>
<td>15</td>
<td>336</td>
<td>22.7</td>
<td>270</td>
<td>30.0</td>
</tr>
<tr>
<td>SH1</td>
<td>2 h in cold room</td>
<td>15</td>
<td>329</td>
<td>10</td>
<td>120</td>
<td>23.8</td>
</tr>
<tr>
<td>SH2</td>
<td>in cold room until Trect &lt; 20 °C</td>
<td>15</td>
<td>333</td>
<td>10</td>
<td>277</td>
<td>19.9</td>
</tr>
<tr>
<td>SHRW1</td>
<td>2 h in cold room, 2 h at RT</td>
<td>15</td>
<td>332</td>
<td>10</td>
<td>120</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>120</td>
</tr>
<tr>
<td>SHRW2</td>
<td>2 h in cold room, 3 h in incubator</td>
<td>15</td>
<td>310</td>
<td>10</td>
<td>120</td>
<td>180</td>
</tr>
</tbody>
</table>

MH1–2, mild hypothermia 1–2; RT, room temperature; SH1–2, severe hypothermia 1–2; SHRW1–2, severe hypothermia and rewarming 1–2; Trect, rectal temperature

4.4 Sample analysis and measurements

All sample analyses and measurements were performed in the laboratory of the Department of Forensic Medicine, Institute of Diagnostics, University of Oulu, unless stated otherwise.
4.4.1 Catecholamines

Prior to November 2010, the isolation of urine catecholamines was carried out with aluminium oxide extraction after adjusting the pH to 1 with hydrochloric acid, and the measurements were done with high-pressure liquid chromatography with an electrochemical detector (Davidson & Fitzpatrick 1985) (I). Thereafter, the technique of analysing catecholamine concentrations in serum/plasma and urine was changed to an enzyme-linked immunosorbent assay (ELISA) kit (Cat Combi kit, DRG, Marburg, Germany), and the measurements were performed with a Multiscan EX microplate reader (Thermo Scientific, Waltham, MA, USA) (II–IV).

4.4.2 Thrombomodulin

Myocardial thrombomodulin mRNA expression

Total RNA was extracted from myocardial tissue with miRNeasy mini kit (Qiagen, Hilden, Germany) using automated QIAcube sample preparation instrument (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. RNA integrity score illustrating the quality of RNA was defined for the samples by the QIAxcel capillary electrophoresis system and RNA QC kit. High Capacity complementary DNA (cDNA) RT kit (Applied Biosystems, Foster City, CA, USA) was used to reverse-transcribe the RNAs with random primers according to the manufacturers’ protocols.

The cDNAs were amplified in duplicate with a Rotor-Gene 3000 (Corbett Life Science, Sydney, Australia) using gene-specific primers (Sigma, Haverhill, UK) and the Maxima SYBR Green qPCR Master Mix (Fermentas, Glen Burnie, MD, USA) real-time PCR system according to the manufacturers’ protocols. The cDNAs were diluted 1:20 for amplification of endogenous reference genes, amphiregulin (III) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (IV). The amplification was carried out as follows: 1 cycle for denaturation (+95 °C 10 min) followed by 40 cycles for three-stage PCR (THBD and amphiregulin: +95 °C 15 s, +57 °C 30 s, +72 °C 30 s; HSF1 and GAPDH: 95 °C 15 s, 60 °C 30 s, 72 °C 30 s). Fluorescence signals were measured continuously during repetitive cycles. THBD and HSF1 gene expressions in human and rat were normalised to reference genes GAPDH and amphiregulin, respectively, using the \(2^{-\Delta\Delta CT}\) method.
The primer sequences were: human *THBD*, forward 5′-TTC ACT TTT CCT CCC TCA GT-3′, reverse 5′-GTG CCA TCA CCA GAC AAT AGA-3′ (GeneBank Accession number J02973, amplicon size 126 bp); human *HSF1*, forward 5′-CAT GAA GCA TGA GAA TGA GGC T-3′, reverse 5′-ACT GCA CCA GTG AGA TCA GGA-3′ (GeneBank Accession number NM_005526.2, amplicon size 116 bp); human *GAPDH*, forward 5′-TGG AAG GAC TCA TGA CCA CA-3′, reverse 5′-CCA TCA CGC CAC AGT TT-3′ (GeneBank Accession number BC029618, amplicon size 85 bp); rat *THBD*, forward 5′-GAT CTC CAT TGC CAG CCT-3′, reverse 5′-CAC GTG CTG CAG TAC TAC CT-3′ (GeneBank Accession number NM_031771.2, amplicon size 140 bp); rat *HSF1*, forward 5′-CCA TGA AGC ACG AGA ACG AG-3′, reverse 5′-ACT GCA CCA GTG AGA TCA GGA-3′ (GeneBank Accession number XM_006241890.1, amplicon size 117 bp); rat *amphiregulin*, forward 5′-GTG CAT GCC ATT GCC TAG CTG A-3′, reverse 5′-TCA TTT CCG GTG TGG CTT GCC A-3′ (GeneBank Accession number NM_017123.1, amplicon size 78 bp).

