Immobilization as a risk factor for arterial and venous thrombosis
To my dear parents, Lena and Jörgen Arinell

"Kunskap är ingen börd att bära."
Mormor
Immobilization as a risk factor for arterial and venous thrombosis
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Abstract


Aim: Immobilization and a sedentary lifestyle are correlated with an elevated risk of both arterial and venous thrombosis. The goal of this research was to investigate whether markers associated with cardiovascular disease risk are altered during long term immobilization in a human model and in the brown bear, which survives annual cycles of long-term immobilization.

Methods: In study populations assigned to 20-60 days of strict head-down-tilt bed rest 24h a day, we analysed blood levels of the emerging cardiovascular disease marker cystatin C, soluble markers of in vivo platelet activation P-selectin and PDGF-BB, and platelet aggregation. Blood samples were taken from free-ranging brown bears in summer and again during hibernation for analysis of lipid profile and platelet aggregation. Histological examination was performed on the left anterior descending coronary artery and aortic arches of bears harvested during the hunting season.

Results: During prolonged bed rest in humans, levels of cystatin C and platelet aggregation remained unchanged, but we observed a significant decrease in platelet activation markers. Brown bear plasma lipids were elevated during hibernation compared with the active state and cholesterol levels were generally considerably higher than normal human values. The arterial specimens showed no signs of atherosclerosis. Platelet aggregation was halved during hibernation compared to the active state.

Conclusions: Long-term immobilization has effects on several cardiovascular risk factors in both humans and bears. Increased knowledge and understanding of the protective mechanisms that allows the brown bear to survive repeated periods of immobilization could contribute to new strategies for prevention and treatment of cardiovascular disease in humans.

Keywords: venous thrombosis, arterial thrombosis, atherosclerosis, cystatin C, cholesterol, platelet activation, platelet aggregation, immobilization

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This thesis is based on the following publications:

I. Arinell K, Christensen K, Blanc S, Larsson A, Fröbert O.

II. Arinell K, Sahdo B, Evans AL, Arnemo JM, Baandrup U, Fröbert O.

III. Arinell K, Fröbert O, Blanc S, Larsson A, Christensen K.


Roman numerals are used throughout the text to reference these studies. Reprints were made with permission of the publishers.
Additional publications not included in this thesis
**Abbreviations**

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<th>Full Form</th>
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<tbody>
<tr>
<td>ACS</td>
<td>Acute coronary syndrome</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMI</td>
<td>Acute myocardial infarction</td>
</tr>
<tr>
<td>APTT</td>
<td>Activated partial thromboplastin time</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep vein thrombosis</td>
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<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PT</td>
<td>Prothrombin time</td>
</tr>
<tr>
<td>STEMI</td>
<td>ST-elevation myocardial infarction</td>
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<tr>
<td>VTE</td>
<td>Venous thromboembolism</td>
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</table>
Introduction

Epidemiology
Worldwide, approximately 60 million people die annually, about 30% from causes related to cardiovascular disease (CVD). In 2015, 34,780 cases of acute coronary syndrome in persons ≥20 years were recorded in Sweden. Within 28 days following diagnosis, 23.5% of men and 28% of women had died (1).

Venous thromboembolism (VTE) includes deep vein thrombosis (DVT) and pulmonary embolism. It is the third most prevalent cardiovascular disease in Sweden, with an overall annual incidence of 100–200 per 100,000 population (2, 3). Approximately one third of individuals with symptomatic VTE manifest pulmonary embolism, while the remainder experience DVT only. Six percent of DVT cases and twelve percent of pulmonary embolism cases have a fatal outcome within one month of diagnosis (4). Coronary artery disease and VTE may be fatal in the acute phase or lead to chronic disease and disability, but they are largely preventable.

The shift from acute communicable disease to chronic degenerative disease as the leading cause of death has been described as an epidemiological transition (5). The health, economic, and social consequences of CVD are extensive, but the disease is a preventable and treatable condition, and the contributing factors need to be more completely identified and addressed.

Sedentary lifestyle and immobilization as a risk factor for venous and arterial thrombosis
Bed rest is probably the single most frequently applied therapy for hospitalized patients regardless of the underlying condition. In the 1930s, bed rest of at least 6 weeks was a cornerstone of therapy for patients with myocardial infarction (6). The goal was to reduce myocardial workload, avoid additional perfusion injury to the myocardium, and minimize remodelling (7).

The period of confinement to bed has been greatly reduced over the years, but adequate clinical trials to determine an optimal duration of bed rest following acute myocardial infarct (AMI) are lacking. The American Heart Association (AHA) scientific statement on cardiac rehabilitation and secondary prevention of coronary heart disease advises that patients with uncomplicated AMI should not be confined to bed rest for more than 12 to 24 hours during hospitalization (8). Nevertheless, a recent study has
reported that patients who experience uncomplicated AMI spend the majority of the first 72 hours in a coronary care unit in bed (9).

Morris et al. (1953) (10) published the first study of activity level and CVD in the Lancet entitled ‘Coronary heart disease and physical activity of work.’ He reported that conductors/ticket collectors working on the typical English double-decker buses had an incidence of cardiac attack lower than the drivers of the same buses, in a precise ratio of 1:2.2. Sedentary lifestyle is a well-known major risk factor for cardiovascular disease (11) and may be responsible for about one-third of deaths attributed to coronary disease (12). Physical activity is associated with a reduction in cardiovascular mortality risk of 35% (13).

Prolonged bed rest also increases the risk of VTE (14), and as does inactivity during travel of long duration (15). A meta-analysis of 43 studies of the risk for VTE in bedridden patients showed an overall relative risk of 1.86 in cohort studies and an overall odds ratio of 2.52 in case-controlled studies (16). Gibbs (17) found that 15% of patients confined to bed rest for <1 week prior to death exhibited venous thrombosis at autopsy, while the incidence rose to 80% in patients in bed for a longer period but the role of other underlying contributing mechanisms is difficult to assess. Isma et al. (18) evaluated the prevalence and incidence of risk factors in a prospective study of patients with VTE. Immobilization was identified as a risk factor in 17% of patients with DVT and in 18% of patients with pulmonary embolism.

Fewer than half of adults in the USA meet recommended physical activity standards. As lifestyles are becoming increasingly sedentary (19), understanding the physiological effects of physical inactivity and how they contribute to increased cardiovascular risk is crucial.

Pathophysiological changes during immobilization

Haemostasis, inflammation, and endothelial function

Virchow (1856) described a triad of explanations for VTE: vascular stasis, intravascular injury, and changes in blood composition (20). Bed rest impairs blood flow, particularly in the arterial system (21). Muscle atrophy after prolonged bed rest contributes to venous pooling of blood (22).

Studies of venous thrombosis associated with both ground and air travel investigated their possible effects on the coagulation system by assessing differences in various parameters before and after immobilization for 6-16 hours (23). Results were inconclusive, in part due to the broad inter-
individual range of normal coagulation parameters and the fact that circadian rhythms were not taken into account.

While physical activity is associated with a decrease in cardiovascular risk of ~30%, magnitude similar to that of pharmacological strategies (13), its effects on the established cardiovascular risk factors dyslipidaemia, hypertension, diabetes, and obesity are relatively modest and do not completely counteract the impact of inactivity on vascular risk. Mora et al. (24) estimated that 60% of the risk reduction associated with activity may be explained by the modification of traditional risk factors, especially those relating to inflammation/haemostasis and blood pressure. Studies of long-term exercise training demonstrate a long-lasting anti-inflammatory effect (25), while bed rest is associated with increase in markers of low-grade inflammation (26). It has also been suggested that 40% of the exercise-related risk reduction may be the direct effect of conditioning of the vascular endothelium (27). Inactivity stimulates a vascular “deconditioned” state characterized by impaired endothelial function, leading to arterial stiffness and increased arterial tone (28). Exercise maintains endothelial function and enhanced vagal tone, reflected in increased heart rate variability, which could partly explain the reduced risk (29).

**Cholesterol**

Lipoproteins are water-soluble molecules that transport lipids in plasma. They consist of a triglyceride and cholesteryl ester core coated with a surface monolayer of phospholipids, free cholesterol, and apolipoproteins. Dyslipidaemia is defined as abnormal levels of lipid or lipoprotein fractions due to genetic or environmental conditions (30).

The ‘cholesterol theory’ was one of the first models addressing the pathophysiology of atherosclerosis. A century ago, dietary cholesterol was shown to induce atherosclerosis in rabbits, and cholesterol studies still dominate atherosclerosis research (31, 32). In the early stage of the atherosclerotic process, low density lipoprotein (LDL) accumulates in the arterial wall. Following oxidation, LDL stimulates the immune system and induces inflammation. Leukocytes invade the intima and mature into macrophages that absorb lipids and expand, creating “foam cells.” The lesion grows with proliferation of intimal smooth muscle cells. Apoptosis of cells within the lesion leads to further leukocyte accumulation and calcification. A *fibrous cap atheroma* is defined as a plaque with a well-defined lipid core, covered by a fibrous cap that is acellular or rich in smooth muscle cells (33).
For humans with cardiovascular disease, the prevailing principle is that the lower the low-density lipoprotein cholesterol (LDL-C), the better. Based on pathobiology and evolution research, as well as extensive data from clinical trials, low levels of LDL-C are beneficial and are associated with regression of atherosclerosis, as well as with protection from cardiovascular events (34). In contrast, high-density lipoprotein cholesterol (HDL-C) has shown an anti-atherogenic effect by facilitating reverse cholesterol transport, i.e. transfer of cholesterol from foam cells to the liver for excretion into the bile, and reducing vascular inflammation and thrombosis, as well as improving endothelial function (35).

Bed rest of 20-35 days has been reported to result in a decrease in HDL and an increase in triglycerides (36, 37). The impact of exercise training on LDL is typically a decrease of <5% (38).

**Cystatin C**

Cystatin C is a non-glycosylated cysteine protease inhibitor of low molecular weight (13kDa) produced at a continuous rate (housekeeping protein) by most nucleated cells. More than 99% of cystatin C is cleared from the circulation by glomerular ultrafiltration and tubular reabsorption, and, in the clinical setting, its level is used as a marker of kidney failure (39).

In addition to reflecting kidney function, elevated levels of cystatin C are related to chronic low grade inflammation and atherosclerosis (40). A sedentary lifestyle has been shown to be associated with an inflammatory response in population-based studies (41). Cathepsins and their inhibitor, cystatin C, can exhibit either pro- or anti-atherogenic action in the various stages of atherosclerosis by affecting cell adhesion, migration, proliferation, plaque rupture and induction of apoptosis (42). Elevated levels of cystatin C are found in patients with coronary artery disease (43) and stroke (44). High cystatin C levels correlate with a substantially increased risk of cardiovascular events even in persons who do not meet the criterion of eGFR <60mL/min/1.73m², a definition of chronic kidney disease (45). In a prospective population-based study, a high cystatin C level was identified as a risk factor for VTE in subjects with normal kidney function (eGFRcrea >90mL/min/1.73m²). An increase in cystatin C of one standard deviation (0.11 mg/L) was associated with a 46% increased risk of VTE (46). During hospitalization for ST-elevation myocardial infarction (STEMI), cystatin C increased by 19%, disproportional to creatinine elevation. Hypothetically, this could be explained by early detection of renal injury by cystatin C and not by creatinine. Evaluation of kidney function
by the gold standard iohexol clearance was not in agreement with cystatin C GFR, suggesting other physiological explanations for the increase in cystatin C (47).

Physical inactivity and high cystatin C levels are both cardiovascular risk factors, but the effect of prolonged bed rest on levels of cystatin C is unknown.

**Platelets**
Platelets are circulating anucleate disc-shaped cells, responsible for initiation of the haemostatic mechanisms that repair damage in the vascular endothelium. Their primary functions include platelet adhesion, activation, secretion, and aggregation and interaction with coagulation factors (48).

Broadley *et al.* (49) observed a decrease in platelet aggregation after 45 minutes in a supine position. Numerous studies have shown that both acute exercise and cardiorespiratory fitness affect platelet function, as assessed by aggregation (50). This is of particular interest in light of an increased recognition of the inflammatory and immunomodulatory consequences of platelet activation. Platelet function may be fundamental, not only to the final stages of cardiovascular disease, but to its development.

In recent years, preclinical research has demonstrated that platelets may adhere to the vascular endothelium of the carotid artery in ApoE deficient mice prior to the development of manifest atherosclerotic lesions (51). Following adhesion, platelets become activated and release potent inflammatory and mitogenic substances leading to chemotaxis, and transmigration of monocytes to the site of inflammation. This platelet-induced chronic inflammatory process results in atherosclerosis (52).

Increased platelet activation, as indicated by elevated levels of soluble P-selectin, has been observed in atherosclerosis (53), ischemic heart disease (54), acute ischemic stroke (55), and VTE (56). In a recent multi-ethnic study of atherosclerosis, P-selectin was positively associated with adiposity, systolic and diastolic blood pressure, current smoking, LDL, and triglycerides and inversely associated with HDL. A positive linear association of P-selectin levels with rate of incident CHD was observed after adjustment for traditional risk factors (57).

Venous thromboembolism may be regarded as part of the cardiovascular disease continuum. Common cardiovascular risk factors such as smoking, obesity, hypercholesterolemia, hypertension, and diabetes mellitus are shared with arterial disease (58-60). Patients with myocardial infarction
and heart failure exhibit an increased risk for pulmonary embolism (61, 62). Conversely, individuals with VTE have an augmented risk for myocardial infarction and stroke (63).

Arterial and venous thrombosis have been traditionally considered distinct and unrelated disorders, but they are linked both epidemiologically and physiologically. Secondary haemostasis has been the primary focus of treatment and prevention of venous thrombosis, but platelets are of interest, not only in arterial clots, but in venous clot formation as well. The combined results of two randomized placebo-controlled trials showed a 42% reduction of recurrent VTE with long-term treatment with low-dose aspirin (100 mg daily) after completed acute anticoagulation therapy in patients with unprovoked VTE (64). It has been suggested that aspirin might reduce the risk of VTE in physically inactive medical patients (65).

Patients diagnosed with unexplained VTE, exhibited an enhanced maximal platelet aggregation with low concentrations of thrombin receptor activating peptide 6 (TRAP-6) and collagen, compared to a control group, indicating that enhanced aggregation could be a risk factor (66).

Elevated levels of soluble P-selectin are of predictive value in the diagnosis of VTE (67). In addition to blood sampling in the acute setting of venous thrombosis, soluble P-selectin levels were prospectively followed in a study of patients with unprovoked VTE after cessation of anticoagulation treatment. The 12% of the patients that experienced recurrence of VTE showed significantly higher P-selectin levels than observed in patients with no recurrence (68). This provides an indication that excess platelet activation may be related to the development, or be a consequence, of VTE.

A translational model

Current basic medical research often comprises testing of a drug, diet, or genetic modification in a mouse model to understand complex disease mechanisms. An alternative is to study a species that, despite extreme physical conditions, does not develop common human diseases. Biomimetics is the imitation of models in nature for the purpose of unravelling complex human issues.

Through natural selection, living organisms have acquired mechanisms that protect against disease. The study of favourable outcomes of millions of years of evolution provides information valuable in the development of disease prevention and treatment strategies. Commonly used drugs such as acetylsalicylic acid and the angiotensin-converting enzyme inhibitors used
for treatment of heart failure and hypertension have their origins in nature. Angiotensin-converting enzyme inhibitors were developed from a peptide found in the venom of the Brazilian sniper *Bothrops jararaca* snake (69). Joseph Buchner, in 1928, extracted the active ingredient salicin from willow. The chemical structure was analysed and produced as acetylsalicylic acid currently one of the most commonly-used pain killers and platelet inhibitors (70).

While inactivity is a thromboembolic risk factor in humans, this is not the case with hibernating animals. To learn more about this biological paradox we employed a biological translational model method to study the hibernating brown bear *Ursus arctos*, which spends 5-7 months of the year inside its winter den. Throughout hibernation, the bear does not eat, drink, defecate, or urinate, and shows almost no physical activity (71, 72). In such circumstances, cardiovascular disease, kidney failure, muscle loss, and osteoporosis would develop in humans. Yet bears show no signs of organ damage upon awakening in spring (Figure 1).
<table>
<thead>
<tr>
<th></th>
<th><strong>Man</strong></th>
<th><strong>Brown bear</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Circulatory system</strong></td>
<td>Blood cloths</td>
<td>No blood cloths</td>
</tr>
<tr>
<td></td>
<td>Heart failure</td>
<td>No heart failure</td>
</tr>
<tr>
<td></td>
<td>Oxidative and energetic stress</td>
<td>Oxidative and energetic stress tolerance</td>
</tr>
<tr>
<td></td>
<td>Endothelial dysfunction</td>
<td>No endothelial dysfunction</td>
</tr>
<tr>
<td><strong>Muscle</strong></td>
<td>90% reduction in muscle mass</td>
<td>15% reduction in muscle mass</td>
</tr>
<tr>
<td><strong>Bone</strong></td>
<td>Severe disuse osteoporosis</td>
<td>No osteoporosis</td>
</tr>
<tr>
<td><strong>Metabolism</strong></td>
<td>Carbohydrate, protein and fat breakdown</td>
<td>Primarily fat breakdown</td>
</tr>
<tr>
<td><strong>Skin</strong></td>
<td>Bed sores</td>
<td>No bed sores</td>
</tr>
</tbody>
</table>

*Figure 1. A comparison of the pathophysiological changes during immobilization in human and brown bear.*
The evolutionary divergence of bears and humans is relatively recent. In contrast to most other hibernators, brown bears and black bears (*Ursus americanus*), maintain near-normal body temperature (31-35°C) during winter sleep and have probably developed unique temperature-independent strategies to protect them from the effects of immobilization (73).

Brown bears exhibit several characteristics that are cardiovascular risk factors in humans. During hibernation, heart rate and cardiac output decreases considerably. The heart rate of five hibernating black bears with implanted data recorders ranged from 13.1 to 38.8 beats per minute with sinus pauses of up to 13 seconds (74). Ultrasonography of hibernating brown bears has shown that this results in a “spontaneous contrast” phenomenon in all cardiac chambers that, in humans, is an ominous sign of risk for thromboembolic disease (75). When blood flow is reduced, fluid shear stress is also typically reduced, and low shear stress in blood vessels is related to the formation of atherosclerotic plaques (76). In contrast to the effects of chronic bradycardia in non-hibernating species, bears maintain cardiac function despite the low heart rate (77).

Fröbert *et al.* (78) found platelet function in brown bears shortly after leaving the den to be lower than values measured in humans.

LDL cholesterol is approximately 1.3 mmol/L in hunter-gatherers, healthy human neonates, and free-living primates and other wild animals (79). In contrast, cholesterol levels in the black bear are higher than human levels (80).

Bears double their caloric intake throughout autumn to build up fat reserves to prepare for the extensive period of nutritional deprivation during hibernation. They become obese, and there are reports of grizzly bears with an increase in body mass of over 50% (81). During hibernation, bears develop hyperinsulinemia and show characteristics of insulin resistance (82). Unlike obese humans and individuals with metabolic syndrome, brown bears show no signs of systemic inflammation or oxidative stress during hibernation (83). Their capacity to survive repeated annual cycles of long periods of immobilization suggests that they are resistant to developing thrombi. Knowledge of how bears tolerate conditions of hibernation may aid in understanding their defence against arterial and venous thromboses.
Figure 2. Ursus arctos in Dalarna, Sweden.
Aims
The overall aim of these studies was to explore potential links between immobilization and the risk of arterial and venous thrombosis in a human and animal model. Additional objectives were to gain further insight into how brown bears endure repeated annual cycles of immobilization without developing thromboses and to investigate relevant differences from humans.