Commercial human myocardial total RNA (Clontech, Mountain View, CA, USA) pooled from the normal hearts of three Caucasian males (ages between 30–39 years; trauma as the COD) was used as a reference sample in human *THBD* and *HSF1* analysis. The relative expressions were obtained by comparing the normalised expressions to that of *THBD* and *HSF1* in the commercial reference sample, which was given the value 1. In the rat study, the relative expression values were calculated by comparing all other normalised expression values to the mean normalised expression value of the control group rats, which was given the value 1.

**Myocardial thrombomodulin protein expression (immunohistochemistry)**

The paraffin-embedded myocardial tissue specimens were sectioned at a thickness of 5 μm. The sections were deparaffinised and rehydrated prior to automatic epitope retrieval by the PT-link system (DAKO, Glostrup, Denmark). Immunohistochemical staining was carried out in an automated instrument (DAKO, Autostainer Plus, Glostrup, Denmark) using En Vision (Peroxidase/DAP) detection system (Dako, Glostrup, Denmark) according to standard procedures and manufacturers’ instructions. Monoclonal antibody MCAG41 (mouse antihuman CD141; AbD Serotec, Raleigh, NC, USA) was used so as to detect TM protein at 1:30 dilution for the heart sections. The slides were counterstained with haematoxylin (Reagen, Toivala, Finland).
Soluble thrombomodulin

Soluble TM concentrations in plasma/serum and urine were measured with ELISA (human: Quantikine Human Thrombomodulin Immunoassay kit, R&D Systems, Inc., Minneapolis, MN, USA; rat: Usen Life Science Inc., Houston, TX, USA). The measurements were performed with a Multiscan EX microplate reader (Thermo Scientific, Waltham, MA, USA).

4.4.3 Toxicological analysis

Toxicological analyses were performed from autopsy samples (I, IV) as part of the routine autopsy protocol when it was deemed necessary for the medico-legal investigation. The analyses were carried out in the forensic toxicology unit of the Department of Forensic Medicine, Hjelt Institute, University of Helsinki.

4.5 Statistical analyses

The statistical analyses were carried out using the IBM SPSS version 21 (IBM, Armonk, NY, USA). Comparisons of median values between individual groups were made with Mann-Whitney non-parametric test, and the Benjamini-Hochberg procedure was used to control false discovery rate in multiple comparisons. Mean values in multiple group comparisons were analysed with one-way analysis of variance and Tukey’s test was used for post hoc analysis. Correlations were determined with Spearman’s correlation coefficient.
5 Results

5.1 Catecholamines in cold exposure and hypothermia (I–IV)

The catecholamine concentrations in urine were significantly higher in hypothermia deaths than in other causes of death. The results presented here include all cases in studies I and IV combined using the grouping of the latter. Changes were also observed in the cold exposure studies of living subjects.

5.1.1 Adrenaline

Median (range) urinary adrenaline concentration was 61.0 ng/ml (0.0–655.0 ng/ml) and 30.0 ng/ml (0.0–346.0 ng/ml) when hypothermia had been the main and a contributory COD, respectively. The values were significantly higher compared with deaths from CVDs, traumas and other causes of death (Fig. 5).

In rats, the highest urinary adrenaline concentrations were seen after severe hypothermia and rewarming to normothermia. Mild hypothermia did not affect urinary adrenaline levels markedly. In human subjects, however, the post-immersion urinary adrenaline level was higher at +10 °C than +30 °C exposure.

5.1.2 Noradrenaline

The urinary noradrenaline levels were generally higher than the corresponding adrenaline levels. The median plasma noradrenaline level was increased in human subjects after immersion to +10 °C compared with +30 °C. The noradrenaline levels showed no clear responses to hypothermia in the rat subjects, and there were no significant differences between different causes of death.

5.1.3 Adrenaline-to-noradrenaline ratio

Proportionally greater differences were seen in the adrenaline than noradrenaline concentrations in the cadaver study. The median ANR levels were higher in hypothermia deaths — main cause, 0.33 (0.00–3.89); contributory cause, 0.21 (0.00–2.04) — than in the control groups (Fig. 6).
5.2 Cold-induced changes in thrombomodulin levels

5.2.1 Myocardial thrombomodulin mRNA and protein levels (III–IV)

Severe hypothermia increased the mean THBD mRNA level 2.6-fold compared with the control group in the rat study (III). Mild hypothermia did not cause any significant changes in the expression levels. Also, the rewarmed groups showed a comparable expression level with the control group. All hypothermia deaths (IV) showed a significantly lower median expression level compared with the control groups altogether (p = 0.007), but there were no significant differences between individual groups (Fig. 7).
Fig. 6. Median urinary adrenaline-to-noradrenaline ratio (ANR) in different causes of death (COD). Total n = 476. Error bars represent 95% confidence interval. *** p < 0.001 compared with hypothermia as main COD; ¤¤¤ p < 0.001 compared with hypothermia as contributory COD; # p < 0.05 compared with cardiovascular disease (CVD) deaths.

Positive myocardial immunohistochemical staining of TM protein was seen in all causes of death (Fig. 8). However, the expression levels (Table 4) were significantly lower when hypothermia was the main COD (IV) than in the control groups. In rats, mild or severe hypothermia did not cause significant changes compared with the controls. Rewarming caused, however, a transient increase in the TM protein level in the first rewarming group, followed by a decrease in further rewarming.
Fig. 7. Median relative thrombomodulin (THBD) mRNA levels in different causes of death (COD). Total n = 79. Error bars represent 95% confidence interval. CVD, cardiovascular disease.

Table 4. Mean grades of immunohistochemical staining (scale 1–4) for human myocardial thrombomodulin protein (total n = 96).

<table>
<thead>
<tr>
<th>Cause of death</th>
<th>Small capillaries</th>
<th>Arterioles/venules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothermia as main cause</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Hypothermia as a contributory cause</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>2.1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Trauma</td>
<td>2.4&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.6&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Other</td>
<td>2.3&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2.3&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> p < 0.001, <sup>2</sup> p = 0.001 compared with hypothermia as main cause
Fig. 8. Human myocardial samples showing positive immunohistochemical staining of thrombomodulin protein in small capillaries (#) and arterioles/venules (¤). The grade of staining is more intensive (+++) in the sample taken from a cardiovascular disease death (a) whereas only minor staining (+) can be observed in the sample from hypothermia death (b). Original magnification was 100x.

5.2.2 Circulation (II–IV)

The median (range) pre-immersion TM concentration in the plasma of healthy human subjects (II) was 4.54 ng/ml (3.34–6.41 ng/ml). In the rat study (III), a significantly lower plasma TM level was observed after severe hypothermia (SH1) compared with mild hypothermia (MH1). A higher level was obtained in further exposure (SH2), while the differences levelled off in the rewarming groups.

In hypothermia deaths (IV), the median (range) TM concentration in post-mortem serum was 11.8 ng/ml (0.4–36.1 ng/ml) when the cases with hypothermia as a contributory COD were included. Deaths from CVD, traumas and other
causes had a higher median TM level of 22.2 ng/ml (1.1–16.4 ng/ml), but the difference was not statistically significant (Fig. 9).

Fig. 9. Median thrombomodulin (TM) concentrations in post-mortem serum (n = 56) and urine (n = 129) in different causes of death (COD). Error bars represent 95% confidence interval. * p < 0.05, *** p < 0.001 compared with cardiovascular disease (CVD) deaths.

5.2.3 Urine (II–IV)

The median (range) pre-immersion urinary TM concentration of healthy human subjects (II) was 15.97 ng/ml (1.05–32.04 ng/ml). Severe hypothermia (III) increased the TM level significantly compared with mild hypothermia (MH1) in rats. Increased levels were also observable in the rewarmed groups.

Hypothermia deaths (IV) had a lower (p < 0.001) median urinary TM level (11.8 ng/ml, 4.4–36.1 ng/ml) than the control groups (24.5 ng/ml, 1.8–134.1
ng/ml). The CVD deaths group had the highest median TM level in urine, and the differences were statistically significant in all comparisons (Fig. 9).

5.3 Thrombomodulin and catecholamines as hypothermia indicators

5.3.1 Temperature data (II–III)

The TM level in plasma correlated negatively ($\rho = -0.570$, $p = 0.002$) with the endpoint $T_{\text{rect}}$ in rats exposed to severe hypothermia (SH1 and SH2). Furthermore, the myocardial TM protein level correlated negatively ($\rho = -0.670$, $p < 0.001$) and urinary adrenaline level positively ($\rho = 0.750$, $p < 0.001$) with the endpoint body temperature in the rewarming groups. There were no significant correlations between temperature and other parameters in the short-term cold exposure data (II).

5.3.2 Post-mortem interval (I, IV)

The median PMIs were 4 (0–195) days (I) and 3 (0–24) days (IV). There was no correlation between PMI and urinary catecholamine or TM levels. The TM concentration in the post-mortem serum correlated, however, weakly with PMI ($\rho = 0.349$, $p = 0.008$)