Specific aims
- To assess the effect of bed rest on the emerging cardiovascular marker cystatin C as well as on kidney function and markers of inflammation/atherosclerosis in humans (Study I).

- To determine plasma concentrations of lipids in free-ranging brown bears during hibernation and in the same bears in their active state (Study II).

- To establish whether the presumably high plasma concentrations of lipids in bears result in atherosclerotic fatty streaks and plaque build-up leading to coronary and aortic pathology (Study II).

- To assess the effects of immobilization on platelet activation in a human bed rest model (Study III)

- To evaluate whether proteins involved in secondary haemostasis differ in brown bears during hibernation compared to the active state (Study IV)

- To investigate how long-term immobilization affects platelet aggregation in a standardized human bed rest model compared to in the hibernating brown bear (Study V).
Subjects and Methods

Study populations

Brown bears (Studies II, IV and V)
Blood samples were taken from the same free-ranging Scandinavian brown bears during winter hibernation (February) and summer (June) in Dalarna County, Sweden, 2010-2012, as previously described (78). The bears were immobilized in the den in February using a dart containing a mixture of tiletamine-zolazepam (1.1 mg/kg), medetomidine (0.03 mg/kg), and ketamine (1.3 mg/kg) (12). In June, bears were darted from a helicopter with a mixture of tiletamine-zolazepam (4.7 mg/kg) and medetomidine (0.09 mg/kg). Blood samples were drawn from the jugular vein within approximately 20 min of darting. This research was approved by the Swedish Ethical Committee on Animal Research (C212/9) and the Swedish Environmental Protection Agency.

Figure 3 and 4. Work in the field.
Human models

Investigation of the effects of bed rest is challenging, as a good biological model is difficult to obtain. Interpreting results from hospitalized patients may be biased by the disease state and surgery or other invasive procedures. In addition, the regimen of bed rest is not easy to standardize. International and national space agencies fund strictly-controlled bed rest studies of healthy adults, as it is necessary to study the effects of immobilization and weightlessness for long-term missions aboard space stations. Bed rest in a head-down tilt position (-6°) simulates the effects of microgravity (84).

Women International Space simulation for Exploration (WISE) study (Studies I and III)

Sixteen healthy female volunteers participated in a 60-day bed rest study in 2005. All were non-smokers, free of any clinical illness, and had not taken oral contraceptives in the three months prior to the study. The participants were required to have engaged in moderate activity such as structured exercise or activities of daily living 30 minutes per day prior to inclusion in the study.

A 20-day ambulatory control period was followed by 60 days of bed rest in a head-down tilt position (-6°) 24 hours/day and a subsequent 20-day recovery period. Baseline data were collected during the ambulatory control period. The subjects were randomized to two groups (n = 8 per group) for the bed rest trial: a control group that remained physically inactive and an exercise group that participated in supine resistance and aerobic exercise. Our study was a secondary observation study of the larger bed rest project that did not include the analysis of platelet activation at the time the study was conducted. We obtained plasma from 15 of the 16 subjects enrolled to conduct our analysis.

The resistance training included 19 sessions of 45 minutes on a flywheel ergometer, including a 10-minute warm up period. The aerobic training consisted of 29 sessions using a specially designed vertical treadmill. Each session lasted 50 ± 2 minutes at intensity ranging from 40% to 80% of pre-bed rest maximum oxygen uptake. All sessions were equally distributed throughout the entire bed rest period. Details of the protocol of the WISE experiment and the training sessions are available (85). The study was approved by the local institutional review board (Midi-Pyrénées I, France).
DLR German Aerospace Center study (Study V)
In 2011 and 2012 the European Space Agency and the German Aerospace Center (DLR) funded a 21-day head-down tilt bed rest study. Ten healthy male volunteers were confined to the metabolic ward of the DLR for a seven-day ambulatory control period followed by 21 days of bed rest in head-down tilt position (-6°) 24 hours a day, and a subsequent seven-day recovery period. One subject withdrew from the study for medical reasons. Nine male volunteers (age 31.0 ± 6.4 years and weight 77.2 ± 5.7 kg) participated.

All were non-smokers and free of any clinical illness. Test subjects were required to exhibit negative results on a thrombophilia screening panel: antithrombin, proteins C and S, factor V Leiden, prothrombin mutation, and antiphospholipid antibody causing prolonged activated partial thromboplastin time. The Ethics Commission of the AertzeKammer Nordrhein (Düsseldorf, Germany) approved the study protocol (Ifd. Number 2010426), and all subjects gave written informed consent. Subject recruitment, screening, participants, and metabolic ward conditions are described by Buehlmeier et al. (86). This prospective, cross-over study was conducted under several dietary regimes, but our study focused exclusively on data derived from the control group.

Laboratory methods

Study I
Blood samples were collected in heparinized tubes at baseline five days prior to bed rest, after 44 days of bed rest, and on day 8 of the recovery period. All samples were taken within 15 min following breakfast. The samples were centrifuged immediately at 3000 rpm for 10 min, plasma was frozen within 30 min and stored in aliquots at -80°C.

Biochemical analyses
Plasma apolipoprotein A1 (reagent: 9D92-01), apolipoprotein B (reagent: 9D92-01), creatine kinase (reagent: 7D63-20), creatinine (reagent: 8L24-01), C-reactive protein (CRP) (reagent: 6K2601), and cystatin C (reagent: 1014; Gentian, Moss, Norway) were analysed on an Architect Ci8200 (Abbott Laboratories, IL, USA) and reported using SI units. The total analytical imprecision of the assays was 1.8% at 0.68 g/L and 1.2% at 2.0 g/L for apolipoprotein A; 4.5% at 0.47 g/L and 2.4% at 168 g/L for apolipoprotein B; 0.7% at 2.8 μkat/L and 0.8% at 13 μkat/L for creatine kinase;
4.8% at 70 mmol/L and 4.8% at 94 mmol/L for creatinine; 0.8% at 8 mg/L for CRP; and 1.7% at 0.77 mg/L and 1.1% at 1.25 mg/L for cystatin C.

GFR was calculated using the Cockcroft-Gault formula, which estimates GFR in mL/min.

\[
\text{GFR} = \frac{(140 - \text{Age}) \times \text{Mass (kilograms)} \times \text{constant}}{\text{Serum creatinine (μmol/L)}}
\]

in which the constant is 1.23 for males and 1.04 for females.

We used a cystatin C immunoassay from Gentian (Gentian, Moss, Norway) on Architect ci8200 (Abbott Laboratories, Abbott Park, Ill., USA) to calculate GFR with cystatin C, using the formula eGFR (mL/min/1.73 m²) = 79.901* (cystatin C value in mg/L) -1.4389 (87).

**Study II**

Blood samples were collected from three female and four male 2–3-year-old free-ranging brown bears during hibernation (February 2010) and during the active period in summer (June 2010).

Material for histological investigation was obtained from six females and six male brown bears from 1.5 to 12 years old (mean 4.6 ± 0.7 years), mean weight 101.6 ± 14.5 kg, harvested by hunting in late summer and early fall of 2010. These were different animals from those used for whole blood biochemical analysis. We collected specimens of the left anterior descending coronary artery (LAD) and aortic arches 1.5 to 7 hours post-mortem (mean 3.0 ± 0.4 hours). The tissue samples were immediately stored in formalin for histological examination.

**Biochemical analyses**

Blood plasma collected in Li-Heparin tubes (Vacuette®, Greiner Bio-one, Kremsmünster, Austria) was used to measure the concentrations of total cholesterol, triglycerides, and LDL and HDL cholesterol. The levels were measured by enzymatic methods using the VITROS 5.1 FS Chemistry System instrument (Ortho-clinical Diagnostics, Rochester, NY, USA) according to the manufacturer’s instructions. Briefly, total cholesterol, triglycerides, and HDL cholesterol were analysed by multilayer film dry-slide
chemistry with colorimetric detection (88). Low density lipoprotein was measured in a two-step enzymatic-colorimetric reaction sequence (88, 89).

Haematocrit was determined in EDTA whole blood (Vacuette®, Greiner Bio-one) with an automated haematology analyser (XE-5000, Sysmex Corporation, Kobe, Japan).

Histology
Tissue was fixed in buffered formalin and paraffin-embedded. All LAD specimens for histological analysis were collected from within 2–3 cm of the LAD ostium, and all aorta specimens were taken from the arch. Representative paraffin blocks were cut in ~3μm sections. Haematoxylin-eosin, elastic van Gieson, and trichrome staining was conducted on all specimens.

Study III
Blood samples for analysis of platelet activation markers were collected in heparin-containing tubes five days prior to bed rest (baseline), after 44 days of bed rest, and eight days into the recovery period. All samples were taken within 15 minutes following breakfast.

Blood samples used for platelet count were collected in EDTA-containing tubes at baseline, after 30 and 58 days of bed rest, and 6 days into the recovery period. The samples were immediately centrifuged at 2000 x g for 10 minutes, and plasma was stored in aliquots at -80°C within 30 minutes. The dates for platelet count and platelet activation differed, because the quantity of blood obtained from each day was insufficient for both analyses.

Biochemical analyses
Sandwich enzyme-linked immunosorbent assays (DY137 and DY220, R&D Systems, Minneapolis, MN, USA) were used to determine P-selectin and PDGF-BB according to manufacturer’s recommendations. The assays have a coefficient of variation of approximately 7%. Briefly, an immobilized monoclonal antibody specific for P-selectin or PDGF-BB was coated onto 96-well microtiter plates, and standards and plasma samples were pipetted into the wells. After incubation and washing, a biotinylated primary antibody was added, and, following a second incubation and washing step, a streptavidin horseradish peroxidase complex was added. The plates were incubated and washed. A substrate was added to enable quantification of bound P-selectin and PDGF-BB by measurement of the ab-
sorbance using a microtiter plate reader. Platelet counts were made with a Sysmex haematology analyser (Siemens, Kobe, Japan).

**Study IV**
I participated in Study IV by quantifying blood coagulation factors in bears via function analyses, therefore only that aspect of the study is included in this thesis. Blood was collected from fourteen free-ranging subadult anesthetized hibernating brown bears in February 2010-2012, and from the same animals when active in June, 2010-2012 (16).

ACL TOP (Instrumentation Laboratory, Bedford, MA, USA) was used to assess fibrinogen, factor II, factor VII, factor VIII, antithrombin, protein S, and von Willebrand factor levels.

**Study V**
Blood was drawn from thirteen free-ranging brown bears (7 females and 6 males, age 2.8 ± 0.6 years) during winter hibernation in February (weight 48.1 ± 14.0 kg) and from the same bears when active in June (weight 50.9 ± 16.6 kg) 2010 and 2011 in Dalarna, Sweden, according to the aforementioned protocol for capture and anaesthesia. Blood samples were taken within approximately 20 min of darting.

Blood cell count was conducted at the accredited Clinical Chemical Laboratory at Örebro University Hospital, Sweden using a fully automated haematology analyser (XE-5000, Sysmex Corporation, Kobe, Japan).

Blood samples for human aggregometry were drawn shortly after awakening during the baseline period and after 19 days of bed rest.

**Platelet aggregometry**
Aggregometry was carried out within 45 minutes of sampling. Whole blood was drawn into 3 mL plastic syringes containing lepirudin (25 µg/mL, Refludan, recombinant hirudin blood collection tubes, Dynabyte, München, Germany) and analysed by multiple electrode platelet aggregometry at 37°C, with constant stir-bar speed of 1000 rpm, on a multiple platelet function analyser (Multiplate impedance aggregometer, Dynabyte, Munich, Germany) as previously described (78). Whole blood and 300 µL of 0.9% NaCl and 300 µL were mixed in a polycarbonate cuvette, and, after three minutes of incubation, 20 µL of agonist was added. Agonists were adenosine diphosphate (ADP) to obtain a concentration of 6.4 µmol/L, arachidonic acid (ASPI-test) to a concentration of 0.5 mM, thrombin PAR1 receptor activating peptide (TRAP, amino acid sequence
SFLLRN, PAR1-AP) to a concentration of 32 µM, thrombin protease-activated receptor 4 (PAR-4)-activating peptide (amino acid sequence AYPGKF, PAR4-AP) to a concentration of 662 µM, or collagen to obtain a concentration of 1µg/mL. To calculate platelet aggregation, impedance was continuously recorded for 6 minutes. Increase in impedance, and thus aggregation, was quantified as area under the curve in arbitrary units (AUC).

To assess the effect of temperature on platelet function in bears, impedance aggregometry was conducted on blood from a subset of animals (n = 6) during winter. Each sample was examined at 33°C and at 37°C.

**Statistical methods**

Statistical analysis was performed using SigmaStat 3.5 software (Systat, San Jose, CA) in Study I – III and Excel 2016 in Study IV-V.

**Study I**

Blood sample results were compared by a two-way repeated measures analysis of variance (ANOVA). Differences were considered significant when $P < 0.05$.

**Study II**

A paired $t$-test was used for the comparison between plasma lipid levels during hibernation and in the active state. Differences were considered significant when $P < 0.05$.

**Study III**

Blood sample results were compared by a two-way repeated measures ANOVA. Differences were considered significant when $P < 0.05$.

**Study IV**

We calculated winter/summer (W/S) ratios of proteins involved in blood coagulation for individual bears, after which we calculated the mean and the $P$ value from paired t tests for each factor. Differences were considered significant when $P < 0.05$.

**Study V**

For comparison between platelet aggregation and blood cell count in humans and brown bears during active and inactive states, a paired t-test was used. Differences were considered significant when $P < 0.05$. 
Results

Study I
Baseline characteristics of the volunteers in Studies I and III including age, height, weight, body mass index (BMI), and blood pressure are summarized in Table 1 in Study I.

We observed a significant increase in cystatin C in both groups on day 8 of recovery compared to baseline (16%, \(P < 0.0001\)) and at 44 days of bed rest (12%, \(P < 0.0004\)). Glomerular filtration rate calculated with cystatin C was significantly lower in both groups after bed rest completion compared to baseline (26% lower, \(P < 0.0001\)) and to 44 days of bed rest (19% lower, \(P = 0.0002\)). Similarly, GFR calculated with the Cockcroft-Gault formula was decreased after bed rest completion compared to baseline (11%, \(P = 0.001\)) and 44 days of bed rest (2%, \(P = 0.005\). C-reactive protein did not change during the bed rest period in the exercise group, but showed a significant increase in the control group during the recovery period compared to both the baseline period (46%) and the bed rest period (39%). All values were low, not exceeding 0.7 mmol/L. No differences were seen in creatinine and creatine kinase levels.

Study II
Values are presented as mean ± standard deviation. Total plasma cholesterol decreased in all bears from hibernation to the active summer period (11.08 ± 1.04 mmol/L vs. 7.89 ± 1.96 mmol/L, \(P = 0.0028\)) as did triglycerides (3.16 ± 0.62 mmol/L vs. 1.44 ± 0.27 mmol/L, \(P = 0.00012\)) and LDL cholesterol (4.30 ± 0.71 mmol/L vs. 2.02 ± 1.03 mmol/L, \(P = 0.0075\)). There was no significant difference between winter and summer in HDL cholesterol (4.78 ± 0.59 mmol/L vs. 4.95 ± 0.51 mmol/L, \(P = 0.18\)). Blood haematocrit was higher during hibernation (56.8% vs. 45.0%, \(P = 0.00013\)).

Aortas of 11 animals killed during hunting were examined. The average wall thickness was ~5 mm with slight tapering toward the aortic arch, thinning to 2–3 mm when reaching the neck arteries. Left anterior descending coronary artery sections were obtained from all 12 bears. The wall thickness was ~1.0–1.5 mm.

Aortas consisted of the typical three vessel wall layers, internal, medial, and adventitial. No atherosclerosis, fatty streaks, foam cell infiltration, or inflammation were seen. Appearance of the internal layer was similar to
the normal human aorta. The medial layer was thick, with a considerably greater quantity of smooth muscle cells compared to that in humans. Groups of smooth muscle cells running oblique to the long axis of the aorta were more pronounced in bears than in humans. The elastic laminae and fibres appeared normal and uninterrupted, and the appearance of the adventitia did not differ from the normal human counterpart. The left anterior descending coronary arteries were free from atherosclerotic changes and presented the morphology of a muscular artery, as is found in nonatherosclerotic healthy humans.

**Study III**
Compared to baseline, mean levels of P-selectin and PDGF-BB decreased after 44 days bed rest by 55% \((P = 0.01)\) and 73% \((P < 0.03)\), respectively, and, in the recovery period, were 76% \((P < 0.001)\) and 78% \((P < 0.02)\) lower than baseline, respectively (Figures 1 and 2 in Study III). Platelet count did not change from baseline value (exercise group = 260 000/µl ± 34 000; control group = 210 000/µl ± 30 000) during the bed rest study, and there was no significant difference in platelet count between the two groups at any sampled time.

**Study IV**
The levels of the major clotting effector proteins FX and FII (prothrombin, which converts to thrombin when activated) and the end product, fibrinogen, increased in blood of hibernating bears compared to active at approximately equal rates (Table 1). In contrast, all other studied pro- and anticoagulant proteins in the coagulation cascade showed moderately decreased levels in winter. We observed no differences between male and female bears.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mean W/S</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin</td>
<td>0.49 n = 13</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Protein C</td>
<td>0.77 n = 13</td>
<td>0.002</td>
</tr>
<tr>
<td>Factor II</td>
<td>1.18 n = 13</td>
<td>0.013</td>
</tr>
<tr>
<td>Factor VII</td>
<td>0.65 n = 14</td>
<td>0.022</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>0.30 n = 13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VWF</td>
<td>0.75 n = 10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>1.2 n = 13</td>
<td>0.24</td>
</tr>
</tbody>
</table>

*Table 1. A comparison of coagulation factors in winter and summer.*
Study V

We found a small but significant increase in haemoglobin during bed rest compared to baseline, most likely due to hypovolemia resulting from the head down tilt.

Brown bears had significantly higher levels of haemoglobin and lower platelet counts in winter compared to summer.

In humans, we observed no differences in aggregation in baseline blood samples from those collected after 19 days of bed rest. In bears, ADP, ASPI, PAR1-AP, PAR4-AP, and collagen impedance aggregometry tests all showed aggregation during winter at half the summer value \((P < 0.05)\) (Table 1 in Study V). Rectal temperature in the bears was 33.4 ± 1.1°C in winter and 39.8 ± 0.8°C in summer. This was approximately 2°C higher than normal summer body temperature, likely the result of the running during helicopter capture (90). To test for the effect of temperature, aggregometry was performed in a subset of blood samples \((n = 6)\) collected during winter, with portions of each sample examined at 33°C and 37°C. No significant differences were observed.
Discussion

Immobilization and Cystatin C

In Study I, we measured the effect of bed rest on plasma concentrations of cystatin C, an emerging marker of cardiovascular disease, along with traditional markers of inflammation and kidney function. We found no change in cystatin C during a bed rest period of 60 days, but, eight days after bed rest was completed, cystatin C levels were increased and GFR decreased, regardless of assay method used.