5.3.3 Sensitivity and specificity (I, IV)

In order to determine the sensitivity and specificity of the catecholamine and TM assays to detect hypothermia deaths, cut-off values were determined. In the catecholamine study (I), the median levels of the group with suspected hypothermia deaths were selected as cut-off values. An optimal cut-off level was determined for the TM assay with a receiver operating characteristics curve. For urinary TM, the area under curve was at a fair level, 0.727 ($p < 0.001$). The cut-off values as well as the sensitivity and specificity of each assay are presented in Table 5 together with data from previously published studies.
Table 5. The sensitivity and specificity of proposed hypothermia death indicators.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Cut-off value for hypothermia</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Maximum PMI (d)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine thrombomodulin</td>
<td>≤ 15.5 ng/ml</td>
<td>70.8</td>
<td>70.3</td>
<td>24</td>
<td>IV</td>
</tr>
<tr>
<td>Adrenaline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>&gt; 20 ng/ml</td>
<td>73.7</td>
<td>65.8</td>
<td>195</td>
<td>I</td>
</tr>
<tr>
<td>Serum</td>
<td>&lt; 100 ng/ml</td>
<td>68</td>
<td>83</td>
<td>&lt; 2</td>
<td>Zhu et al. 2007a</td>
</tr>
<tr>
<td>PCF</td>
<td>&lt; 20 ng/ml</td>
<td>60</td>
<td>79</td>
<td>2</td>
<td>Ishikawa et al. 2013</td>
</tr>
<tr>
<td>CSF</td>
<td>&lt; 2 ng/ml</td>
<td>55</td>
<td>90</td>
<td>2</td>
<td>Ishikawa et al. 2013</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>&gt; 105 ng/ml</td>
<td>76.3</td>
<td>30.3</td>
<td>195</td>
<td>I</td>
</tr>
<tr>
<td>Serum</td>
<td>&gt; 0.5 ng/ml</td>
<td>60</td>
<td>75</td>
<td>10</td>
<td>Ishikawa et al. 2014</td>
</tr>
<tr>
<td>PCF</td>
<td>&lt; 125 ng/ml</td>
<td>60</td>
<td>85</td>
<td>2</td>
<td>Ishikawa et al. 2013</td>
</tr>
<tr>
<td>CSF</td>
<td>&lt; 20 ng/ml</td>
<td>80</td>
<td>83</td>
<td>2</td>
<td>Ishikawa et al. 2013</td>
</tr>
<tr>
<td>Urine ANR</td>
<td>&gt; 0.19</td>
<td>68.9</td>
<td>78.1</td>
<td>195</td>
<td>I</td>
</tr>
<tr>
<td>Serum ACTH</td>
<td>&gt; 0.01 ng/ml</td>
<td>69</td>
<td>74</td>
<td>~3.5</td>
<td>Ishikawa et al. 2008</td>
</tr>
<tr>
<td>CSF ACTH</td>
<td>&lt; 0.1 ng/ml</td>
<td>84</td>
<td>93</td>
<td>~3.5</td>
<td>Ishikawa et al. 2008</td>
</tr>
<tr>
<td>Serum TSH</td>
<td>&lt; 4 μU/ml</td>
<td>58</td>
<td>68</td>
<td>2</td>
<td>Ishikawa et al. 2009</td>
</tr>
<tr>
<td>CSF TSH</td>
<td>&lt; 10 μU/ml</td>
<td>75</td>
<td>79</td>
<td>2</td>
<td>Ishikawa et al. 2009</td>
</tr>
<tr>
<td>Serum GH</td>
<td>&gt; 7 ng/ml</td>
<td>69</td>
<td>74</td>
<td>2</td>
<td>Ishikawa et al. 2011</td>
</tr>
<tr>
<td>Serum CgA</td>
<td>&lt; 20 pmol/ml</td>
<td>81</td>
<td>100</td>
<td>&lt; 3</td>
<td>Yoshida et al. 2011</td>
</tr>
<tr>
<td>CSF CgA</td>
<td>&gt; 10 pmol/ml</td>
<td>90</td>
<td>100</td>
<td>&lt; 3</td>
<td>Yoshida et al. 2011</td>
</tr>
</tbody>
</table>

1 Not exclusively hypothermia deaths; ACTH, adrenocorticotropic hormone; ANR, adrenaline-to-noradrenaline ratio; CgA, chromogranin A; CSF, cerebrospinal fluid; GH, growth hormone; PCF, pericardial fluid; PMI, post-mortem interval; TSH, thyroid-stimulating hormone

5.3.4 Correlations between thrombomodulin and catecholamines (II–IV)

Significant correlations were found between catecholamine and TM levels in both living subjects (II–III) and post-mortem examination (IV). In rats exposed to severe hypothermia (SH1 and SH2), high adrenaline concentrations in plasma were associated with low myocardial THBD transcript levels. A similar negative correlation was also seen regarding urinary noradrenaline in the hypothermia deaths ($\rho = -0.707$, $p < 0.001$). In the severely hypothermic rat groups, both the circulating and urinary adrenaline concentration correlated with the respective TM concentration (urine: $\rho = 0.650$, $p = 0.001$; plasma: $\rho = 0.610$, $p = 0.026$). In
human subjects, strong positive correlations were detected in urinary adrenaline and TM ($\rho = 0.806$, $p < 0.001$) as well as between noradrenaline and TM ($\rho = 0.760$, $p < 0.001$). Similar correlations in the urinary assays were observed in hypothermia deaths (adrenaline – TM, $\rho = 0.539$, $p < 0.001$; noradrenaline – TM, $\rho = 0.735$, $p < 0.001$). When the CVD deaths were excluded, these correlations, although weaker, were seen in the whole cadaver data (adrenaline – TM, $\rho = 0.353$, $p = 0.001$; noradrenaline – TM, $\rho = 0.627$, $p < 0.001$).

5.4 Other observations

5.4.1 Heat shock factor 1

The myocardial $HSF1$ transcript level increased 1.9-fold in rats exposed to severe hypothermia compared with the control group (III). The mean relative $HSF1$ transcript levels were significantly higher in the severe hypothermia than the mild hypothermia groups (SH1 vs. MH1, $p < 0.001$; SH2 vs. MH2, $p = 0.007$). Furthermore, the myocardial $HSF1$ and $THBD$ transcript levels correlated strongly ($\rho = 0.830$, $p < 0.001$) in the severe hypothermia groups. In the autopsy material (IV), there were no significant differences in the $HSF1$ transcript levels between different causes of death.

5.4.2 Ethanol and medications

The use of alcohol was considered a contributory COD more frequently in hypothermia deaths than in the other groups (I). The ethanol levels measured from blood had moderate negative correlations with the urinary TM concentration and the myocardial TM protein levels (IV).