High cystatin C levels have been reported to be associated with elevated levels of CRP (91) and other inflammatory markers, including IL-6, tumour necrosis factor alpha (TNF-α), and two soluble TNF-α receptors, even with creatinine-based eGFR ≥ 60 mL/min/1.73 m² (92). Levels of cystatin C are linearly positively associated with cardiovascular mortality. In contrast, elevated creatinine level only predicts poor outcome in patients with severe kidney dysfunction (93). Whether cystatin C is an independent risk factor or reflects an association of mildly impaired kidney function with enhanced cardiovascular risk has not been clarified. Assessment of cystatin C levels is superior to conventional estimates based on serum creatinine measurement for detecting very early impairment of renal function (94). The hypothesis of “shrunken pore syndrome” defined by Grubb et al. (95) proposes that reduction in diameter of the glomerular membrane pores decreases permeability to cystatin C, a much larger molecule than creatinine, while permeability to creatinine remains intact. This could explain the observation that a decrease in eGFR (cystatin C) is associated with a considerably higher risk of cardiovascular disease than is a decrease in eGFR (creatinine). Hence, cystatin C might be a marker of early atherosclerotic changes in the glomerular filtration barrier.

Bed rest is linked to cardiovascular risk. Elevation of cystatin C is a marker of cardiovascular risk, and an elevation of cystatin C increases the risk of both arterial and venous thrombosis (43-46). However, we saw no change in cystatin C during 60 days bed rest. It is unlikely that the elevated level of cystatin C seen after completion of bed rest reflected impaired kidney function or an inflammatory response. Large cohort studies have found an association of cystatin C level with inflammation. In a longitudinal study of individuals undergoing elective surgery, the markers of inflammation CRP, SAA, haptoglobin, and orosomucoid increased postoperatively, but cystatin C remained unchanged (96). In our study, CRP was
unchanged during bed rest, with a small significant increase seen in the non-exercise control group after completion of the bed rest study. We attribute this finding to chance, with no physiological relevance, since the absolute values were within a range considered clinically normal.

A rise in cystatin C is seen in both subclinical hypothyroidism and subclinical hyperthyroidism (97) as well as in response to steroid treatment (98). The source of our finding of increased levels of cystatin C after prolonged bed rest could be a temporary adaptive hormone change. However, that is outside the scope and methodology of our study.

Brown bears: lipids and atherosclerosis

In Study II, we found brown bear plasma lipids to be elevated during hibernation compared to the active state and total cholesterol levels higher than normal human values. These findings corroborate previous findings of increased cholesterol levels during hibernation in captive American black bears. Nelson et al. (80) measured their plasma cholesterol and reported total cholesterol of 13.4 mmol/L in winter and 9.6 mmol/L in summer, observations similar to ours. In Study IV our results were confirmed by proteomics.

Hibernating bears exhibit elevated levels of non-esterified fatty acids, indicating greater mobilization of lipid reserves from adipose tissue during fasting (99). In humans, one week of fasting was shown to result in a 27% increase in plasma cholesterol (100).

The association of plasma lipid concentration with atherosclerosis seen in humans has also been observed in some animal models (101-104). In our study, bears up to the age of 12 years showed no indications of atherosclerosis, including the earliest sign, fatty streaks (105), despite elevated cholesterol, indicating relative resistance to atherosclerosis. There was no significant difference in HDL cholesterol in summer and winter, but mean values were generally high, 4.78 and 4.95 mmol/L, respectively. Epidemiological studies over the past several decades have shown an inverse association between HDL cholesterol level and atherosclerotic cardiovascular disease (ASCVD) in humans (106-108). In humans, plasma HDL cholesterol above 1.9 mmol/L was reported associated with longevity and relative freedom from coronary heart disease (109).

The premise that HDL cholesterol levels are inversely related to ASCVD is referred to as the “HDL hypothesis,” and is the basis of intervention to elevate plasma HDL cholesterol. Evidence of regression of atherosclerotic lesions was observed with intravenous infusion of HDL cholesterol in
rabbits fed a high cholesterol diet (110). Transgenic mice with high plasma Apo-A1 and HDL did not develop fatty streak lesions despite an atherogenic diet (111). However, several meta-analyses have shown that HDL-raising drugs fail to reduce cardiovascular events (112, 113), casting doubt on the HDL hypothesis. The latest research indicates that HDL function, as opposed to level, serves as a better explanatory model for its benefits. High density lipoprotein particles exhibit a variety of actions that can counteract atherogenesis, including cholesterol efflux, maintenance of endothelial function, and protection against inflammation and LDL oxidation. The ability of HDL to promote cholesterol efflux from cells is a newly described concept, termed the HDL cholesterol efflux capacity (114). Cholesterol efflux capacity is inversely associated with incident coronary events after adjustment for HDL cholesterol (115). Perhaps this is the source of the absence of atherosclerosis in brown bears. Numerous novel interventions that manipulate the function of HDL cholesterol are current targets as promising drugs (116-118).

Platelet activation and aggregation in humans during bed rest

In Study III, we found reduced levels of soluble platelet activation markers P-selectin and PDGF-BB in plasma during long-term bed rest. We had expected an increase in platelet activation markers during immobilization as an indication of increased risk of thrombosis and atherosclerosis. We also hypothesized that the exercise group would have lower levels of P-selectin and PDGF-BB than the non-exercise control group, but that was not confirmed. There was no difference in platelet activation between the control and exercise groups during bed rest. In Study V, three weeks of bed rest did not affect platelet aggregation. Multiplate aggregometry was designed as an instrument to evaluate patient compliance and response to platelet inhibitor medication, e.g. acetylsalicylic acid and ADP inhibitors such as clopidogrel. It allows a full aggregation response to be observed despite differences in platelet activation, and is a more sensitive marker of active platelets. The Multiplate analysis reveals reactions of circulating platelets in peripheral blood to agonist stimulation. Therefore, our findings in Studies III and V of decrease in platelet activation markers along with maintained platelet aggregation are not conflicting.

A recent review of the effects of physical activity on platelet function found that 8-12 weeks of regular exercise decreased platelet aggregation (50). This may be the result of increased levels of nitric oxide due to physical training, as nitric oxide suppresses platelet aggregation (119). In con-
contrast, concentrations of degradation products of nitric oxide, plasma nitrite and nitrate, are shown to decrease with long-term bed rest (120).

In a small study of healthy individuals, Broadley et al. (49) showed that lying supine for 45 minutes significantly decreased platelet aggregation, from 7.9 to 4.4 ohms, as well as platelet activation as indicated by a drop in soluble P-selectin from 51.9 to 44.7 ng/mL. The authors suggested that this has clinical implications both in laboratory practice and in standard care of patients with acute coronary syndrome, and perhaps contributes to the pharmacological inhibition of platelet aggregation. They proposed that the reduction can be partly explained by a fall in plasma catecholamines, which was also demonstrated in the study. The reverse, an increase in platelet aggregability and an increase in catecholamine levels, has been observed after assumption of upright posture (121). Catecholamines can act on alpha-2 adrenoreceptors and promote platelet aggregation (122). In longer bed rest studies, psychological stress has been reported (123) but no changes in catecholamines (124). Broadley et al. used a lower concentration of ADP (2 µM) than we used, precluding direct comparison. A biphasic curve would be expected, as low concentrations of weak agonists such as ADP may lead to a secondary aggregation wave reflecting the release of ADP from platelet storage granules. Responses were measured when the curve plateaued and for no longer than eight minutes, likely not including the secondary wave of aggregation. We used the standard Multiplate agonist dose of 6 µM ADP assigned to evaluate the effect of pharmaceutical inhibition of ADP on the P2Y12 receptor. A lower dose may have shown different aggregation results.

The majority of studies conclude that exercise training exerts antithrombotic effects on platelet function. The converse, increased platelet activation and aggregation, was not seen in our study of bed rest. Two European space project studies using healthy volunteers showed no alterations in coagulation factors involved in secondary haemostasis at the end of prolonged bed rest compared to baseline (125). In the WISE study, ultrasound examinations did not reveal evidence of venous clot formation (126). The combined results of our studies indicate that long-term immobilization per se does not affect primary or secondary haemostasis in a manner that promotes thrombosis.

In contrast, evidence of decreased platelet activation was shown in Study II. A combination of risk factors is likely necessary for bed rest to induce venous thrombosis and atherosclerosis. Numerous prospective studies have reported an association between platelet function and cardio-
vascular risk in patients with established coronary artery disease, but, in healthy individuals, data on platelet aggregation are not conclusive (127). Many of the relevant studies were conducted several decades ago and varied in platelet aggregation methods and agonists and concentrations used.

However, increased levels of soluble P-selectin have been linked to cardiovascular events in healthy populations (128, 129). A prospective trial of healthy women evaluating soluble P-selectin as a potential indicator of cardiovascular risk found mean plasma levels of P-selectin to be significantly higher among those who developed CHD, independent of other coexisting risk factors (128), corroborating findings in other studies (129). The pro-atherogenic effect of P-selectin has been verified in gene-knockout animal models. P-selectin knockout mice crossed with ApoE-deficient mice exhibited significantly reduced atherosclerosis and leukocyte recruitment in atherosclerotic plaques (130). Most researchers analyse soluble P-selectin with ELISA assays rather than membrane P-selectin expression with flow cytometry assays. Michelson et al. (131) demonstrated that circulating degranulated platelets in baboons rapidly lose surface P-selectin, leading to a rise in soluble P-selectin. The P-selectin-negative platelets remain in the vascular system. Membrane P-selectin may therefore not be as reliable as a platelet activation marker as soluble P-selectin.

**Brown bears and haemostasis**

In hibernating brown bears, platelet aggregation was halved compared to that found in the active state (Study V). We therefore speculate that brown bears have developed reduced platelet aggregation as a protection against thromboses during six months in a low circulatory state. With respect to secondary haemostasis, the key components of coagulation, factor II, fibrinogen, and factor X [mass spectrometry (MS) data] (Study IV), increased during hibernation; whereas anticoagulant proteins antithrombin and protein C, as well as the procoagulant proteins factor VII in the extrinsic pathway and factor VIII in the intrinsic pathway, were downregulated during hibernation (Figure 5).

**KARIN ARINELL.** *Immobilization as a risk factor for arterial and venous thrombosis* 37
Figure 5. A schematic presentation of the coagulation cascade and how protein levels measured (in red boxes) were altered in winter (in comparison to summer) in brown bears.

A general suppression of clotting factor activity is not seen in brown bears or other hibernating animals, and the mechanisms by which brown bears selectively and reversibly regulate coagulation protein transcription is unclear. Studies of coagulation factor alterations during hibernation in ground squirrels and hedgehogs have shown a decrease in factors in the intrinsic and extrinsic pathways. The Franklin’s ground squirrel *Spermophilus franklini* exhibited a decrease in factor V during hibernation (132), but levels were unchanged in hedgehogs (133) and the thirteen-lined ground squirrel *Spermophilus (Citellus) tridecemlineatus*. Decrease in factor VIII and IX was observed in hibernating thirteen-lined ground squirrels. In contrast to this apparent hypo-coagulability, the authors also found an increase in factor II levels (134).

In a study of American black bears, plasma concentration of D-dimer was analysed as a marker of excessive clotting in unmatched bears in winter and summer. The concentration did not exceed 250 ng/mL, currently
considered the threshold for venous thromboembolism in mammals, during hibernation or in the active state. In winter, activated partial thromboplastin time (APTT) was prolonged compared to summer, but prothrombin time (PT) was unaffected. The authors considered this a hibernation adaptation to avoid formation of intravascular blood clots (prolongation of APTT) in combination with a maintained extrinsic pathway to prevent extreme blood loss when injured (135). The initial step in healing of an acute wound involves haemostasis. In a study of ten wild American black bears, normal wound healing was maintained during hibernation, despite reduced skin temperature (136). Hypothermia is known to decrease wound healing rate in humans (137, 138).

The cited studies are not in conflict with our results. As factors influencing prothrombin time are both up- and down-regulated in brown bears, PT could be expected to be unchanged, as was shown in black bears. As the metabolic rate during hibernation is only 25% of the summer active rate (73), protein synthesis is reduced and central processes appear to be maintained by increased levels of a few key or broad-specificity proteins. Thus, all coagulation factor levels were reduced to <80% except for the three key components, factors II, X, and fibrinogen, which were increased by 20%, as shown by quantitative proteomics and verified by function analyses (Study IV). This could be the most economical means of preserving the coagulation response during hibernation while avoiding thrombus formation. The remarkable increase in factor II and fibrinogen was demonstrated by both MS and function analyses in Study IV. As haematocrit during hibernation increased to 1.3-fold the summer levels, the higher levels of these components may reflect a difference in plasma volume due to dehydration, as opposed to an increase in production.

The high agreement between factor levels evaluated by MS and by coagulation function demonstrated that the molecules were active and provided validation of the quantitative data.

Possible explanations for the decrease in platelet aggregation include downregulation of platelet receptors, as receptor density is correlated to platelet activation (139). Adenosine diphosphate from dense granulae is a crucial moderator of platelet activation in the secondary activation loop. During thrombin activation, blocking the ADP receptor P2Y12 has been reported to result in 70% platelet inhibition (140). Thus, alteration in platelet granule packaging during hibernation may potentially explain the lower aggregation response.
Another possible explanation could be a fall in catecholamines during hibernation, as previously mentioned. This has not been thoroughly investigated, but a 5-fold decrease in catecholamines during hibernation compared to the active state was reported in a Finnish study of a 3-year-old bear (141). Measuring catecholamines in animals is difficult due to unavoidable stress when sampling. Both abrupt awakening in the den and a helicopter chase are likely to induce a catecholamine response.

We found bears to exhibit lower platelet aggregation compared to humans in response to all agonists except ADP, even in summer. Species-specific variation in the potentiation of platelet aggregation cannot be excluded. This could be due to species difference in amino acid sequence of the platelet receptor, enabling only partial activation of the agonist. TRAP (PAR1-AP, SFLLRN) is based on human peptide sequences of the PAR1 receptor. PAR4-AP (AYPGKF) is based on a murine sequence of the PAR4 receptor and was developed as the most potent human platelet PAR4 agonist (142). They were, as might be expected, less effective in bears than in human platelet activation. Adenosine diphosphate and collagen provided the most consistent cross-species results in an earlier animal study (143), but direct comparison among species is problematic. Substantial intra-individual seasonal variation was evident in our study.

The observed decrease in platelet count corresponds with findings in studies of hibernating hamsters (144, 145). Upon arousal, thrombocytopenia was reversed, suggesting storage and release, possibly by margination of platelets during hibernation (144). As haematocrit plays a role in the degree of margination (146), the reduced platelet count is likely an effect of higher haematocrit due to dehydration in the den and the overall decrease in protein synthesis in winter.

In ground squirrels, von Willebrand factor was shown to be reduced both quantitatively and qualitatively during hibernation, which should reduce primary haemostasis by preventing platelet adhesion to collagen (147). We also observed decreased levels of von Willebrand factor.

In humans, mild and moderate hypothermia (down to 31°C) was shown to enhance agonist-induced platelet aggregation in a whole blood in vitro study (148). However, we found reduced platelet aggregation during moderate hypothermia in hibernating bears and no differences in aggregation when we investigated in vitro platelet function at different temperatures. In comatose survivors of cardiac arrest randomized to 24 (standard group) or 48 hours (prolonged group) of induced hypothermia at a target core temperature of 33±1°C, platelet aggregation assessed by
the Multiplate method was below the normal range, independent of core temperature. No significant difference was observed in platelet aggregation of the standard and prolonged hypothermia groups (149). It has been suggested that the decrease in platelet reactivity is due to post-resuscitation syndrome, rather than to hypothermia per se (150).

**Study design and methodological considerations**

**Study populations**

Strengths regarding our study population include a well-standardized human bed rest regime, minimizing potential bias. Blood samples were obtained from the same bears in summer and winter, eliminating bias due to inter-individual differences. The bears were free-ranging, thus reflecting natural processes, in contrast to studies using wild animals kept in animal parks.

The population in the DLR German Aerospace Center study included only males, and the WISE study comprised only females. Possible sex differences are thus unknown. All participants were healthy and relatively young, and extrapolating results to both sexes and other age groups is not possible. The inherent increased risk of complications makes it unethical to expose sick or elderly individuals to long periods of standardized bed rest.

A further limitation is that plasma lipid measurements were conducted in one group of bears, while histology was investigated in another group. However, the artery samples were collected from older animals, thus increasing the chance of detecting atherosclerotic changes. As cholesterol in humans rises with age (151), we have no reason to believe that cholesterol would be lower in the bears harvested by hunting. One could argue that the histological examinations were conducted in bears too young to have developed atherosclerosis, but brown bears typically reach reproductive maturity before age five and rarely live longer than 25 years (152). Human data reports findings of atherosclerosis at a young age. Fatty streaks are found in the majority of children 10-14 years of age, and the development of fibrous plaques begins in the 20s (153). In a previous study, multiple saccular aneurysms were found in the descending thoracic aorta of two elderly black bears. There was no trace of thrombotic material in the aneurysms. The bears were housed in a zoo and primarily fed a horsemeat diet, and findings are therefore not comparable to wild bears. The location of the aortic abnormalities was not in accordance with a “stress and
strain” aetiology, since hemodynamic forces are believed to be maximal in the aortic arch, suggesting a parasite aetiology (154, 155). We found one case report of cerebrovascular atherosclerosis in a 38-year-old animal park grizzly bear *Ursus arctos horribilis* (156). (It should be mentioned that a co-investigator in our group, A. Ewans, called the animal park from where this finding was reported, and the administrator could not confirm that grizzly bears have been kept in the park.) The bears from our study were all free-ranging and from the same geographic area.

Our prospective study design, in which every bear and human served as its own control, eliminates bias due to individual characteristics. Blood samples were collected from sub-adult animals, as larger bears would have been more difficult to handle in the field. An advantage of using younger bears was reduced risk of interference in reproductive activity and pregnancy and limited influence of past disease in the analyses while a limitation of using young bears is that they are on trajectory of growth.

The limited quantity of blood obtained from the brown bears precluded analysis of all coagulation factors, which is essential for a clearer understanding of alterations in secondary haemostasis. The same limitation applied to the human studies. We sampled at two time points in bears and two to three time points in humans and can therefore not distinguish when our observed changes took place. Comparing two species complicates the interpretation of our results. Protein function in bears and humans appears to be 99% similar (KG Welinder, Professor Emerita in Chemistry and Bioscience) pers. comm., 2017). In bears, one novel complement C3-type protein was found in addition to the ordinary C3 known in humans. However, protein sequences present a range of 50% to 100% identity; hence, immunochemical cross-reactivity between bear and human protein can range from high (identity >80%) to non-existent (Study IV). The high correlation between MS-assessed factor levels and the coagulation function in Study IV also supports reliability of the results, despite the use of different species.

**Methodology considerations**

All human samples were taken in the morning, and systematic error due to circadian variation in platelet activity can be excluded. Aggregation was analysed immediately after sampling, reducing the risk of spontaneous aggregation. Our obtained levels of P-selectin and PDGF-BB are difficult to compare with other studies of platelet markers, as there is no standard
calibration for ELISA kits, and each manufacturer uses different references. Establishment of calibration standards would be valuable.