The results of the toxicological analyses are presented in Table 6 (IV). Substances affecting haemostasis, such as anticoagulants and NSAIDs, were detected in 11 cases (6.2% of those included in the toxicological analysis) of the cadaver material involved in the TM study. These cases did not have any significance in the results. Statins and ACE inhibitors were not detected in the material.
### Table 6. The results of the toxicological analyses (IV).

<table>
<thead>
<tr>
<th>Substance group</th>
<th>Hypothermia main COD n (%)</th>
<th>Hypothermia contributory COD n (%)</th>
<th>CVD deaths n (%)</th>
<th>Trauma main COD n (%)</th>
<th>Other causes n (%)</th>
<th>All groups n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>36 (54.5)</td>
<td>11 (50.0)</td>
<td>21 (72.4)</td>
<td>31 (79.5)</td>
<td>12 (57.1)</td>
<td>111 (62.7)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>27 (40.9)</td>
<td>12 (54.5)</td>
<td>6 (20.7)</td>
<td>16 (41.0)</td>
<td>9 (42.9)</td>
<td>70 (39.5)</td>
</tr>
<tr>
<td>BZDs and their derivatives</td>
<td>18 (27.3)</td>
<td>4 (18.2)</td>
<td>4 (13.8)</td>
<td>8 (20.5)</td>
<td>8 (38.1)</td>
<td>42 (23.7)</td>
</tr>
<tr>
<td>Antidepressants</td>
<td>7 (10.6)</td>
<td>3 (13.6)</td>
<td>6 (20.7)</td>
<td>4 (10.3)</td>
<td>1 (4.8)</td>
<td>24 (13.6)</td>
</tr>
<tr>
<td>Beta blockers</td>
<td>10 (15.2)</td>
<td>3 (13.6)</td>
<td>6 (20.7)</td>
<td>4 (10.3)</td>
<td>1 (4.8)</td>
<td>24 (13.6)</td>
</tr>
<tr>
<td>Antipsychotics</td>
<td>7 (10.6)</td>
<td>1 (4.5)</td>
<td>3 (10.3)</td>
<td>1 (2.6)</td>
<td>7 (33.3)</td>
<td>19 (10.7)</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>3 (4.5)</td>
<td>3 (13.6)</td>
<td>3 (10.3)</td>
<td>2 (5.1)</td>
<td>1 (4.8)</td>
<td>12 (6.8)</td>
</tr>
<tr>
<td>NSAIDs²</td>
<td>3 (4.5)</td>
<td>0 (0.0)</td>
<td>4 (13.8)</td>
<td>1 (2.6)</td>
<td>2 (9.5)</td>
<td>10 (5.6)</td>
</tr>
<tr>
<td>Opioids</td>
<td>3 (4.5)</td>
<td>1 (4.5)</td>
<td>1 (3.4)</td>
<td>1 (2.6)</td>
<td>3 (14.3)</td>
<td>9 (5.1)</td>
</tr>
<tr>
<td>Tricyclic antidepressants</td>
<td>4 (6.1)</td>
<td>2 (9.1)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>3 (14.3)</td>
<td>9 (5.1)</td>
</tr>
<tr>
<td>Anthypertensive and cardiac drugs</td>
<td>1 (1.5)</td>
<td>2 (9.1)</td>
<td>3 (10.3)</td>
<td>1 (2.6)</td>
<td>0 (0.0)</td>
<td>7 (4.0)</td>
</tr>
<tr>
<td>Antiepileptics</td>
<td>1 (1.5)</td>
<td>0 (0.0)</td>
<td>3 (10.3)</td>
<td>0 (0.0)</td>
<td>1 (4.8)</td>
<td>5 (2.8)</td>
</tr>
<tr>
<td>Anticoagulants</td>
<td>2 (3.0)</td>
<td>0 (0.0)</td>
<td>2 (6.9)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>4 (2.3)</td>
</tr>
<tr>
<td>Illicit drugs</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (2.6)</td>
<td>1 (4.8)</td>
<td>2 (1.1)</td>
</tr>
<tr>
<td>Other</td>
<td>14 (21.2)</td>
<td>5 (22.7)</td>
<td>2 (6.9)</td>
<td>1 (2.6)</td>
<td>3 (14.3)</td>
<td>25 (14.1)</td>
</tr>
</tbody>
</table>

1 excluding tricyclic antidepressants, 2 including salicylates, 3 excluding beta blockers, 4 excluding opioids; BZD, benzodiazepines; COD, cause of death; NSAID, non-steroidal anti-inflammatory drug

### 5.4.3 Wischnewski’s spots

The frequency of Wischnewski’s spots was significantly higher (p < 0.001) among the hypothermia deaths than the other groups studied (I). The sensitivity of detecting hypothermia deaths based on the presence of these spots was 63.9%, and the specificity was 88.3%.
6 Discussion

6.1 Hypothermia-induced changes in thrombomodulin and catecholamine levels

A short-term cold exposure or mild hypothermia seemed to have no significant effects on the TM levels, which is supported by previous findings (Matsuda et al. 1992). Severe hypothermia, however, was shown to cause a transient decrease in the circulating TM level and an increase in the urinary TM level in healthy living rats, while changes in the opposite directions took place in prolonged exposure. A delayed increase in the circulating TM concentration has also been shown about two hours after the start of induced hypothermia (Boldt et al. 1996). These findings could be confirmed in the cadaver material, where a severe and most likely prolonged hypothermia was indeed present.