Importantly, pre-analytical handling, including venous stasis during blood collection and agitation during transport (157), can lead to artefact platelet activation, potentially altering results. Blood sampling and laboratory methods were conducted in a standardized manner, and we assume no systematic bias from sample handling. In bear sampling both summer and winter, aggregation was conducted within 45 minutes of sampling, minimizing the risk of spontaneous aggregation. Temperature shifts were avoided by insulating the blood samples in a blanket when transported from the collection site to the lab by snowmobile and automobile.

Blood samples from the WISE project were obtained in 2005 and analysed five years later, which may have influenced results. However, Larsson et al. (158) showed that, when using the Gentian method, cystatin C levels remained stable in blood samples assayed over a four year span. All samples were analysed in a single batch and in a random mode, using the same reagent batch and the same calibration on a single instrument. The instrument had a high assay capacity, and the time interval between the first and the final assay was less than 30 min. This, in combination with the low assay coefficient of variation, makes it unlikely that the obtained differences were due to variation in the assay or to sample evaporation during the assay.

Plasma volume reacts dynamically to postural changes due to alterations in venous pressure in the lower parts of the body. Small filterable elements travel with water into the interstitial space, while larger components remain in the blood vessels. Change from supine to a sitting position induces a modest increase of biochemical parameters, e.g. in the concentration of iron from 14.1 umol/L (12.0-17.7) to 14.3 umol/L (12.1-18.9), \((P = 0.008)\) (159). As cystatin C (molecular weight 13kDa) molecules are smaller than iron, postural effects on cystatin C levels are likely minimal and not the source of the observed increase in our study.

It is a limitation of our study that a gold standard measurement, such as iohexol clearance, was not used. We did, however, use two methods to calculate GFR, the Cockcroft-Gault formula and cystatin C, with both revealing a decrease in GFR following bed rest.

**Potential sources of bias**

In the WISE study, individuals in both groups exhibited significant weight reduction after 44 days of bed rest with no gain during the recovery peri-
od, compared to baseline. According to Knight et al. (160), lower weight is correlated with lower cystatin C levels. However, we found an increase in cystatin C levels.

Season-dependent levels of platelets, haematocrit, and catecholamines could, hypothetically, have affected platelet aggregation in brown bears. We observed a reduction in platelet count from approximately 260 x 10⁹/L in summer to 170 x 10⁹/L during hibernation. Studies of the association of platelet count and platelet aggregometry have shown decreased aggregation when platelet count drops below 150 x 10⁹/L (161), lower than our mean winter finding of 174 x 10⁹/L ± 51. We therefore suggest that the reduction in platelet aggregation in brown bears was not linked to the reduction in platelet concentration. In a previous study (78), aggregometry data obtained 7-10 days after leaving the den (mid-April 2009) showed platelet aggregation similar to that observed during hibernation in the present study, although mean platelet count was higher at 207 10⁹/L ± 24.

Haematocrit was significantly higher in winter (54.8% ± 3.8) compared to summer (44.0 ± 3.3%). No influence of haematocrit on platelet reactivity was observed by multiple electrode aggregometry in whole blood drawn into hirudinized aspiration systems (162, 163). Danielak et al. (162) found haematocrit levels from 28–52% to have no effect on Multiplate results. Data of higher haematocrit levels is lacking.

A potential source of inaccuracy in our assessment of platelet function in brown bears is the effect of the anaesthetics used. In an in vitro study, the alpha-2 agonist medetomidine at a concentration of 10 ng/mL reduced platelet aggregation to the agonist epinephrine but did not change the response to collagen. This effect was not seen with exposure to a clinically relevant concentration of 1 ng/mL (164). In winter, we used a lower dose of medetomidine than in summer, so our reduced levels of platelet aggregation cannot be attributed to the anaesthetic. Yokota et al. (165) found no effect of therapeutic doses of medetomidine on aggregation in cattle or horses.

Ketamine (used in winter) has also been shown to suppress aggregation by various agonists at concentrations of 150–500 μM (166). In baboons, intramuscular injection of ketamine hydrochloride at 10 mg/kg body weight inhibited platelet aggregation stimulated by ADP, arachidonic acid, epinephrine, and collagen (167). However, tiletamine, also an N-methyl-D-aspartate receptor antagonist and chemically related to ketamine, was used in both summer and winter and likely has the same effect on platelet...
aggregation, although this was not specifically studied. In the study of platelet aggregation in April (78), aggregation level was similar to our findings during hibernation, although the former did not use ketamine. Benzodiazepines also reduce platelet aggregation (168), but we used a higher zolazepam dose in summer than in winter; hence, it does not explain our findings. Therefore, we conclude that our observations are not merely a result of anaesthetics.

The instruments and methods used in Study II are routinely employed to assess levels of total cholesterol, LDL, HDL, and triglycerides in human blood samples. The methods have not been validated for use in bears. However, veterinary clinics conduct identical analyses with the same instruments and reagents in a wide variety of animal species, including horses, monkeys, and lizards.
Conclusions

(i) During prolonged bed rest the emerging marker of cardiovascular risk, cystatin C, was unchanged.

(ii) Free-ranging brown bears tolerate elevated cholesterol levels, obesity, physical inactivity, and slowed circulation during hibernation without developing signs of atherosclerosis. The brown bear may serve as a reverse translational model for atherosclerosis resistance.

(iii) During long-term bed rest, a known risk factor for thrombosis, the levels of P-selectin and PDGF-BB decreased, indicating down-regulation of platelet activation. This could represent a protective physiological mechanism in humans to prevent thrombosis during immobilization.

(iv) During brown bear hibernation, coagulation factor levels were reduced to <80%, with the exception of the three key factors II, X, and fibrinogen, which were increased by 20% as shown by quantitative proteomics and verified by functional analyses. This could be the most efficient means of preserving a coagulation response during hibernation while avoiding thrombus formation.

(v) In hibernating brown bears, platelet aggregation was halved compared to summer. We hypothesize that this is a protective measure that inhibits formation of thrombi during periods of low blood flow. In humans, platelet aggregation was unaffected by 20 days of bed rest.
Future perspectives

Given the low daily energy expenditure characteristic of modern living (169), the consequences of physical inactivity seem likely to worsen. The obesity epidemic has been paralleled by a proportionate rise in chronic kidney disease (CKD), and obesity is a documented independent risk factor for CKD (170). In a study of obese adolescents undergoing bariatric surgery, eGFR (cystatin C) increased by 3.9 mL/min/1.73m² for each 10-unit loss of BMI in an adjusted analysis (171). Although cystatin C was unchanged during our bed rest study, it might be a marker of the consequences of a sedentary lifestyle. As an early marker of hypertensive end-organ damage and small-vessel disease, cystatin C may potentially serve as a risk marker to be monitored in primary prevention of cardiovascular disease and to inhibit early CKD progression.

Although bed rest is associated with thrombosis in humans, the results of studies, including ours, indicate that long-term immobilization per se does not affect primary or secondary haemostasis in a manner that induces thrombosis in healthy humans. Further investigation using subjects exhibiting risk factors for thrombosis would be interesting, but challenging.

Compared to healthy humans, brown bears are obese, and have low blood flow and higher cholesterol levels. Questions of how bears tolerate the extreme conditions of months of immobilization without presenting signs of atherosclerosis and thrombosis remain unanswered. Further studies with flow cytometry on platelet receptors could reveal whether platelet aggregation is downregulated in response to differences in receptor levels in summer and winter. Addition of the ADP-cleaving enzyme apyrase when analysing platelet aggregation may provide further insight into the importance of ADP in dense granulae as a second loop of activation. Investigation of cholesterol reflux capacity in brown bears would give additional information of the possible protective role of HDL cholesterol against atherosclerosis. Knowledge of the adaptive mechanisms of hibernators may aid in development of strategies to prevent thrombosis in humans. Flow cytometry could give a hint of differences in platelet receptor expression summer versus winter telling us which receptors could be possible medical targets of inhibition. Humans on antiplatelet drugs can suffer from bleeding complications, but hibernating bears do not seem to have bleeding problems. Perhaps this “natural” way of downregulating platelet reactivity is a more physiological way of maintaining adequate haemostasis without bleeding complications and therefore a therapeutic option in humans.
**Swedish summary**

**Titel:** Immobilisering som en riskfaktor för arteriella och venösa tromboser

**Bakgrund:** 2000-talets stillasittande livsstil och immobilisering bidrar till en ökad risk för både arteriella och venösa tromboser. Det övergripande syftet med den här avhandlingen var att utforska hur olika markörer associerade med ökad kardiovaskulär risk, påverkas av långvarig immobilisering hos både en mänsklig försöksmodell och hos den svenska brunbjörnen som överlever årliga cyklar av långa perioder av immobilisering.

**Syfte:** Från två skilda studiepopulationer som låg i strikt “head-down-tilt” sängvila 24 timmar per dygn under 20 till 60 dagar, analyserade vi den framväxande kardiovaskulära riskmarkören Cystatin C, lösliga markörer för trombocytaktivering i kroppen (P-selectin and PDGF-BB) och trombocytaggregation med hjälp av Multiplate aggregometri. Blodprover togs från vilda brunbjörnar under deras aktiva period under sommaren och under hibernering under vintern för att mäta och jämföra skillnader i lipidprofil och trombocytaggregation. Från björnar skjutna under jakt genomfördes histopatologiska undersökningar på artärgrenar från vänster kranskärl och aortabågen.

**Resultat:** Hos människor var nivåerna av Cystatin C och trombocytaggregation oförändrade under långvarig sängvila, men vi såg en signifikant sänkning av trombocytaktiveringsmarkörer. Brunbjörnens plasmalipidnivåer var ökade under hibernering jämfört med deras aktiva tillstånd och kolesterolnivåerna var generellt mycket högre jämfört med människor. De histopatologiska undersökningarna av kärlen visade inga tecken på ateroskleros. Trombocytaggregationen halverades under hibernering jämfört med i björnarnas aktiva tillstånd.

**Sammanfattning:** Långvarig immobilisering har effekter på flera kardiovaskulära riskmarkörer och processer hos både människa och björn. Björnen har sannolikt utvecklat skyddande mekanismer under hiberneringen för att överleva årliga långa perioder av vila. Ökad förståelse av dessa mekanismer skulle i framtiden kunna bidra till nya strategier för prevention och behandling av kardiovaskulära sjukdomar hos människan.
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Effect of prolonged standardized bed rest on cystatin C and other markers of cardiovascular risk

Karin Arinell1*
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Abstract

Background: Sedentary lifestyle is associated with coronary artery disease but even shorter periods of physical inactivity may increase cardiovascular risk. Cystatin C is independently associated with cardiovascular disease and our objective was to investigate the relation between this novel biomarker and standardized bed rest. Research of immobilization physiology in humans is challenging because good biological models are in short supply. From the Women International Space simulation for Exploration study (WISE) we studied markers of atherosclerosis and kidney function, including cystatin C, in a standardized bed rest study on healthy volunteers. Fifteen healthy female volunteers participated in a 20-day ambulatory control period followed by 60 days of bed rest in head-down tilt position (-6°) 24 h a day, finalized by 20 days of recovery. The subjects were randomized into two groups during bed rest: a control group (n = 8) that remained physically inactive and an exercise group (n = 7) that participated in both supine resistance and aerobic exercise training.

Results: Compared to baseline values there was a statistically significant increase in cystatin C in both groups after bed rest (P < 0.001). Glomerular filtration rate (GFR), calculated by both cystatin C and Cockcroft-Gault equation, decreased after bed rest while there were no differences in creatinine or creatine kinase levels. CRP did not change during bed rest in the exercise group, but there was an increase of CRP in the control group during recovery compared to both the baseline and the bed rest periods. The apo-B/apo-Ai ratio increased during bed rest and decreased again in the recovery period. Subjects experienced a small but statistically significant reduction in weight during bed rest and compared to baseline weights remained lower at day 8 of recovery.

Conclusion: During and following prolonged standardized bed rest the concentrations of several clinically relevant cardiovascular risk markers change.

Background

Sedentary lifestyle is associated with inflammation in population-based studies [1,2] and increases cardiovascular risk [3]. Bed rest causes muscle atrophy, which in turn leads to lower creatinine levels and decreased glomerular filtration rate (GFR), when calculated by the Modification of Diet in Renal Disease (MDRD) formula or Cockcroft-Gault formula dependant on creatinine [4]. Cystatin C is also a marker of GFR but is unaffected by muscle mass. However, age, sex, weight, smoking and high concentrations of CRP affect the plasma level of cystatin C [5]. Because bed rest and cystatin C levels are both cardiovascular risk factors we found it of interest to investigate how cystatin C, together with other risk markers, are affected by prolonged standardized bed rest.

It has been proposed that elevated cystatin C levels are directly correlated to both inflammation and atherosclerosis [6]. High cystatin C levels are independently associated with cardiovascular risk factors such as BMI, low HDL cholesterol and smoking even in patients without chronic kidney disease or microalbuminuria [7]. Cystatin C is independently associated with cardiovascular disease after adjustment for major cardiovascular risk factors [8]. Jernberg et al. demonstrated an association between cystatin C level and mortality in patients with...
Effect of prolonged standardized bed rest on cystatin C and other markers of cardiovascular risk

Karin Arinell1†, Kjeld Christensen1†, Stéphane Blanc2†, Anders Larsson3† and Ole Fröbert1†

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suspected or confirmed non-ST-elevation acute coronary syndrome [9]. Research of immobilisation and human physiology is challenging because good biological models are in short supply. Interpretation of biological samples in hospitalised patients may be biased by various disease states, surgical and other invasive procedures and non-standardized bed rest regimens. In collaboration with the Women International Space simulation for Exploration study (WISE) conducted in 2005 [10] we studied kidney function and atherosclerosis/inflammation markers in a two-month bed rest study on healthy volunteers enabling research on the physiopathology of immobilization.

Methods
Study design
Sixteen healthy female volunteers participated in a 60-day bed rest study. All were non-smokers, free of any clinical/biomedical sicknesses and had not taken any contraceptive pills 3 months prior to the study. An additional inclusion criterion was that the participants were required to exercise 30 minutes per day (moderate activity as structured exercise or activities in daily living) prior to the study.

A 20-day ambulatory control period was followed by 60 days of bed rest in head-down tilt position (-6°) 24 h a day and the study was finalised by a 20 day recovery period (Figure 1). Baseline data collection was performed in the ambulatory control period. The subjects were randomized into two groups (n = 8, each) during bed rest: a control group that remained physically inactive and an exercise group that participated in both supine resistance and aerobic exercise training. Fifteen of the participants gave informed consent to participate in our study.

The resistance training included 19 sessions of 45 minutes training on a flywheel ergometer (including 10 min of warm up). The aerobic training was designed as 29 sessions using a specially designed vertical treadmill. Each session lasted 50 ± 2 min at varying intensities between 40 and 80% of pre-bed rest maximum oxygen uptake. All sessions were equally distributed during the entire bed rest period. The detailed protocol of the WISE experiment and the training sessions are described in detail in previous reports [10].

Laboratory analysis
Blood samples were collected in heparin-containing tubes at baseline (five days before bed rest), after 44 days of bed rest and 8 days into the recovery period (Figure 1). All samples were taken within 15 min after breakfast. The samples were centrifuged immediately at 3000 rpm for 10 min, and plasma was frozen within 30 min and stored in aliquots at -80°C.

Plasma apolipoprotein A1 (reagent: 9D92-01), apolipoprotein B (reagent: 9D92-01), creatine kinase (reagent: 7D63-20), creatinine (reagent: 8L24-01), C-reactive

![Figure 1](https://example.com/figure1.png)  
**Figure 1** Overview of the bed rest study. A 20-day ambulatory control period was followed by 60 days of bed rest in head-down tilt position (-6°) and the study was finalised by 20 days recovery period.
Sixteen healthy female volunteers participated in a 60-day two-month bed rest study on healthy volunteers immobilized. In collaboration with the WISE experiment and the training sessions are described in detail in previous reports [10].

**Methods**

During bed rest: a control group that remained physically inactive and an exercise group that participated in 29 sessions using a specially designed vertical treadmill. Each session lasted 50 ± 2 min at varying intensities.

**Laboratory analysis**

We used a cystatin C immunoassay from Gentian (Gentian, Moss, Norway) on Architect ci8200 (Abbott Laboratories, Abbott Park, Ill., USA) to calculate GFR in mL/min/1.73 m². The formula for calculating GFR with cystatin C is eGFR (mL/min/1.73 m²) = 79.901* (cystatin C value in mg/L) 1.4389 [11].

**Statistical analysis**

Statistical analysis was performed using SigmaStat 3.5 software (Systat, San Jose, Ca). Blood sample results were compared by a two way repeated measures analysis of variance and a p-value of < 0.05 was considered statistically significant.

**Results**

Baseline characteristics of the volunteers: age, height, weight, body mass index (BMI) and blood pressure are summarized in Table 1. Weight decreased in both groups after 44 days of bed rest and the weight reduction was still statistically significant in the recovery period when compared to baseline. After 8 days of bed rest there was a small but significant weight increase in the control group, but not in the exercise group, compared to the bed rest period.

CRP did not change during the bed rest period in the exercise group, but there was a statistically significant increase in CRP in the control group in the recovery period compared to both the control period (46%, p-value 0.008) and the bed rest period (39%, p-value 0.021) (Figure 2). During the recovery period there was a statistically significant difference in CRP between groups (56%, p-value 0.025). There was no difference in creatinine and creatine kinetic levels (Figure 3 and 4).

There was no statistically significant difference in cystatin C in both groups 8 days after bed rest compared to baseline (16%, p-value < 0.0001) and 44 days of bed rest (12%, p-value < 0.0004) (Figure 5). GFR calculated with cystatin C decreased significantly in both groups after bed rest completion (Figure 6) compared to baseline (26% decrease, p-value < 0.0001) and 44 days of bed rest (19% decrease, p-value 0.0002). Similarly GFR calculated with the Cockcroft-Gault formula decreased after bed rest completion (Figure 7) compared to baseline (11%, p-value 0.001) and 44 days of bed rest (2%, p-value 0.005).

The apo-B/apo-Ai ratio increased after 44 days of bed rest (15%, p-value < 0.0001) and decreased again 8 days into the recovery period (25%, p-value < 0.0001) (Figure 8).

**Discussion**

In this study we measured the effect of bed rest on plasma concentrations of cystatin C, which is an emerging marker of cardiovascular disease. We also measured traditional markers of inflammation and kidney function. Eight days after a bed rest period of 60 days was completed cystatin C levels increased and GFR decreased.

### Table 1 Baseline data of age, height, weight, BMI, systolic and diastolic blood pressure.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After 44 d of bed rest</th>
<th>After 8 days of recovery</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Exercise</td>
<td>Control</td>
</tr>
<tr>
<td>N</td>
<td>8</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>34 ± 4</td>
<td>34 ± 3</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.63 ± 0.06</td>
<td>1.67 ± 0.05</td>
<td>1.63 ± 0.06</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>55.6 ± 3.9</td>
<td>59.3 ± 2.7</td>
<td>52.6 ± 4.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.2 ± 1.2</td>
<td>21.3 ± 1.6</td>
<td>19.9 ± 1.2</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>97 ± 8</td>
<td>96 ± 8</td>
<td>98 ± 11</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>59 ± 6</td>
<td>55 ± 8</td>
<td>63 ± 11</td>
</tr>
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</table>

Weight decreased during bed rest in both groups. The exercise group had not gained weight 8 days into the recovery period, but the control group gained some weight.

* = statistically significant difference compared to baseline
* = statistically significant difference compared to recovery period

There was no statistically significant difference between the groups.
Figure 2 CRP levels during bed rest study. CRP did not change during the bed rest period in the exercise group, but there was a statistically significant increase in CRP in the control group in the recovery period compared to both the control period and the bed rest period.

Figure 3 Creatinine levels during bed rest study. There was no difference in creatinine levels.

Figure 4 Creatine kinase levels during bed rest study. There was no difference in creatine kinase levels.

Figure 5 Cystatin C levels during bed rest study. There was a statistically significant increase in cystatin C after the bed rest completion.
Figure 4 Creatine kinase levels during bed rest study. There was no difference in creatine kinase levels.

Figure 5 Cystatin C levels during bed rest study. There was a statistically significant increase in cystatin C after the bed rest completion.
Figure 6 GFR levels during bed rest study. There was a statistically significant decrease in GFR, calculated with cystatin C, in both groups after the bed rest completion.

Figure 7 GFR levels during bed rest study. There was a statistically significant decrease in GFR, calculated with the Cockcroft-Gault formula in both groups after the bed rest completion.
Figure 8 Lipid levels during bed rest study. The apo-B/apo-Ai ratio increased after 44 days of bed rest and decreased again 8 days into the recovery period.

decreased and for GFR this was independent of calculation method.

Bed rest is probably the single most applied therapy for hospitalized patients no matter the underlying disease. It is therefore of great importance to understand the pathophysiological changes during immobilization as it may influence treatment. The growing problem of “sedentary lifestyle” could also be considered as a global immobilisation experiment. Future trips to Mars require further information of the effect of immobilization to the human body.

Patients with elevated levels of cystatin C are at higher risk of developing cardiovascular diseases [8,9]. Low glomerular filtration rate is a risk factor for cardiovascular mortality, independent of other cardiovascular risk factors [12]. Peralta et al discussed the probability that cystatin C might be a better parameter for identifying patients with chronic kidney disease at risk of developing cardiovascular complications than a creatinine-based equation [13]. Other researchers found that elderly people with the highest quintile of cystatin C (1.29 mg/l) have a significantly elevated risk of death from cardiovascular causes, myocardial infarction, and stroke after multivariate adjustment [14]. Cystatin C is linearly associated with cardiovascular mortality, but creatinine on the other hand predicts worse outcome only in patients with severe kidney dysfunction [15].

It has been described that high cystatin C levels correlate to an extensively increased risk of cardiovascular events in persons who do not meet the criterion of eGFR ≤ 60 mL/min/1.73 m2, a definition of chronic kidney disease [16]. High cystatin C levels have been found to be associated with elevated levels of CRP and [5,17] and other inflammatory markers such as IL-6, tumour necrosis factor alpha (TNF-α), and two soluble TNF-α receptors, even with creatinine-based eGFR ≥ 60 mL/min/1.73 m2 [18]. How non-renal factors influence cystatin C concentrations need further research.

In our study CRP was elevated after bed rest in the non-exercise group. Our finding might reflect that bed rest increases inflammatory activity, which in turn advances the atherosclerotic process leading to enhanced risk of CVD as well as elevated cystatin C levels by atherosclerosis in the kidneys and thereby a decreased glomerular filtration rate. In a recent study of overweight/obese postmenopausal women practising physical activity, it was shown that women with the highest tertile of physical activity energy expenditure had lower concentrations of hsCRP after adjustment for fat mass [19]. This can give an explanation to the difference in CRP between the control group and the exercise group in our study.

Cystatin C also has a different role in relation to inflammation/atherosclerosis. Inflammatory cytokines associated with atherosclerosis stimulate the production
of lysosomal cathepsins and increase the plasma concentration of cystatin C. Cystatin C is a cathepsin inhibitor and might therefore play a roll in counterbalancing a potentially destructive greater elastolytic activity [20]. Mice deficient in cystatin C have increased elastic lamina degradation and greater atherosclerotic plaque formation. Studies have shown that both cathepsins and their inhibitor cystatin C could act either pro- or anti-atherogenic in the different stages of atherosclerosis [21]. This role of cystatin C probably plays a lesser part in our study.

A limitation to our study is that the blood samples were taken in 2005 and analysed five years later. This might have influenced our results. A recent study however, has shown that when using the Gentian method the cystatin C levels were stable when comparing blood samples over four years of time [22].

All samples were analyzed in a single batch and in a random mode with the same reagent batch and the same calibration on a single instrument. The instrument has a high assay capacity so the time interval between first and last assay were less than 30 min. This in combination with the low assay CVs makes it unlikely that the differences in this study are due to variation in the assay or sample evaporation during the assay. Our findings were done in women only and the groups were relatively small. Knight et al. found in a cross-sectional study that male gender was independently associated with higher serum cystatin C after adjusting for creatinine clearance. Older age, greater height and weight have a similar effect. Previously, serum cystatin C levels have been found to correlate somewhat with weight [5]. In our study there was a statistically significant weight reduction in both groups after 44 days of bed rest and in the recovery period compared to baseline. According to Knight et al. because of weight loss a reduction in cystatin C levels could have been expected. In vertebrates both metabolic rate and glomerular filtration rate are positively correlated to body size [23]. However, we found the opposite - an increase in cystatin C levels. This could indicate that cystatin C is a marker of other physiological processes than kidney function. Knight et al. showed that high CRP levels are independently associated with increased cystatin C levels after adjustment for creatinine clearance. Cystatin C can also be a biomarker for inflammation [24,25]. In another substudy of WISE it was shown that bed rest causes both mechanistic and functional impairment of endothelial function [26]. These results could serve as a possible explanation for the cystatin C findings in our study - because early changes in endothelial function are part of the pathogenesis of atherosclerosis.

The decrease in GFR calculated with the Cockcroft-Gault formula can be explained by a combination of weight reduction and stable creatinine values. Weight reduction in the control group was most likely due to muscle atrophy and stable fat mass. Unexpectedly, Bergouignan et al. showed that the desire to eat was reduced in the exercise group - leading to a negative energy balance and a decrease in fat mass [27]. It is a limitation to our study that a golden standard measurement, as e.g. iohexol clearance, was not used. We did, however, use two different methods to calculate GFR, both dependent (the Cockcroft-Gault formula) and independent of weight (cystatin C) and the findings were identical - a decrease in GFR after bed rest. In a recent cross sectional study of persons with early stages of chronic kidney disease, light and total physical activity was positively correlated to kidney function when measured by MDRD. This relationship lost statistical significance after adjustment for BMI, cholesterol, CRP and mean arterial blood pressure. Increased physical activity may reduce the progression of chronic kidney disease by decreasing oxidative stress and inflammation and reducing blood pressure besides the positive effect of weight loss [28]. Conceivably decreased physical activity, in our study bed rest, has the opposite effect with an increase in oxidative stress and inflammation.

We cannot explain why the changes were seen first after the bed rest study was completed and not during bed rest. The effect could be due to the change from resting to standing position as it has previously been shown that body position per se influences renal perfusion [29]. Further studies are required to determine the mechanism of the effect of bed rest on cystatin C as well as other cardiovascular risk markers.

Conclusion

In conclusion, cystatin C increased and cystatin C estimated GFR decreased in healthy female volunteers after a standardized bed rest period of 60 days. As cystatin C is a cardiovascular risk marker, this study may implicate that longer time periods of immobilization augment the risk of atherosclerosis. This is however only a hypothesis. Further studies are warranted to explore the role of cystatin C as a link between inactivity and cardiovascular risk.

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KA, KC, AL and OF were involved in the conception and design of this project. SB contributed with acquisition of data. KA and OF analyzed data. KA, KC and AL were responsible for interpretation of data. The manuscript was drafted by KA and all other authors revised it critically. All approved of publication.

Competing interests
The authors declare that they have no competing interests.

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References
Despite the lack of information from free-ranging bears, no sampling was done during hibernation, lipid concentrations were found to vary significantly among populations of black bears. 4 Despite the dependency on lipid metabolism during hibernation, lipids are only are plasma lipid levels raised during hibernation, lipids are associated, as is also the case for various mammal models of atherosclerosis. 5–8 We have only been able to find one published account on atherosclerosis in bears, a case report of cerebrovascular fatty streaks and plaque building. 9

The objective of this study was to ascertain plasma lipid concentrations of hibernating and active free-ranging brown bears and relate them to arterial histopathology. Blood was drawn from seven immobilized free-ranging brown bears (three females, 2–3 years old) during hibernation in February in the left descending anterior coronary artery (LAD) and aortic arches within 3 hours from the killing of the animals. The tissue samples were immediately stored in formalin for histopathologic examination.

Plasma from blood, collected in Li-Heparin tubes (Vacuette®, Greiner Bio-one, Kremsmünster, Austria) were used to measure cholesterol (4.30 ± 0.71 mmol/L vs. 2.02 ± 1.03 mmol/L, P = 0.0028) as did triglyceride (3.16 ± 0.62 mmol/L vs. 1.44 ± 0.27 mmol/L, P = 0.0075), whereas HDL cholesterol was unchanged. No atherosclerosis, obesity, physical inactivity, and circulatory slow flow during hibernation without signs of atherosclerosis. This species might serve as a reverse translational model for atherosclerosis resistance. Clin Trans Sci 2012; Volume 5: 269–272

Introduction

Hibernation is an extreme physiological challenge for the brown bear. Despite highly elevated plasma lipids during hibernation and brown bears (Ursus arctos), 1 account on atherosclerosis in an aged grizzly bear (Ursus arctos horribilis) 9 different species of bears. 10 The study of bears was approved by the Swedish Ethical Committee on animal research (C212/9). All procedures described in the study were in compliance with Swedish laws and regulations.

Methods

Material

Blood samples were collected from free-ranging brown bears in summer (June 2010). The bears were immobilized in the den during February and during their active period in June 2010. The experimental design of recapturing provides controls for both internal and external factors that affect the metabolic rate (body and external temperature, feeding status, denning site with the highest precision possible). The experimental denning site with the highest precision possible. Th e tissue samples were immediately stored in formalin for histopathologic examination.

Plasma from blood, collected in Li-Heparin tubes (Vacuette®, Greiner Bio-one, Kremsmünster, Austria) were used to measure cholesterol (4.30 ± 0.71 mmol/L vs. 2.02 ± 1.03 mmol/L, P = 0.0028) as did triglyceride (3.16 ± 0.62 mmol/L vs. 1.44 ± 0.27 mmol/L, P = 0.0075), whereas HDL cholesterol was unchanged. No atherosclerosis, obesity, physical inactivity, and circulatory slow flow during hibernation without signs of atherosclerosis. This species might serve as a reverse translational model for atherosclerosis resistance. Clin Trans Sci 2012; Volume 5: 269–272

The objective was to compare plasma lipids in hibernating and active free-ranging brown bears and relate them to arterial histopathology. ALE carried out and supervised sampling of bears shot during hunting. BS carried out blood sample laboratory experiments and UB designed and carried out histopathology. KA and OF analyzed the data, interpreted the results and wrote the paper. All authors have contributed to, seen and approved the manuscript.
Brown Bears (*Ursus arctos*) Seem Resistant to Atherosclerosis Despite Highly Elevated Plasma Lipids during Hibernation and Active State

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**Abstract**

Hibernation is an extreme physiological challenge for the brown bear (*Ursus arctos*) in which metabolism is based mainly on lipids. The study objective was to compare plasma lipids in hibernating and active free-ranging brown bears and relate them to arterial histopathology. Blood was drawn from seven immobilized free-ranging brown bears (three females, 2–3 years old) during hibernation in February and from the same bears while active in June and analyzed by enzymatic and automated hematology methods within 48 hours of sampling. Left anterior descending coronary arteries and aortic arches from 12 bears (six females, 1.5–12 years old) killed in hunting were examined by histopathology. Total plasma cholesterol decreased from hibernation to the active period (11.08 ± 1.04 mmol/L vs. 7.89 ± 1.96 mmol/L, \( P = 0.0028 \)) as did triglyceride (3.16 ± 0.62 mmol/L vs. 1.44 ± 0.27 mmol/L, \( P = 0.00012 \)) and LDL cholesterol (4.30 ± 0.71 mmol/L vs. 2.02 ± 1.03 mmol/L, \( P = 0.0075 \)), whereas HDL cholesterol was unchanged. No atherosclerosis, fatty streaks, foam cell infiltration, or inflammation were seen in any arterial samples. Brown bears tolerate elevated cholesterol levels, obesity, physical inactivity, and circulatory slow flow during hibernation without signs of atherosclerosis. This species might serve as a reverse translational model for atherosclerosis resistance. Clin Trans Sci 2012; Volume 5: 269–272

**Keywords:** apolipoproteins, cholesterol, hibernation physiology, triglycerides

**Introduction**

Hibernation is an extreme physiological challenge for the brown bear. For 5–7 months of the year metabolism is based mainly on lipids from stored fat while protein synthesis is reduced. The dependency on lipid metabolism during hibernation increases plasma lipids as documented in two American black bears (*Ursus americanus*) and one Asiatic black bear (*Ursus thibetanus*).<sup>1</sup> Four American black bears<sup>1</sup> and two brown bears<sup>3</sup>—all kept in captivity. A recent study compared plasma lipids in seven captive and nine free-ranging adult American black bears and although no sampling was done during hibernation, lipid concentrations were found to vary significantly among populations of black bears. Despite the lack of information from free-ranging bears, the overall conclusions from these previous studies are that not only are plasma lipid levels raised during hibernation, lipids are also generally elevated compared to human values.

In humans, plasma lipid concentrations and atherosclerosis are associated, as is also the case for various mammal models of atherosclerosis.<sup>5–8</sup> We have only been able to find one published account on atherosclerosis in bears, a case report of cerebrovascular atherosclerosis in an aged grizzly bear (*Ursus arctos horribilis*).<sup>9</sup>

The objective of this study was to ascertain plasma concentrations of lipids in free-ranging brown bears during hibernation and in the same bears while active. To establish whether the presumably high plasma concentrations of lipids cause atherosclerotic fatty streaks and plaque building we related the biochemical findings to coronary and aortic histopathology from bears shot in Sweden during the legal hunting season.

**Methods**

**Material**

Blood samples were collected from free-ranging brown bears during hibernation (February 2010) and during their active period in summer (June 2010). The bears were immobilized in the den during February and from a helicopter during June by darting with a mixture of tiletamine-zolazepam and medetomidine.<sup>10</sup> Blood was drawn from the jugular vein as described previously.<sup>11</sup> Only already radio-collared bears were studied to engage the bear at the denning site with the highest precision possible. The experimental design of recapturing provides controls for both internal and external effects (body and external temperature, feeding status, metabolic rate) in accordance with recommendations by Carey et al.<sup>12</sup> The study of bears was approved by the Swedish Ethical Committee on animal research (C212/9). All procedures described were in compliance with Swedish laws and regulations.

From bears shot during hunting during late summer and early fall of 2010 we collected myocardial specimens containing the left descending anterior coronary artery (LAD) and aortic arches within 3 hours from the killing of the animals. The tissue samples were immediately stored in formalin for histopathologic examination.

**Biochemical analyses**

Plasma from blood, collected in Li-Heparin tubes (Vacutette<sup>®</sup>, Greiner Bio-one, Kremsmünster, Austria) were used to measure...
the concentrations of total cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol. The levels were measured by enzymatic methods using VITROS 5.1 FS Chemistry systems instrument (Ortho-clinical diagnostics, Rochester, NY, USA) according to the manufacturer’s instructions. Briefly, total cholesterol, triglyceride and HDL cholesterol were analyzed by multilayer film dry-slide chemistry with colorimetric detection. Lipoprotein analysis of LDL was measured in a two-step reaction sequence, i.e., enzymatic–colorimetric reaction.

Hematocrit concentration was determined in EDTA whole blood (Vacutette*, Greiner Bio-one), by an automated hematology analyzer (XE-5000, Sysmex Corporation, Kobe, Japan).

**Histopathologic examination**

Tissue was fixed in buffered formalin and paraffin-embedded. All LAD specimens for histological analysis were taken within 2–3 cm from the LAD ostium and all aortic specimens were sections of the arch. Representative paraffin blocks were cut in sections with an approximate thickness of 3 μm. On all specimens haematoxylin-eosin, elastic van Gieson, and trichrome reactions were performed.

**Statistics**

Values are presented as mean ± standard deviation (s.d.). A paired \( t \)-test was used for the statistical comparison between plasma lipid levels during hibernation and during active state. Differences were considered statistically significant when \( P < 0.05 \).

**Results**

Blood samples were collected from seven free-ranging brown bears, (three females and four males, 2–3 years old). Material for histological investigation was obtained from 12 brown bears (six females, six males), between 1.5 and 12 years old (mean 4.6 ± 0.7 years), weighing 101.6 ± 14.5 kg. Samples from these bears shot during hunting were collected between 1.5 and 7 hours post mortem (mean 3.0 ± 0.4 hours).

Total plasma cholesterol decreased in all bears from hibernation to the active summer period (11.08 ± 1.04 mmol/L vs. 7.89 ± 1.96 mmol/L, \( P = 0.0028 \), Figure 1A) as did triglyceride (3.16 ± 0.62 mmol/L vs. 1.44 ± 0.27 mmol/L, \( P = 0.00012 \), Figure 1D) and LDL cholesterol (4.30 ± 0.71 mmol/L vs. 2.02 ± 1.03 mmol/L, \( P = 0.0075 \), Figure 1B). There was no statistically significant difference between the two conditions regarding HDL cholesterol (4.78 ± 0.59 mmol/L vs. 4.95 ± 0.51 mmol/L, \( P = 0.18 \), Figure 1C). Blood
hematocrit was higher during hibernation (56.8% vs. 45.0%, \( P = 0.00015 \)).

Aortas were examined from 11 animals. The average wall thickness was around 5 mm with slight tapering toward the aortic arch; there was a definite thinning when reaching neck arteries, the thickness of those were 2–3 mm. Left anterior descending coronary artery sections were obtained from all 12 bears. The wall thickness was about 1–1.5 mm.

**Histology**

Aortas consisted of the typical three vessel wall layers, internal, medial, and adventitial. No atherosclerosis, fatty streaks, foam cell infiltration, or inflammation were seen (Figure 2). The internal layer was of quite normal appearance compared with human aortas. The media was thick and with considerably more smooth muscle cells compared with human histology. It is evident that groupings of smooth muscle cells running obliquely to the long axis of the aorta is more pronounced in bears than in humans. The elastic laminae and fibers appeared normal and uninterrupted and the histological appearance of the adventitia did not differ from human equivalents.

The left anterior descending coronary artery was free of atherosclerotic changes and had the morphology of a muscular artery as is also found in nonatherosclerotic healthy humans (Figure 3).

**Discussion**

This study represents the first systematic comparison of plasma lipids in hibernating and active free-ranging brown bears and the first description of aorta and coronary artery histopathology in this species. Our main findings were that brown bear plasma lipids are elevated during hibernation compared with active state and that cholesterol levels generally are much higher than normal human values. Furthermore, bears up to 12 years old show no signs of atherosclerosis—not even fatty streaks.

The most probable source of increased plasma lipids is from stored triglycerides deposited in adipose tissue. Lipid oxidation of this fat is the primary fuel in bears during hibernation and the increase in cholesterol and triglycerides may be viewed as an alteration in the metabolism of lipoproteins responsible for clearance of these lipids.\(^1\) Hematocrits were higher during hibernation and hemoconcentration likely contributed to the increase in cholesterol but can only explain part of the rise in this parameter.