At cell level, THBD mRNA and TM protein expression levels were low in hypothermia deaths. The low transcript level could be considered to represent an end-stage where all cellular activities eventually cease. This conclusion is supported by the rat study data where transcriptional activation was seen in severe hypothermia, but prolonged exposure was rather associated with suppressed expression levels. Rewarming increased myocardial TM protein levels in rats after a 4-hour period. A similar reaction has been seen after a 24-hour recovery period (Rücker et al. 2009), suggesting a compensatory mechanism. These changes could not be confirmed in the cadaver material as there was no recovery period present. The observed TM protein levels were, however, lower compared with other causes of death, which could be related to the supposed increased need for TM in the circulation.

Previous findings about the influence of cold stress on catecholamine levels were confirmed in the present study. Circulating noradrenaline level reacted even to short-term cold exposure. Possibly because of this, there were no clear differences in the noradrenaline levels in different rat groups or CODs. The adrenaline levels in urine were, however, higher in severely hypothermic rats and hypothermia deaths, reflecting the more severe exposure needed for adrenomedullary activation (Frank et al. 2002). Interestingly, a short-term cold exposure without hypothermia was associated with higher urinary adrenaline levels in the living subjects during a follow-up period. This might be explained by a compensatory mechanism triggered by the exposure. An increased ANR level
was confirmed to be related to lethal hypothermia, as originally proposed by Hirvonen & Huttunen (1982). The urinary ANR levels of hypothermia deaths (0.33 and 0.21 for hypothermia as main and contributory COD, respectively) in this study were comparable to those reported for deaths with a long agonal period, 0.21 (Wilke et al. 2007). A similar ratio (0.21, based on the average reported concentrations) has also been reported induced by long-distance swimming in ice-cold water (Noakes et al. 2009), perhaps giving some idea of the stress level related to this activity.

**Possible mechanisms linking hypothermia and thrombomodulin**

As reviewed previously, TM is mainly considered to act as an anticoagulant via activating PC (Esmon & Owen 1981). However, TM also exhibits procoagulant properties via the activation of TAFI (Bajzar et al. 1998). As for cold exposure and hypothermia, alterations to coagulation potential in both ways have been described. Decrease in body temperature has been shown to impair blood coagulation (Rajagopalan et al. 2008) and enhance bleeding (Heinius et al. 2011). Suggested explanations have been the inhibition of enzymatic reactions (Rohrer & Natale 1992) and dysfunction in thrombocyte activation (Valeri et al. 1987). On the other hand, surface cooling enhances coagulative potential also in healthy subjects by increasing blood viscosity (Keatinge et al. 1984) as well as thrombin-antithrombin III complex concentration (Nagelkirk et al. 2012), and APC resistance (Mercer et al. 1999). Moreover, cold has been shown to shorten the coagulation time of blood in the presence of endotoxins (Ferraro et al. 1992), possibly contributing to the development of DIC.

Based on these observations, it is difficult to draw firm conclusions about the exact role of TM in the hypothermic state. It is obvious, however, that hypothermia causes changes both in the blood coagulation and TM levels. Thus, maintaining the delicate balance in coagulation provides a plausible link between hypothermia and the altered TM levels. Nonetheless, it cannot be concluded with certainty that the hypothermia-induced changes in TM expression and secretion are purely physiological.

**Thrombomodulin and catecholamines**

Strong correlations were seen between TM and catecholamine levels throughout the data in both living subjects and cadaver material. High adrenaline levels in
plasma were connected to low myocardial $THBD$ transcript levels in rats. Also, positive correlations were observed between catecholamine and TM levels in plasma and urine. Hence, it would be reasonable to assume that these parameters are interrelated, possibly by a regulatory relationship.

Similar findings have been reported in settings not related to hypothermia or cold exposure (Johansson et al. 2012). Interestingly, adrenaline administration has been shown to exert a strong anticoagulative effect during endotoxaemia (van der Poll et al. 1997). This could, at least partly, be mediated through TM, as septicaemia is also known to increase TM levels in plasma (Boldt et al. 1995).

A possible mechanism could be through HSF1 during hypothermia, as there was a clear inverse correlation between plasma adrenaline concentration and myocardial $HSF1$ transcript levels in rats exposed to severe hypothermia. Additionally, both $HSF1$ and $THBD$ transcript levels were high in this group. Multiple binding sites for heat shock factors have been found in the $THBD$ gene (Conway et al. 1994, Fu et al. 2008), suggesting that cold and heat stress-related transcriptional changes are possible. In the cadaver material, the relation to HSF1 could not be demonstrated. This might be because the time window for the peak activation of HSF1 has been passed in the prolonged exposure in lethal hypothermia. The lack of significant differences in $HSF1$ levels between the study groups could also be explained by an increased expression of $HSF1$ in atherosclerotic lesions (Metzler et al. 2003), which were present in the majority of the CVD deaths group. The myocardial $HSF1$ levels have not been studied in different causes of death. However, there were no clear differences in the glomerular expression of $HSF1$ in deaths that had occurred in different ambient temperatures (Sakurada et al. 2013).