Previous studies have documented increasing cholesterol levels in captive American black bears during hibernation.\(^12\) Nelson et al. measured cholesterol in plasma and found levels in the same range as we (total cholesterol of 13.4 mmol/L in winter and 9.6 mmol/L in summer)\(^1\). In contrast, Hissa et al. reported higher cholesterol values during summer than winter in two captive brown bears.\(^5\) Although Frank et al. measured cholesterol in wild American black bears they did not include hibernation sampling.\(^7\) Chauhan et al.\(^7\) analyzed serum and not plasma and used a different analysis technology (thin layer chromatography) thus hindering direct comparisons with our findings. Despite considerable variation in food patterns and physical activity between free-ranging and captive bears, the findings of the present study corroborate that brown bear plasma lipids are elevated during hibernation and that the levels generally are considerably higher than what is normally found in humans. The bears in this study had very high plasma concentrations of HDL cholesterol compared to humans. Although low HDL concentrations are associated with increased risk of cardiovascular disease in humans, recent findings question the protective role of high HDL levels.\(^15\)

Although seasonal variations and inactivity influence lipid levels, this is not confined to hibernators. In healthy humans total cholesterol peaks in December–January although the total seasonal variation is around 0.1 mmol/L only with changes in plasma volume accounting for much of the variation.\(^14\) Two bed rest studies in humans, one lasting 35 days\(^17\) and one lasting 20 days\(^18\) both documented an HDL cholesterol decrease and a triglycerides increase in relation to bed rest. However, these changes are far from the drastic alterations in the same variables in brown bears. Although sedentary life style and high cholesterol levels are powerful risk factors for cardiovascular disease in humans, our histopathological findings indicate that brown bears are relatively resistant to atherosclerosis. This observation seems even more remarkable when considering that during hibernation heart rate, and thus blood flow, is reduced dramatically. In captive grizzly bears average resting heart rate is reduced from 84 to 17 beats per minute from active state to hibernation despite unchanged...
stroke volume.29 When blood flow is reduced fluid shear stress is also typically reduced, and low shear stress in blood vessels is related to the forming of atherosclerotic plaques.22 But another risk factor in the atherosclerotic process—oxidative stress—is increased in hibernating American black bears23 and adds to the picture of bears as unique biological models.

The instrument and methods used in this study are routinely used for measuring blood levels of total cholesterol, LDL, HDL, and triglycerides in human samples. To the best of our knowledge, the methods have not yet been validated for use in bears. However, in veterinary clinics identical analyses, using the same instruments and reagents, are routinely performed in a wide variety of animal species (e.g., horse, monkey, and lizard). Our methodology for measuring LDL cholesterol is not a routine veterinary technique, but is also an enzymatic colorimetric method, which must be considered reliable irrespective of species. It is a limitation to our study that plasma lipid measurements were done in one group of bears whereas histopathology was investigated in another group. However, the last group was older, thus increasing the chance of detecting atherosclerotic changes. As cholesterol levels rise with age in humans22 we have no reason to believe that cholesterol levels were lower in the bears shot during hunting. One could argue that the histopathological examinations were done in bears too young to develop atherosclerosis. However, brown bears typically reach reproductive maturity before the age of 5 years and rarely live to after the age of 25.23 Human data supports that early findings of atherosclerosis can be present at a very young age.23 Had we also measured additional fatty acids and lipoprotein lipase (lipogenic) or hormone-sensitive lipase (lipolytic) we could have speculated in the causes of raised cholesterol in bears, but this was outside our scope.

Conclusion
We conclude that brown bear total cholesterol and triglyceride levels peak during hibernation and are considerably higher than human levels. Despite this and regardless of exposure to other factors constituting risks for development of cardiovascular disease in humans (obesity, physical inactivity, and circulatory slow flow during hibernation) we found no signs of atherosclerosis in brown bears. We hypothesize that brown bear resistance to atherosclerosis may serve as a biological model for prevention of cardiovascular disease in humans. Future studies must determine the biological mechanisms behind this resistance and whether for example the inflammatory or antioxidant defense systems, cholesterol recycling, endocannabinoid changes, or perhaps even how bears handle mental stress could improve our understanding.

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Conflict of Interests
The authors declare that they have no financial, personal, or professional associations that could be perceived as interfering with the objectivity of the study.

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Abstract

Downregulation of platelet activation markers during long-term immobilization.

Introduction

Immobilization and sedentary lifestyle are risk factors for venous thromboembolism and cardiovascular disease, yet little is known about the underlying mechanisms for venous thromboembolism. The prevalence and incidence of possible underdiagnosed venous thromboembolism have been estimated at 1% during short-term immobilization and up to 20% during longer bed rest regimens. In collaboration with the Women’s Imaging Research in Space (WISE) study, we investigated two platelet activation markers: P-selectin, a glycoprotein on the platelet cell membrane, and PDGF-BB, a platelet-derived growth factor. These markers are released during platelet activation and are therefore potential markers for platelet activation during immobilization.

Methods

The study design was a prospective, randomized, and controlled study of 10 women who participated in a 84-day study. The study was divided into three phases: a 5-day baseline, a 60-day immobilization period, and a 24-hour recovery period. Blood samples were taken before the start of bed rest, after 44 days of bed rest, and after 8 days of recovery. The platelet count and the levels of P-selectin and PDGF-BB were measured.

Results

The platelet count did not change during the bed rest period. The levels of P-selectin and PDGF-BB decreased significantly after bed rest and remained decreased during the recovery period.

Conclusion

Our findings indicate downregulation of platelet activation during immobilization. Further research is needed to investigate the underlying mechanisms for venous thromboembolism.
Downregulation of platelet activation markers during long-term immobilization

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Abstract

Immobilization and sedentary lifestyle are risk factors for venous thromboembolism and cardiovascular disease, yet little is known about platelet function during long-term physical inactivity. Our aim was to investigate platelet activation markers and their coupling to standardized immobilization: platelet-derived growth factor (PDGF-BB) and P-selectin. We studied 15 healthy females participating in the Women International Space simulation for Exploration study. Following a 20-day ambulatory control period, the subjects underwent 60 days of bed rest in head-down tilt position (−6°) 24 hours a day, finalized by 20 days of recovery. The subjects were randomized into two groups during bed rest: a control group (n = 8) that remained physically inactive and an exercise group (n = 7) that participated in both supine resistance and aerobic exercise training. Blood samples for the analysis of platelet activation markers were collected at baseline (5 days before bed rest), after 44 days of bed rest and 8 days into the recovery period. Compared to baseline, the levels of P-selectin and PDGF-BB decreased after bed rest (by 55%, p = 0.01 and 73%, p < 0.001, respectively) and remained decreased in the recovery period (by 76%, p < 0.001 and 78%, p < 0.02, respectively, compared to baseline). Platelet count (baseline value for the exercise group 260 000/microl ± 34 000 and baseline value for the control group 210 000/microl ± 30 000) did not change during the bed rest study (two-way repeated measurements ANOVA, p = ns). There were no statistical differences between the physically inactive and the exercise group. During long-term immobilization, a known risk factor for thrombosis, the levels of P-selectin and PDGF-BB decreased. Our findings indicate downregulation of platelet activation during immobilization.

Keywords: Platelets, P-selectin, PDGF, immobilization, thrombosis

Introduction

Bed rest is probably the single most applied therapy for hospitalized patients regardless of the underlying disease. Prolonged bed rest is also a known risk factor for venous thromboembolism [1], as is inactivity during travels of long duration [2].

Old data suggest that 15% of patients exposed to bed rest during the last week before death had venous thrombosis at autopsy, whereas the incidence rose to 80% in patients confined to bed for longer time periods [3]. The role of different risk factors for venous thromboembolism is difficult to evaluate. The prevalence and incidence of possible underlying mechanisms for venous thromboembolism were investigated in a prospective study of patients with this diagnosis. Immobilization was identified as a risk factor in 17% of patients with deep venous thromboembolism and in 18% of patients with pulmonary embolism [4].

Evolving research indicates that bed rest and physical inactivity also increase the risk for arterial thrombosis, possibly due to alterations in endothelial function [5]. Sedentary lifestyle can be viewed as a long-term process of social evolution toward inactivity causing inflammation [6, 7], endothelial dysfunction, and increasing cardiovascular risk [8].

The aim of this study was to investigate two essential markers of platelet activation and their coupling to immobilization: platelet-derived growth factor (PDGF) and P-selectin. Both are low molecular weight proteins, released from α-granules in platelets upon platelet stimulation [9, 10]. PDGF is a dimeric molecule consisting of structurally similar A- and B-polypeptide chains, which combine to homo- and heterodimers via disulfide-bonds. Interaction of PDGF isoforms on cells is accomplished by binding and activating tyrosine kinase receptors [11]. P-selectin is a cellular adhesive molecule, which can be attached to P-selectin glycoprotein ligand-1 (PSGL-1) on the platelet cell membrane or exist as a soluble form in serum [9].

Research of immobilization and human physiology is challenging because good biological models are not available. Interpretation of biological samples in hospitalized patients is difficult because of various disease states, surgical and other invasive procedures, and non-standardized bed rest regimens. In collaboration with the Women International Space simulation for Exploration study (WISE) conducted in 2005 [12], we studied the effect of 2 months of standardized bed rest on PDGF, P-selectin, and platelet count in healthy volunteers.
Methods

Study design

Sixteen healthy females volunteered in a 60-day bed rest study (for details in the study design, please refer to our previous publication [13]). The participants were non-smokers, free of any clinical/biomedical sicknesses, and had not taken any contraceptive pills 3 months prior to the study. An additional inclusion criterion was that the subjects were obliged to exercise 30 minutes per day (moderate activity as structured exercise or activities in daily living) prior to the study.

A 20-day ambulatory control period was followed by 60 days of bed rest in the head-down tilt position (−6°) 24 hours a day and the study was completed by a 20-day recovery period (Figure 1). Baseline data were collected in the ambulatory control period. During the ambulatory control period, the subjects were still obliged to exercise 30 minutes per day. The subjects were asked, under professional supervision, to stay active to prevent possible detraining occurring while living confined to the institute. The participants were randomized into two groups (n = 8, each) during bed rest: a control group that was strictly physically inactive and an exercise group that participated in both supine resistance and aerobic exercise training. The subjects were randomized by selecting masked names in a hat. Our study is a secondary observation study of a larger bed rest project that did not initially include the analysis of platelet activation. We received enough plasma to conduct our analysis on 15 of the 16 subjects enrolled.

The resistance training consisted of 19 sessions of 45 minutes training on a flywheel ergometer (including 10 minutes of warm up). The aerobic training included 29 sessions using a specially designed vertical treadmill. Every session lasted for 50 ± 2 minutes at varying intensities between 40% and 80% of pre-bed rest maximum oxygen uptake. All sessions were equally distributed during the complete bed rest period. The specific protocol of the WISE experiment and the training sessions are described in detail in previous reports [12].

The study was approved by the local institutional review board (Midi-Pyrénées I, France).

Laboratory analysis

Blood samples were collected in heparin-containing tubes at baseline (5 days before bed rest), after 44 days of bed rest and 8 days into the recovery period (Figure 1). All samples were taken within 15 minutes after breakfast. For platelet count, the blood sampling was done in EDTA-containing tubes at −80°C within 30 minutes. The dates for platelet count and platelet activation differ because of insufficient amounts of blood for both analyses from the same dates.

Sandwich enzyme-linked immunosorbent assays (DY137 and DY220, R&D Systems, Minneapolis, MN) were used to determine P-selectin and PDGF-BB according to manufacturer’s recommendations. The assays have a total coefficient of variation of approximately 7%. Briefly, an immobilized monoclonal antibody specific for P-selectin or PDGF-BB was coated to 96-well microtiter plates; standards and serum samples were pipetted into the wells. After incubation and washing, a biotinylated primary antibody was added and after another incubation and washing step, a streptavidin–horseradish peroxidase complex was added. The plates were then incubated and washed. A substrate was added enabling quantization of bound P-selectin and PDGF-BB by measurements of the absorbance using a microtiter plate reader. Platelet count was measured by using Sysmex hematology analyzer (Siemens, Kobe, Japan).

Statistical analysis

Statistical analysis was performed using SigmaStat 3.5 software (Systat, San Jose, CA). Blood sample results were compared by a two-way repeated measures analysis of
variance (ANOVA) and a p-value of <0.05 was considered statistically significant.

Results
Baseline characteristics of the volunteers: age, height, weight, body mass index (BMI), and blood pressure are summarized in Table 1.

Compared to baseline, the levels of P-selectin (Figure 2) and PDGF-BB (Figure 3) decreased after bed rest (by 55%, $p = 0.01$ and 73%, $p < 0.03$, respectively) and remained decreased in the recovery period (by 76%, $p < 0.001$ and 78%, $p < 0.02$, respectively, compared to baseline). Platelet count (baseline value for the exercise group 260 000/µl ± 34 000 and baseline value for the control group 210 000/µl ± 30 000) did not change during the bed rest study (two-way repeated measurements ANOVA, $p = ns$). There were no statistical differences between the physically inactive and the exercise group.

Discussion
In this study we investigated how 60 days of bed rest affects platelet activation, by measuring the levels of the platelet activation markers, P-selectin and PDGF-BB. Both markers decreased significantly during bed rest and the levels remained reduced for 8 days in the recovery period while platelet count did not change during immobilization.

In the 1930s, bed rest of at least 6 weeks was a cornerstone in the therapy for patients with myocardial infarction [14]. The goal was to reduce myocardial workload, avoid additional perfusion injury of the myocardium, and minimize remodeling [15]. The time period confined to bed has been greatly reduced over the years, but adequate clinical trials investigating a possible optimal amount of time in bed during disease are lacking. In a systematic review of randomized and quasi-randomized controlled trials of patients with uncomplicated myocardial infarction, short (median 6 days) and longer (median 13 days) periods of bed rest were compared. A longer bed rest regimen did not improve all-cause mortality, cardiac mortality, or reinfarction [16]. Guidelines from the European (ESC) [17] and American cardiac societies (ACC/AHA) [18] advise a bed rest duration of 12–24 hours after uncomplicated acute ST-elevation myocardial infarction, but this regime has been criticized to be ‘consensus-based’ rather than evidence based [16]. The pathophysiological burden including the thrombogenic risk of immobilization is not fully understood.

In our study, there was no difference in platelet activation reduction between the control and the exercise group during bed rest. We expected an increase in platelet activation markers during immobilization as a marker of increased risk of thrombosis. We also hypothesized that the exercise group to some degree would have lower levels of P-selectin and PDGF-BB than the control group. The affects of acute exercise and longer periods of physical training on platelet function diverge. Both a decrease and an increase of platelet activation and aggregation have been reported [19, 20]. The discrepancies may be explained by methodological variations and circadian rhythm [21]. It has therefore been hard to draw any conclusion on the effect of exercise on platelet function [19]. Consequently, it is doubtful whether the moderate exercise program in this study was sufficient to affect platelet function, including parameters we did not assess. It has been shown that marathon running, but not long-distance cycling, induces significant platelet activation. This may be due to mechanical alteration [22].

A previous study showed that a short supine bed rest of 45 minutes significantly decreases platelet aggregation (from 7.9 to 4.4 Ω) and platelet activation (a drop in P-selectin from 51.9 to 44.7 ng/ml). The authors suggested that the result has clinical implications both in laboratory practice and in the standard care of patients with acute coronary syndromes. When comparing platelet aggregation and activation between groups of subjects and within subjects over time, the duration of bed rest should be considered. Bed rest may also contribute to the pharmacological inhibition of platelet aggregation [23]. As hospitalized patients to a great extent are bed ridden, it is essential to investigate the pathophysiological implications of bed rest.

Is the increased risk of thrombosis during bed rest due to increased platelet activation? Or is there perhaps a ‘protective’ mechanism of decreased platelet activity that enables us to rest safely every night?

The Scandinavian brown bear (Ursus arctos) is in a hibernating state for 5–7 months all through winter. During this period, the bear has no physical activity [24] and is apparently free from thromboembolic events. Our group recently found that platelet aggregation is reduced in brown
bears shortly after leaving the den compared to humans [25]. Unpublished data confirms that platelet aggregation is significantly reduced during hibernation compared to active state.

Elevated concentrations of P-selectin have been observed with atherosclerosis [26, 27], ischemic heart disease [28], acute ischemic stroke [29], and venous thromboembolism [30]. Low-circulating P-selectin levels have been associated with smaller thrombi in genetically modified animals that were deficient in P- and E-selectin [31]. Treatment with a recombinant soluble form of PSGL-1 before and during occlusion of
veins in different animal models prevented and treated venous thrombosis successfully [32, 33].

In recent years, research has shown that platelets may also adhere to an intact but activated endothelial cell monolayer [34–36]. After the adhesion process, platelets become activated and release potent inflammatory and mitogenic substances leading to chemotaxis, adhesion, and transmigration of monocytes to the site of inflammation. This ‘platelet-induced’ chronic inflammatory process results in atherosclerosis [37].

At the site of vascular lesion and platelet adhesion to the exposed matrix platelet activation is considered to be the initial step in thrombus formation [38].

A venous thrombosis consists of two regions – one region contains fibrin and trapped erythrocytes and the other region is platelet-rich and is localized further away from the attachment site [39]. These findings imply that activation of the coagulation system precedes platelet activation and aggregation during venous thrombus formation. This might explain why antithrombotic drugs have a limited effect on venous thrombosis.

It is a limitation to our study that a measurement of platelet function, such as aggregometry, was not studied. In an ongoing bed rest study we specifically address platelet function, and this will be reported in a later publication.

The platelet activation markers did not return to normal after 8 days in the recovery period. It would have been interesting to evaluate the time to normalization with further blood sampling at a later time point in the recovery period, but unfortunately no additional plasma was available.

In conclusion, platelets play a major role in inflammation/atherosclerosis and thrombosis by diverse pathophysiological mechanisms. Our findings of decreased platelet activation during bed rest could represent a protective physiological mechanism to prevent humans from developing thrombosis during immobilization. Further studies are needed to clarify the clinical implications of our findings.

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References


Biochemical Foundations of Health and Energy Conservation in Hibernating Free-Ranging Subadult Brown Bear Ursus arctos

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Running title: Hibernation Survival Strategies in Brown Bear

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Keywords: Antimicrobial proteins, blood constituents, coagulation factors, complement system, hibernation biochemistry, metabolomics, protein turnover, proteomics, sex hormone binding globulin SHBG

ABSTRACT

Brown bears (Ursus arctos) hibernate for 5-7 months without eating, drinking, urinating and defecating at a metabolic rate of only 25% of the summer activity rate. Nonetheless, they emerge healthy and alert in spring. We quantified the biochemical adaptations for hibernation by comparing the proteome, metabolome, and hematologic features of blood from hibernating and active free-ranging subadult brown bears with a focus on conservation of health and energy. We found that total plasma protein concentration increased during hibernation, even though the concentrations of most individual plasma proteins decreased, as did the white blood cell types. Strikingly, antimicrobial defense proteins increased in concentration. Central functions in hibernation involving the coagulation response and protease inhibition, as well as lipid transport and metabolism, were upheld by increased levels of very few key or broad-specificity proteins. The changes in coagulation factor levels matched the changes in activity measurements. A dramatic 45-fold increase in sex-hormone-binding-globulin SHBG levels during hibernation draws, for the first time, attention to its significant but unknown role in maintaining hibernation physiology. We propose that energy for the costly protein synthesis is reduced by three mechanisms, (i) dehydration, which increases protein concentration without de novo synthesis; (ii) reduced protein degradation rates due to a 6 °C reduction in body temperature, and decreased protease activity; and (iii) a marked redistribution of energy resources only increasing de novo synthesis of few key proteins. This comprehensive global data identified novel biochemical strategies for bear adaptations to the extreme condition of hibernation, and have

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Biochemical Foundations of Health and Energy Conservation in Hibernating Free-Ranging Subadult Brown Bear \textit{Ursus arctos}

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Keywords: Antimicrobial proteins, blood constituents, coagulation factors, complement system, hibernation biochemistry, metabolomics, protein turnover, proteomics, sex hormone binding globulin SHBG

ABSTRACT

Brown bears (\textit{Ursus arctos}) hibernate for 5-7 months without eating, drinking, urinating and defecating at a metabolic rate of only 25\% of the summer activity rate. Nonetheless, they emerge healthy and alert in spring. We quantified the biochemical adaptations for hibernation by comparing the proteome, metabolome, and hematologic features of blood from hibernating and active free-ranging subadult brown bears with a focus on conservation of health and energy. We found that total plasma protein concentration increased during hibernation, even though the concentrations of most individual plasma proteins decreased, as did the white blood cell types. Strikingly, antimicrobial defense proteins increased in concentration. Central functions in hibernation involving the coagulation response and protease inhibition, as well as lipid transport and metabolism, were upheld by increased levels of very few key or broad-specificity proteins. The changes in coagulation factor levels matched the changes in activity measurements. A dramatic 45-fold increase in sex-hormone-binding-globulin SHBG levels during hibernation draws, for the first time, attention to its significant but unknown role in maintaining hibernation physiology. We propose that energy for the costly protein synthesis is reduced by three mechanisms, (i) dehydration, which increases protein concentration without \textit{de novo} synthesis; (ii) reduced protein degradation rates due to a 6 °C reduction in body temperature, and decreased protease activity; and (iii) a marked redistribution of energy resources only increasing \textit{de novo} synthesis of few key proteins. This comprehensive global data identified novel biochemical strategies for bear adaptations to the extreme condition of hibernation, and have
Hibernation is a physiological survival strategy when food is limited during winter. The ability to hibernate is an old evolutionary adaptation used by very different families of mammals. To date most physiological and molecular studies have been carried out in small hibernating experimental animals like rodents, bats, and hedgehogs, which however hibernate with body temperatures near the freezing point, and therefore must adapt differently from bears hibernating at > 30 °C. Despite a wealth of information on hibernation and torpor physiology, the trigger of the physiological adaptations remains elusive (1-3). Yet the trigger controls gene regulations, which are the primary driver of the hibernation phenotype (4).

Important physiological responses in hibernating bears include a 6 °C reduction in body temperature (5-7), a reduction to 24% in cardiac output (8) and to 25% in metabolic rate (oxygen consumption) compared to those of active bears (5), and a reduction to 25% in metabolic rate (oxygen consumption) compared to summer activity, when adapted immunity takes over. We propose that energy is conserved in hibernation by decreased protein synthesis, which is counteracted by dehydration and by a reduction in chemical and biochemical degradation rates. Furthermore, our data provide a significant and unexpected clue to the regulation of hibernation physiology by sex-hormone-binding-globulin (SHBG), the levels of which increased 45-fold in hibernation activity over summer activity.

Results

Data—Blood was collected from four female and three male free-ranging subadult anesthetized brown bears during hibernation in February 2010 and from the same seven animals when active in June 2010 (16). Similar paired blood samples collected in later years were used in validation analyses. For proteome analysis, we digested non-enriched, non-diluted blood plasma with trypsin in spin cups to minimize handling errors. Total digests were subjected to high-resolution quadrupole orbitrap mass spectrometry, to obtain label-free quantification of plasma proteins across four orders of magnitude of molar concentration. To determine the metabolic profiles of the samples, we used targeted mass spectrometry to measure 186 phospholipids, amino-containing components, and hexose sugars. A commercially available human reference plasma sample was analyzed in parallel. Analysis of each winter sample was immediately followed by that of the summer sample of the same bear (Supplemental Table S1, protein data; Supplemental Table S2, metabolite data; and Fig. 1). We denoted bear proteins by the most similar human gene names, and the corresponding UniProt consortium accession numbers and function assignments (Supplemental Table S1). The bear protein database we used (Supplemental file bear-protein-db-Febr2013.txt) was derived from the
genome sequence of polar bear *Ursus maritimus* (17), and annotated by BlastP against the non-redundant human NCBI Reference Sequence Database. These data were complemented by standard clinical data (Supplemental Table S3), some of which have been published previously (6, 14, 18), and by quantitative activity analyses of seven coagulation factors (Supplemental Table S4).

We calculated winter/summer (W/S) ratios of protein and metabolite levels and blood cell counts for each individual bear, after which we calculated the mean and the *p*-value from paired t-tests for each factor. Only *p*-values > 0.05 are reported below to indicate a lower significance of the corresponding W/S. We observed no differences between males and females or between the 2-year old bear and the six 3-year old bears (Fig. 1B) reinforcing our conclusion that they are subadult animals (19). We found very few intracellular proteins released from bone and muscle tissues (Supplemental Table S1, sheet Cells&others). Therefore, these are not discussed further.

**Plasma Protein and Hemoglobin Concentrations**—UV and UV-VIS absorption spectra of bear plasma samples showed clear differences between the seven winter and summer samples (Fig. 1A). Protein and hemoglobin concentrations derived from the A280 and A415 peaks are summarized in Supplemental Table S5.

The protein concentration was significantly higher in 2010 winter plasma, 68.9 ± 5.1 g L⁻¹, than in summer plasma, 63.0 ± 4.6 g L⁻¹, corresponding to a 9.6% increase. For one bear we found similar protein concentrations in winter and summer. The significance of increased plasma protein concentration during hibernation was verified on paired samples collected in 2013 from another seven subadult bears. Again, winter plasma, with a concentration of 67.0 ± 2.2 g L⁻¹, was more concentrated than summer plasma, with a concentration of 54.0 ± 2.4 g L⁻¹, corresponding to an increase of 24.1% (Supplemental Table S5). It is notable that plasma concentration during hibernation was similar for the 2010 and 2013 samples, i.e. 67-69 g L⁻¹.

The hemoglobin spectra (Fig. 1A) showed fully oxygenated hemoglobin with the characteristic Soret peak at 415 nm, as predictable for plasma exposed to air. We found high and variable hemoglobin levels in all 2010 and 2013 plasma samples (Supplemental Table S5), most likely introduced by some hemolysis during transport on ice to centrifugation. The natural hemoglobin concentration in bear plasma therefore remains unknown and might be like that in healthy humans, in whom the range is 0.01 - 0.04 g L⁻¹. Protein concentrations in bears measured at A280 were affected < 1.4% by hemoglobin.

In Supplemental Table S1, proteins originating from red blood cell rupture are shown in italics. The hemoglobin alpha (HBA) and beta (HBB) chains showed an increase to 3-fold their summer levels in winter, which is in agreement with the hemoglobin concentration determined by absorption spectroscopy. The levels of peroxiredoxin-2 isoform (PRDX2), also originating from red blood cells, showed a similar increase to 4-fold its summer levels. Carbonic anhydrase 1 (CA1) data indicated an increase of the levels of this protein to 12-fold its summer levels, but this protein was only detected in five bears and with low protein scores; CA1 is known to be less abundant in red blood cells than hemoglobin and PRDX2. No proteins from red blood cells were increased in summer. Thus the independent hemoglobin absorption data provide an initial quality control of the quantitative proteomics data.

**Red Blood Cells, Hemoglobin and Iron Metabolism**—Blood and blood plasma appeared more viscous in winter than in summer, and the plasma yield was lower, which indicated a state of dehydration during hibernation. Red blood cell (RBC) count, total hemoglobin (HB) levels, and the hematocrit (volume of RBCs/volume of blood) during hibernation were 1.3-fold the summer levels (Supplemental Table S3). Total plasma protein and albumin (ALB) concentrations also increased significantly in hibernation (Fig. 1A top; Tables S5, S1), despite a decrease in the concentration of most individual plasma proteins (Fig. 1C top). RBC proteins were excluded from the analyses.

The levels of haptoglobin-2 (HP), which binds potentially harmful HB released naturally from RBC, were increased by a factor of 4.7 in winter. The HP-HB complex has high oxygen affinity, effectively blocking uncontrolled oxygen release and the creation of reactive oxygen species (20). HP permits proteolysis of globin chains and conserves iron as well as ceruloplasmin (CP, W/S ratio 1.6), which oxidizes Fe²⁺ to Fe³⁺. The levels of the Fe³⁺ transporter transferrin (TF has two forms both analyzed in this study), and those of the transferrin
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receptor (TFRC, W/S ratio 0.71), which is required for iron recycling but not oxidation, were lower in winter. The levels of hemopexin (HPX, W/S ratio 0.74), which transports heme to the liver for breakdown and iron recovery, were similarly decreased in winter. Protection against oxidation and preservation of Fe³⁺ seems to be important in hibernation.

Defenses and Immunity—Invading pathogens are first met by an organism’s outer and inner surfaces (skin, lung, intestine) and challenged by antimicrobial peptides in epithelial cells. In hibernating bears, we detected raised levels of the circulating antimicrobial defense proteins CAMP, HP, LYZ, and PGLYRP2 (which increased in winter to 3.7-fold, 4.7-fold, 2.7-fold, and 2.2-fold of their levels in summer, respectively) (Supplemental Table S1). Furthermore, proteins protecting against self-proteins were increased or retained during hibernation, as the levels of serum-amylloid-P-component (APCS, W/S ratio 1.7), which scavenges material released from damaged blood cells like HP, increased and the levels seemed unchanged for clusterin that prevents aggregation of non-native proteins (CLU, W/S ratio 0.90, \( p = 0.10 \)).

By contrast, both the innate and acquired cellular and humoral immune defenses were generally suppressed during hibernation. The white blood cell count (W/S ratio 0.6), and the numbers of the relatively short-lived neutrophils (W/S ratio 0.5), and monocytes (W/S ratio 0.6) were significantly lower, as reported previously (6), whereas the numbers of the more long-lived lymphocytes were unchanged (Supplemental Table S3). The significantly reduced levels of macrophage stimulatory protein MST1 (W/S ratio 0.20), and monocyte differentiation antigen CD14 (W/S ratio 0.22), which recognizes lipopolysaccharide on gram-negative bacteria, emphasized the suppression of innate immunity in hibernation.

The lectin, classic, and alternative pathways of complement activation, as well as components of the membrane attack complex (MAC), were all represented in the proteome data (Fig. 2; Table S1). In the lectin and classic complement activating pathways, bacteria are bound by lectins and antibodies, respectively. The lectin-activating pathway is initiated by lectin FCN1 and protease MASP1, and the levels of these proteins were decreased during hibernation to 0.21-fold and 0.15-fold of the summer levels, respectively. The antibody-initiated classic pathway was also down-regulated, as indicated by decreases in the levels of subunits of complement protease C1 (C1QA, C1QB, C1R, and C1S, which had similar W/S ratios of 0.49, 0.43, 0.40, and 0.39, respectively), and its SERPING1 inhibitor (W/S ratio 0.61), C1, like MASP1 of the lectin pathway, cleaves C2 and C4 generating the active C3 convertase C4C2a. Subunit C1QG and complement C2 could not be identified because they were missing in the bear protein database.

The alternative pathway is initiated by the binding of complement factor B (CFB, W/S ratio 0.76) to hydrolyzed C3 (C3H2O), which is formed slowly by a spontaneous process and present at low levels in plasma. Proteolytic cleavage of CFB in this complex by complement factor D (CFD) produces the alternative pathway C3 convertase complex [(C3H2O)BbP, stabilized by properdin (P)]. CFD was absent from the bear protein database and was therefore not included in the analysis.

Initiation of the three complement pathways (Fig. 2) leads to the cleavage of their common substrate C3 (W/S ratio 0.89) followed by exposure of a reactive thiol ester. The C3b fragment covalently attaches to proximal molecules or cell surfaces and changes C3 convertase to C5 convertase. The MAC complex, which forms pores in cell membranes, is assembled of C5b; C6; C7; the C8A, C8B, C8G subunits of C8; and the C9 multimer (W/S ratios 0.77, 0.39, 0.80, 0.63, 0.65, 0.31, and 0.88, respectively). We conclude that the activity of all pathways of the complement system is significantly down-regulated in hibernation.

We also detected decreased levels of complement regulatory proteins CFHR2 (two dissimilar bear transcripts of complement factor H-related protein 2, with W/S ratios 0.44 and 0.25) in hibernating bears, whereas complement factor H (CFH) was not observed. Decreased levels of complement factor I (CFI, W/S ratio 0.68), were also detected in winter. CFHR2 competes with CFH, a cofactor of CFI, which degrades C3b and C4b. The subunits of C4BP, C4BPA (W/S ratio 1.07, \( p = 0.25 \)) and C4BPP (W/S ratio 1.21, \( p = 0.075 \)), as well as the target component C4 (W/S ratio 1.11, \( p = 0.16 \)) showed non-significant changes in protein levels. C4BP functions in the protection of host cells proximal to an attacking pathogen in complex with partners including CFI and CFH, which explains its
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conservation in hibernation. C-reactive protein (CRP, W/S ratio 0.66) is a prominent marker of inflammation and activates the complement system in complex with phosphocholine and C1Q. Vitronectin (VTN, W/S ratio 0.88), in complex with C5b, C6 and C7, inhibits attachment of the MAC complex to cell membranes. Taken together, these findings suggest that the observed changes in the levels of regulators of the complement pathways correspond with those of their target components.

In addition to the well-studied human complement components, we found a C3-like component (W/S ratio 0.45), which is ~40% identical in amino acid sequence to human C3. This component is present also in giant panda, dog, and pig, but not in human or mouse. In human C3, C4, and A2M proteins, the active site thiol-ester-forming sequences linking Cys and Gln are Cys-Gly-Glu-Gln. In C3-like proteins the homologous sequences are Cys-Pro-Glu-Gln. The larger and rigid proline residue may inhibit spontaneous thiol ester formation. However, our molecular modeling of this C3-like domain was inconclusive in this respect, and further analysis will be needed. Mass spectrometry data indicated C3-like plasma concentrations to be very low compared with that of C3. The function of C3-like component is unknown.

We attempted to distinguish the immunoglobulin classes IgM, IgA, IgG, IgE, and IgD, and light chain types IgL and IgK, via their constant regions. However, the automatic predictions of bear transcripts of the complex IGH, IGK, and IGL loci appeared to be of poor quality. Our manually verified transcripts are included in Supplemental Table S1. The levels of IGHM, IGHG1, IGLC1, IGKC, and IGJ were reduced in hibernation to approximately 90%, whereas the levels of the secretory IGHA1 were doubled. IGHD and IGHE concentrations are normally low and these proteins were not detected. The levels of immunoglobulins varied among the bears, as did that of the lymphocytes producing them.

The levels of the immunoglobulin helper proteins VPREB1, PIGR, ATRN, B2M, and CD5L were reduced in hibernating bears (W/S ratios 0.54, 0.30, 0.70, 0.24, and 0.87, respectively). We included the alpha-1-microglobulin–bikunin precursor (AMB, W/S ratios 0.83) in our analysis of immunity-related proteins because alpha-1-microglobulin inhibits immunological functions of white blood cells in vitro, and bikunin, which is called the light chain of inter-alpha-trypsin inhibitor, exhibits broad serine protease inhibitor activity in complex with the four heavy chains of the inhibitor (ITIH1, ITIH2, ITIH3, and ITIH4; W/S ratios 1.07, p = 0.2; 0.94, p = 0.2; 0.25; and 0.45, respectively). ITIH4 plays a role in the acute-phase reaction.

We conclude that the humoral and cellular immune responses were significantly reduced in hibernation and taken over by non-specific circulating antimicrobial defense proteins and secretory IGHA1, which had their levels raised by 2-fold to 5-fold.

Platelet, Coagulation and Fibrinolysis—Activated platelets attach to a site of tissue injury or to blood vessels, and to each other, to form aggregates. Platelet count was down to 0.67-fold of the summer levels in blood of hibernating bears (Supplemental Table S3). The levels of platelet-activating factor phospholipid 1-O-hexadecyl-2-acetyl-phosphatidylcholine are balanced by synthesis and the inactivation by platelet-activating factor acetylhydrolase (PLA2G7, W/S ratio 0.14) (Supplemental Table S1). The von Willebrand factor (VWF, W/S ratio 0.73) is essential in initial clotting, promoting the adhesion of platelets to the site of vascular injury by bridging the platelet receptor GP1BA and the sub-endothelial collagen matrix. Later in the coagulation cascade, VWF stabilizes coagulation factor VIII (F8). Thrombospondin (THBS1) is a multifunctional protein mediating cell-to-cell and cell-to-matrix interaction. THBS1 (W/S ratio 1.12, p = 0.08) and GP1BA (W/S ratio 1.8) were observed in the plasma of four bears only, indicating levels marginal to our detection. Histidine-rich glycoprotein (HRG, W/S ratio 0.14) binds a multitude of ligands, including THBS1. Both HRG and THBS1 interact with an abundance of biological partners, and the apparent increase in THBS1 could indicate a major role in other pathways.

The blood plasma coagulation cascade is initiated by two pathways that both activate factor X (F10, W/S ratio 1.2) leading to fibrin clot formation (Fig. 2). The intrinsic (contact activation) pathway begins with complex formation of kininogen (KNG1, W/S ratio 0.67), kallikrein (KLKB1, W/S ratio 0.58), and factor XII (F12, W/S ratio 0.86, p = 0.1) on collagen. This results in activation of F12, which cleaves F11 (not detected), which then
cleaves F9. F9 (W/S ratio 0.70), assisted by its cofactor F8 (not detected) cleaves F10. However, the role of F12, if any, in normal hemostasis is unclear as humans and animals totally deficient in F12 have no bleeding tendency. Kallistatin (SERPIN A4, W/S ratio 0.49) inhibits kallikrein. Hence, the intrinsic pathway was significantly down-regulated in hibernation. The extrinsic, or tissue factor pathway components tissue factor (F3) and factor VII (F7) were not detected in winter or summer, which indicates low activity of this pathway. Fibrin coagulation is achieved by thrombin (F2, W/S ratio 1.3), which is activated from prothrombin by F10 and cofactor Va (F5, W/S ratio 0.98, p = 0.44). Activated F5 and F8 are degraded by PROC protease (W/S ratio 0.80). Anticoagulant PROS assists PROC but the levels of PROS increased in winter to 1.5-fold the summer levels, which suggest PROS may have additional unknown roles.