The $THBD$ gene is also known to contain a region responsive to cyclic adenosine monophosphate (cAMP) (Tazawa et al. 1994), and up-regulation of $THBD$ expression by cAMP treatment has been demonstrated (Hirokawa & Aoki 1990, Sunagawa et al. 2006). Adrenaline increases the cAMP level in myocardial cells (Triposkiadis et al. 2009), which would lead to increased $THBD$ transcript levels. The regulatory mechanisms are, however, probably modified during hypothermia, demonstrated by the negative correlations between catecholamine and $THBD$ expression levels in this study.
6.2 Suitability of thrombomodulin as a hypothermia indicator

To assess the usability of a biochemical substance as an indicator in medico-legal investigation, several aspects need to be taken into account. Such things concern the stability of the studied substance in post-mortem samples, unambiguity as for the interpretation of the results and the effects of confounding factors, among other things.

6.2.1 Demands of an optimal indicator

The most important attributes of a post-mortem hypothermia indicator would be high specificity for lethal hypothermia and high sensitivity to detecting these cases in a focused study population, that is, in cases where exposure to a cold environment is suspected in the light of the circumstances. A good correlation of the substance level to the body temperature at the occurrence of death would be extremely valuable to truly estimate the significance of hypothermia in the death process.

Further demands of a biochemical indicator concern the reliability of the analytical procedures to provide accurate results. The longer the PMI is, the further the autolysis of tissues as well as the bacterial and fungal growths progress. Additionally, the outer circumstances may cause the tissues to cool down, freeze, thaw, dampen or become exposed to other destructive forces. The indicator should remain stable regardless of the sample quality in order to provide comparable results.

6.2.2 Comparing thrombomodulin with catecholamines and other suggested indicators

Diagnostic power

The specificity of urinary TM concentration with a cut-off point at 15.5 ng/ml was at a reasonable level, 70.8%, while the sensitivity of this assay was 70.3%. Positive likelihood ratio (LR+) for a low urinary TM concentration was thus 2.4 and negative likelihood ratio (LR−) 0.4. The corresponding figures for the best catecholamine assay, ANR, were: sensitivity, 68.9%; specificity, 78.1%; LR+, 3.1; LR−, 0.4.
The pre-test odds and probability of hypothermia deaths depend on the prevalence in the population examined. The occurrence of hypothermia deaths is quite low (less than 100 per year, Fig. 2) in relation to the overall causes of death (approximately 50,000 deaths per year) in the Finnish population. Hypothermia deaths are relatively infrequent even when examining the deaths where a medico-legal autopsy takes place (approximately 10,000–12,000 deaths per year). However, the population of suspected hypothermia deaths is far more limited, and the prevalence of hypothermia deaths much higher than in the general population in this regard. Thus, if the pre-test probability of a hypothermia death in this focused population is assumed to be 50.0%, for instance, the odds would be 1.0. If the measured urinary TM was below 15.5 ng/ml, and the ANR was above 0.19, the post-test odds would then be 1.0 x 2.4 x 3.1 = 7.4. Hence, the post-test probability of the death being from hypothermia would be 88.2%. Although the exact prevalence of hypothermia deaths in this regard is difficult to estimate, it is evident that the diagnostic power of the studied assays is reasonably good.

Comparing the diagnostic power of different hypothermia assays with each other is challenging. In most published studies, the sample sizes have been limited, the control cases heterogeneous and no characteristic figures have been provided. In terms of sensitivity and specificity, urinary ANR and TM assays are on a par with most of the other indicators studied (Table 5).

An additional problem in the assay comparisons is that most studies have focused only on a selected indicator. However, a comprehensive study including several previously proposed indicators was carried out by Palmiere et al. (2013). The most significant differences between hypothermia and control cases, including mostly deaths from traumas and intoxication, were seen in the levels of blood ketones and IPA, urine adrenaline, serum cortisol, and urine free cortisol (Palmiere et al. 2013). However, no cut-off values were given for any of the parameters.

**Stability**

The stability of a biochemical indicator is an important factor. The PMI may sometimes be very long in cases where hypothermia is suspected to be the COD. Furthermore, freezing and thawing of tissues is often present. These post-mortem changes may cause increases or decreases in the concentrations of the examined substance, and cause false positive or negative findings.
A weak correlation was seen between PMI and TM concentration in post-mortem serum. This finding is probably explained by the autolytic changes in endothelial cells (Maruyama et al. 1985) and thrombocytes (Suzuki et al. 1988). TM is also found in the urothelium (Obama et al. 1999), which could cause changes in the urinary TM concentrations as well. However, no such correlation was seen in the urinary assay with PMIs up to 24 days (median 3 days), suggesting that the urinary TM concentration is more resistant to post-mortem changes.

Catecholamine concentrations also remained stable in urine with PMIs up to 195 days (median 4 days), confirming the results of a previous animal study (Lapinlampi & Hirvonen 1986). Some post-mortem degradation may occur, but cooling markedly slows down the process (Willemsen et al. 2007). In post-mortem serum, the catecholamine levels are prone to increase with time due to diffusion from sympathetic nerve endings and adrenal glands (Hirvonen & Huttunen 1996), at least after a few days (Berg & Bonte 1973). Additionally, there seems to be great variation in the catecholamine concentrations, depending on the sampling site (Zhu et al. 2007a).

A major problem in many hypothermia indicator studies is that the PMIs of the cases included are too short to allow any conclusions about the stability of the substance (Table 5). As an exception, the level of 3HB was not markedly affected by post-mortem decomposition with PMIs up to 14 days (Iten & Meier 2000). Additionally, the 3HB levels can be analysed from different body fluids with comparable results (Palmiere et al. 2014a, Palmiere & Werner 2014). It would, thus, be useful in cases where certain fluids or tissues may not be available because of putrefaction, for instance.

**Alcohol and medication**

The use of ethanol was involved in the majority of hypothermia deaths. Ethanol intoxication impairs thermoregulatory mechanisms (Kalant & Lê 1983), and may alter the biochemical responses to hypothermia. These influences are most evident in the case of 3HB. As previously pointed out, ethanol in blood hinders the metabolism of ketone bodies, and thus normal levels of 3HB cannot be considered as evidence of hypothermia in these cases (Palmiere et al. 2014b). Ethanol levels in blood and urine correlated negatively with urinary soluble TM and myocardial TM protein levels. Low levels of TM in connection with high ethanol levels may thus be inconclusive regarding hypothermia. In blood, there
were no correlations between TM and ethanol levels, and plasma soluble TM concentration has been shown to be unaffected by the intake of approximately three doses of ethanol in short time (Blann et al. 2002). Urinary catecholamine concentrations were unaffected by ethanol levels in the present study. Acute and chronic use of ethanol is known to affect the circulating catecholamine levels (Patel & Pohorecky 1989), and low urinary catecholamine concentrations have been associated with high ethanol levels in some hypothermia deaths (Hirvonen 1976, Hirvonen & Huttunen 1982). Yet, the urinary catecholamine assay is considered quite reliable despite the presence of alcohol (Palmiere et al. 2014b).

Many medications and drugs affect thermoregulation heavily even at therapeutic levels, as reviewed previously. The influences of these substances on the postulated hypothermia indicators have, however, received little attention. It is nearly impossible to estimate the effect of an individual substance or its metabolite on the indicator studied in the post-mortem setting. As with ethanol, it is likely that the multitude of pharmacological agents found in the toxicological analyses cause some ambiguity with the biochemical assays, and their influence on the results should be carefully considered on a case-specific basis.

TM expression is enhanced by statins (Undas et al. 2005). Some ACE inhibitors may also alter TM levels (Boldt et al. 1998). These, as well as medications affecting haemostasis, are frequently used by people with CVDs. Though infrequent in the study population, these substances must be considered when interpreting the results of the TM assays.

**Body temperature and rewarming**

It is impossible to retrieve accurate temperature data in the cadaver studies. In the living subjects, however, the body temperature and the ambient temperature were continually monitored. The most significant correlation with endpoint $T_{rect}$ was found in the plasma TM concentration in the rats exposed to severe hypothermia. In the rewarming groups, the urinary adrenaline concentration showed a significant correlation, suggesting its potential as a marker for hypothermia followed by rewarming. This information may be valuable in examining deaths where the outer circumstances have changed during the death process, or when examining a hypothermia victim after hospitalisation.
6.3 Strengths and limitations of this study

The TM and catecholamine analyses were studied in living subjects and autopsy material. The experimental protocols included different exposure temperatures from physiological to life-threatening, and lethal circumstances to acquire a broad understanding of the changes observed. The autopsy material collected is larger than those used in most previously published studies especially concerning the number of hypothermia deaths.

The catecholamine measurements were included as part of the medico-legal investigation in some autopsy cases. Thus they may have had influence on the diagnoses affecting the grouping in these cases.
7 Conclusions

The influences of cold exposure and hypothermia on the expression and secretion of TM and catecholamines were studied. Based on the results and in the light of literature, the following conclusions can be made.

- Severe hypothermia increases myocardial THBD expression, followed by a decrease in the lethal stage.
- The circulating TM level is transiently decreased in severe hypothermia.
- The myocardial and urinary TM protein levels are decreased during hypothermia.
- Hypothermia increases the urinary adrenaline level, especially after rewarming.
- High urinary ANR levels are associated with hypothermia deaths.
- Significant correlations exist between TM and catecholamine levels.

These conclusions suggest that controlling TM expression and secretion during cold stress and hypothermia is important, possibly related to the regulation of haemostasis, inflammation and other vital processes, threatened by the dangerous environmental circumstances. These observations could be important in developing new forms of therapy involving the use of hypothermia, and in improving the treatment of hypothermic patients.

Adrenaline and noradrenaline, as stress hormones, seem to play an important role in governing TM expression and secretion during hypothermia, possibly via HSF1. Further studies should be conducted to reveal the exact mechanisms between catecholamines and the regulation of TM on cell level.

In the medico-legal point of view, this study proved on a larger scale that measuring the urinary catecholamine concentrations is useful in examining possible victims of lethal hypothermia. A high ANR level can be considered suggestive of hypothermia in the post-mortem examination. Decreased levels of TM in urine and in the myocardium provide further evidence of ante-mortem cold stress.
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