Most remarkably, the levels of the ultimate clotting effector proteins F10 and F2, and those of the fibrinogen substrate subunits FGA (W/S ratio 1.1, p = 0.2), FGB (W/S ratio 1.2, p = 0.12), and FGG (W/S ratio 1.2, p = 0.13) increased in winter, and to approximately the same extent. Indeed, the stoichiometric 2:2:2 ratios of fibrinogen subunits FGA, FGB, and FGG changed to the same extent in the seven individual bears; the non-significant p-values reflect that the W/S ratios among the bears varied between 0.8 and 2.6. The data show that the central clotting mechanism is fully active during hibernation. By contrast, all other components of fibrin clot formation and degradation had moderately decreased levels. Fibrin monomers are cross-linked by factor XIII, which is composed of subunits F13A1 (W/S ratio 0.80, p = 0.23), and F13B (W/S ratio 0.52). Fibrin monomer degradation is reduced by carboxypeptidase B2 (CPB2, W/S ratio 0.50).

Plasmin is essential to fibrin clot hydrolysis (Fig. 2). Plasmin is activated from plasminogen (PLG, W/S ratio 0.81) by a number of proteases and has broad specificity and range of biological substrates in addition to fibrin. The major inhibitor of plasmin, alpha-2-antiplasmin (SERPIN F2, W/S ratio 0.79), had a decrease similar to plasminogen. The serine protease hyaluronan-binding-protein 2 (HABP2, W/S ratio 0.67) is also implicated in fibrinolysis. As collagen is involved in clotting, we included the collagen degrading protease xaa-Pro dipeptidase (PEPD, W/S ratio 0.38) among the list of clot clean-up proteins (Supplemental Table S1). A number of specific serine protease inhibitors were down-regulated or had unchanged levels, including SERPINC1 (W/S ratio 0.56), SERPINA1 (W/S ratio 0.86), SERPIND1 (W/S ratio 0.90, p = 0.13), and SERPINA5 (W/S ratio 1.08, p = 0.19). By contrast, levels of the non-specific abundant protease inhibitor alpha-2-macroglobulin (A2M) were significantly increased to 1.7-fold the summer levels, suggesting that A2M can substitute for the specialized serpins.

The spectacular regulated increases of only the key components of coagulation, F10, F2, and fibrinogen, demonstrated by mass spectrometry (MS), were verified and extended (F7, F8) by functional analyses of six factors and immunochromatographic analysis of VWF (Table 1: Fig. 2). The excellent agreements between MS counted factor levels and coagulation functionality demonstrated that molecules are active, and serves as mutual validations of the quantitative data.

Lipid Transport and Metabolism—In hibernating brown bears, energy and water are obtained from catabolism of lipids in adipose tissue, rather than via the digestive tract. Proteome, metabolome, and hematologic data demonstrated this marked switch (Tables S1, S2, S3). Most remarkable, lipase (CEL, W/S ratio 32), the carrier proteins serum albumin (ALB, W/S ratio 1.2), the apolipoproteins APOB (W/S ratio 1.3), APOC1 (W/S ratio 2.3), APOD (W/S ratio 1.7), and cholesterol (Chol) in form of the "bad" low-density lipoprotein (LDL) particles were significantly up-regulated in hibernation.

CEL, which is almost exclusively expressed in the pancreas, is activated by bile salt. ALB transports free fatty acids to all cell types from adipocytes, where they are produced from triglycerides (W/S ratio 2.3). The increase in APOB levels in hibernation is in agreement with the increase in LDL particles in hibernation found by Arinell et al. (14), as each LDL particle contains one molecule of APOB. The significant increases in the winter levels of APOC1 and APOD, apolipoproteins that play important roles in Chol transfer, are in accordance with the significant increases in levels of total Chol (W/S ratio 1.5), and Chol-LDL (W/S ratio 2.6) during hibernation. The Chol from high-density lipoprotein particles (Chol-HDL) was not significantly different in hibernating and active bears (W/S ratio 0.97, p = 0.18; Supplemental Table
Phosphatidylcholine-sterol acyltransferase (LCAT, which produces cholesteryl esters) and phospholipid transfer protein (PLTP) interact with HDL, and both showed non-significant changes in their levels (LCAT W/S ratio 0.86, \( p = 0.21 \); PLPT W/S ratio 0.76, \( p = 0.21 \)). Phosphatidylinositol-glycan-specific phospholipase D (GPLD1, W/S ratio 0.80) and serum paraoxonase/arylesterase 1 (PON1, W/S ratio 0.66) had significant but moderately lowered levels in hibernation.

The human chromosome 11q23 contains a gene cluster that encodes apolipoproteins APOA1 (W/S ratio 0.60), APOC3 (W/S ratio 0.75, \( p = 0.11 \)), and APOA4 (W/S ratio 0.04). These proteins were all down-regulated in hibernation. The very low levels of APOA4 could indicate an active degradation. APOA1 is the major protein component of HDL in plasma, which also contains APOA4. The levels of proteins that interact with HDL were all decreased in winter to ~0.7-fold their summer levels, suggesting that HDL levels might be similarly reduced despite the non-significant change in Chol-HDL levels. A down-regulation of APOC3 would be in accord with the function of this apolipoprotein as an inhibitor of lipoprotein and hepatic lipases (LPL, LIPC), as higher activity of these lipases is essential to the hydrolysis of triglycerides in hibernation. APOC2, APOA5, and APOF levels were below the limit of detection of the proteome approach, and the APOE and apolipoprotein(a) (LPA) sequences were not present in the bear protein database. The levels of individual lipases, including LPL and LIPC, were also below the limits of detection, whereas the levels of CEL, a general lipase that hydrolyzes both triglycerides and sterol esters, increased in winter to 32-fold the summer levels, which is consistent with a greater than 10-fold total lipase activity increase in blood plasma during bear hibernation (Supplemental Table S3).

It is noteworthy that the increased activity in liver and adipose tissues in hibernation did not result in detectable levels of released intracellular proteins from exhausted cells of these tissues.

In contrast with proteins, which normally require an active vesicular transport out of cells, we consider the concentrations of intracellular metabolites to be reflected in their blood plasma levels. We characterized the extreme changes in metabolic conditions in hibernation by targeted mass spectrometry quantifying 40 carnitines; 90 phosphatidylcholines, including lysoPC with one acyl group, PCaa with two acyl groups, and PCae with one acyl and one alkyl ether group; 15 sphingomyelins; 21 amino acids; 19 biogenic amines; and hexose sugars in plasma of hibernating and active bears and a human plasma reference sample (Supplemental Table S2; and Fig. 1B, C). Soluble metabolites need no transporter proteins, and we found none in the bear proteome for carnitines, amino acids, biogenic amines, or hexoses.

Hexose sugars (predominantly glucose, but also fructose, mannose, galactose and others that have identical masses) levels covered a broad range and were increased moderately in hibernation. The mean hexose levels in winter and summer bear samples, and in the human reference were 10.0 mM, 8.9 mM, and 8.2 mM, respectively. This is notably higher than solely glucose measured in enzymatic assays of bear samples (18) (Supplemental Table S2).

Carnitines are required in all tissues for transport of fatty acids from the cytosol to the mitochondria for fatty acid oxidation. All were up-regulated in hibernation, in accordance with the essential catabolism of lipids that occurs during this state. Sum concentration of all carnitines in winter and summer bear samples, and in human samples were 95 \( \mu \text{M} \), 56 \( \mu \text{M} \), and 61 \( \mu \text{M} \), respectively. Phosphatidylcholines (PC), along with cholesterol, are essential constituents of cell plasma membranes. The levels of lysoPC, the products of hydrolysis of PCaa, were increased by a factor ~1.7 in hibernation, which indicates increased cell membrane turnover, a probable result of shrinking adipocytes as lipid stores are depleted. The sum concentrations of lysoPC of hibernating bears and human were similar, 265 \( \mu \text{M} \) and 251 \( \mu \text{M} \), respectively. PCaa is the dominant phospholipid type in bear plasma and its levels increased (W/S ratio ~1.2), whereas the levels of PCae decreased (W/S ratio ~0.8) on average in hibernation. Concentrations of individual PCaa and PCae in bear followed the pattern of the corresponding concentrations of these products in the human sample. However, the total concentrations in bear plasma were 2.6-fold to 4.6-fold those in human. The concentrations of total PCaa in hibernation, summer, and human samples were 4.3 mM, 3.6 mM, and 1.4 mM, respectively. The total PCae concentrations in winter, summer, and human were lower: 664 \( \mu \text{M} \), 846 \( \mu \text{M} \), and 185 \( \mu \text{M} \), respectively.
Sphingomyelins (SM) are components of the cell membrane that are thought to increase chemical resistance. SM levels were similar in hibernating and active bears. However, they were 2- to 5-fold higher than the levels of these metabolites in human plasma. Total sphingomyelin concentrations in winter, summer, and in the human sample were 745 μM, 710 μM, and 257 μM, respectively. Similarly, the levels of SM-cleaving sphingomyelinase-like-phosphodiesterase-3a (SMPDL3A, W/S ratio 0.95, p = 0.34) seemed to be unchanged in hibernation.

The ratio of total unsaturated/total saturated plasma phospholipids (PC plus SM) was unchanged in hibernation, which suggested that cell membrane fluidity was not adjusted to the 6 °C temperature decrease in hibernation.

Nitrogen Metabolism—It is a major challenge to the physiological adaptations for hibernation in bears to conserve and recycle nitrogen in protein metabolism within the metabolic energy available in this condition, while avoiding intoxication. We analyzed amino acids and biogenic amines as phenylosothiocyanate derivatives by targeted mass spectrometry and found the plasma concentrations of these factors in bear were mostly within 50% of the reference range for humans (Supplemental Table S2). Significant physiological differences included up-regulation of molecules containing several nitrogen atoms during hibernation. These molecules included creatinine [W/S ratio 3.9 determined by mass spectrometry (Supplemental Table S2); W/S ratio 3.8 determined by standard clinical analysis (Supplemental Table S3)], ornithine (W/S ratio 3.6), acetyl-ornithine (W/S ratio 1.9), lysine (W/S ratio 2.0), citrulline (W/S ratio 2.6), glutamine (W/S ratio 1.6), carnosine (W/S ratio 2.5), and histidine (W/S ratio 1.4). In addition, proline levels increased 1.8-fold during winter; an amino acid that has not been quantified before. The aromatic amino acids tryptophan (W/S ratio 0.7) and tyrosine (W/S ratio 0.5), as well as sulfur-containing methionine (W/S ratio 0.7) and methionine sulfoxide (W/S ratio 0.2), had lower levels in hibernation. The latter is remarkable, as it may be taken as an indicator of reduced oxidative stress in hibernation. Our biogenic amine data are unique, and our amino acid data are in agreement with the metabolic changes reported by Stenvinkel et al. (18), and Sommer et al. (21) (Supplemental Table S2). Stenvinkel et al. (18) also reported significant 1.24-fold increases in total protein and albumin concentrations similar to our results (bottom of Supplemental Table S3). It is notable that our data and the available published nitrogen-related data, quantified by different methods, provide reciprocal validations.

Systemic Regulation—Sex-hormone-binding-globulin isoform 1 showed the highest increase in hibernation levels among all plasma proteins (SHGB, W/S ratio 45). This dramatic up-regulation was verified by western blot analysis, showing SHBG immuno-reactive bands in the plasma only from winter bears at ~47 and ~51 kDa (Fig. 1D). Human circulating and recombinant SHBG showed similar bands due to heterogeneous N-glycosylation forms (22). Our analysis of the human reference plasma sample did not reveal any SHGB immuno-reactive bands, suggesting that the SHGB levels in hibernating bears is markedly higher than the average human plasma levels (normal range 20-120 nM in adults). The very high SHGB levels in hibernating bears challenges the general understanding of SHBG’s primary role as regulator of androgen bioavailability.

Seven other carriers of hydrophobic vitamins and hormones were down-regulated: beta-2-glycoprotein 1 (APOH, W/S ratio 0.69), alpha-1-acid-glycoprotein 2 (ORM2, W/S ratio 0.63), afamin (AFM, W/S ratio 0.87, p = 0.07), vitamin D-binding-protein isoform 3 (GC, W/S ratio 0.89, p = 0.16), retinol-binding-protein 4 (RBP4, W/S ratio 0.78), corticosteroid-binding-globulin (SERPIN A6, W/S ratio 0.79), and transthyretin or thyroid-hormone-binding-protein (TTR, W/S ratio 0.67). Also thyroglobulin (TG), the precursor of thyroid hormones, was decreased in winter to 0.15-fold its summer levels. This observation is consistent with the lower levels of free thyroxine and triiodothyronine observed in hibernating captive black bear Ursus americanus and may, at least in part, explain the general lowered basal metabolism that characterizes hibernation (23).

Remarkably, the levels of the lipid mobilization factors adiponectin (ADIPQ, W/S ratio 0.58) and zinc-alpha-glycoprotein (AZGP1), which is present at high levels in cancer cachexia and low in people with obesity; W/S ratio 0.25) were significantly decreased in hibernating bears, suggesting that bears utilize other systemic mediators of lipid mobilization during hibernation.

The decrease in the levels of the insulin-like-growth-factor-binding-protein-complex-acid-
labile-subunit (IGFALS, W/S ratio 0.15) suggests a significant down-regulation of major circulating components of the IGF system in hibernating free-ranging brown bears, as also observed in captive American black bears (24). IGFALS is circulating in complex with IGF-1 and IGF-binding-protein 3 neither of which were identified, possibly due to very few detectable tryptic peptides of these proteins.

Discussion

We compared the levels of 154 plasma proteins covering four orders of magnitude of molar concentration, quantified 144 metabolites and blood cells in the same seven subadult free-ranging brown bears in winter hibernation and summer activity, and discovered a number of specific adaptations to hibernation involving defense against pathogens, lipid transport and metabolism, iron and nitrogen conservation, total protein levels and half-lives. The changes observed suggested a hibernation survival strategy using protective proteins with antimicrobial, clean-up and antioxidation properties, and conservation of energy in hibernation by down-regulating complex multiprotein cascades and up-regulating a small number of generalist and principal effector proteins, such as one lipase (CELL) and one protease inhibitor (A2M), both of broad-specificity. The unique regulation of coagulation factors were verified by functional measurements (Table 1; Fig. 2). SHBG increased 45-fold in hibernation and appears to play a significant role in the maintenance of hibernation physiology. It is also notable that intracellular components from leaking or broken fat, muscle, bone, or liver cells were not detected. The biochemical data have been interpreted in the context of known physiological adaptations.

Blood Characteristics—Red bloods cell count, total hemoglobin levels, and the hematocrit were increased to 1.3-fold of the summer levels in hibernation. The summer levels are in agreement with recently published brown bear reference data (25). Total plasma protein concentration in hibernation was significantly increased by 9.6% in the seven 2010 samples, and by 24.1% in seven 2013 samples (Fig. 1A, Supplemental Table S5). The high hibernation levels of total protein were similar among all bears and similar to the normal human levels, whereas the lower summer levels showed considerable variance, because blood plasma concentration depends on the changing water content of available summer forage. ALB, which composes ~50% of plasma protein, was increased to 1.18-fold its summer levels (Supplemental Table S1). Stenvinkel et al. (18), using different methods, reported increases in total protein and ALB levels to 1.25-fold and 1.24-fold, respectively, in plasma samples from 16 hibernating young brown bears. These data explain both the lower yield and higher viscosity of plasma obtained from hibernating brown bears. The increased hematocrit to 1.3-fold of summer levels in hibernation is probably the best measure of dehydration.

Defenses—Of 36 proteins with defense properties that were measured by mass spectrometry in this study, 32 were significantly decreased in hibernation, including those of the lectin, alternative, and antibody complement pathways, the membrane pore-forming complex, and regulatory proteins including CRP (Fig. 2). In contrast, the levels of four protective antimicrobial proteins, CAMP, HP, LYZ, and PGLYRP2, and the antibody that is found in body secretions, IGA1, were more than doubled in hibernation (Supplemental Table S1). Hence, the hibernating bear can maintain solid first-line defense by de novo synthesis of just a few generalist proteins and a secreted antibody rather than maintaining complex multiprotein pathways, thus saving energy.

HP, besides being antimicrobial, antioxidant and a recycler of iron (20), is most known as the kidney-protecting scavenger of hemoglobin released from bruised blood cells. Winter up-regulated APCS have similar protective scavenging properties. Among these defense proteins, only the increases in HP hibernation levels have been documented previously (2- to 6-fold) (26). Besides HP, this immunochemical study of plasma collected samples at successive months during one year from captive brown bears included A2M (increased in hibernation), SERPINA1 (unchanged), and CRP (unchanged), and showed the same trends as our data (Supplemental Table S1). A comparative study of hibernating versus pre-hibernating captive female American black bear sera found 4-fold increases in HP levels (27). However, among the 15 proteins described in this two-dimensional gel electrophoresis study, our data showed pronounced disagreement with five of the reported changes. We
suggest that the disagreement is due to the experimental design of Chow et al. (27), rather than the investigated bear species.

The innate and acquired cellular and humoral immune defenses were decreased, not abolished, in hibernation. As illustrated in Fig. 2, our data covers most components of the complement system and consistently demonstrated similar levels of reduction, including subunit levels of C1 and of the MAC complexes. Moreover, the complement regulatory proteins were reduced similarly to their targets. Our study is the first to distinguish the complement pathways of activation.

Coagulation and fibrinolysis must be strictly regulated to avoid thrombosis and pulmonary embolism. It appears that most specialized effector and regulator proteins of these pathways decreased in hibernation and were decreased in a balanced way as in the complement cascades. Yet hemostasis was maintained during hibernation increasing only the levels of the key effectors F10, F2 and fibrinogen, and the non-specific regulator A2M (Fig. 2). A recent study in 10 wild American black bears demonstrated that normal wound healing was maintained in hibernation, despite reduced skin temperatures, which were 4-6 °C lower than the hibernation body temperature (28). The first step in healing includes initial coagulation, which we showed is geared up by the slight increased levels of F10, F2 and fibrinogen, which will facilitate rapid initial response. Furthermore, our demonstrated increases in antimicrobial protein levels could indicate protection against wound infection, and might additionally substitute for inflammation, the second normal response in wound healing (28), as we found the levels of innate cells and components much reduced in hibernation.

The concentration of A2M, which has unspecific antiproteolytic activity, increased 1.7-fold in hibernation, whereas the specific SERPIN-type inhibitors, except for the anticoagulant SERPINA5, were significantly decreased in plasma (Fig. 2). Increased levels of A2M protein in hibernating brown bears was also found by Mominoki et al. (26); and Sheikh et al. (29) demonstrated similar increases in serum A2M from wild black bears using anti-bear A2M rabbit polyclonal antibodies. Most studies have applied anti-human antibodies, which in many cases have borderline cross-reactivity with bear proteins in our experience, due to limited amino acid sequence identity (Supplemental Table S1). Bear and human A2M are 81% identical in sequence and do cross-react, because of their large 180 kDa subunit sizes, which increase the probability of conserved immunogenic epitopes.

Intriguingly, Fuster et al. (30) reported a 40% decrease in protein degradation of isolated rat skeletal muscle incubated with plasma of hibernating brown bear versus controls without bear plasma. We propose the most likely inhibitor candidate is A2M. On proteolytic attack by any type of protease, tetrameric A2M changes its conformation due to activation of an internal thiol ester, and catches one molecule of the attacking protease thus inhibiting its activity. Therefore, we propose A2M might have a broad medical potential in reducing plasma protein degradation. Human A2M plasma concentrations are normally high (31), and vary considerably among individuals and with age (32). Hence, humans seem to tolerate increased A2M levels well, like bears in hibernation, which on average benefit from 1.7-fold increase in A2M levels.

Lipids—Some plasma proteins participating in lipid hydrolysis and transport were significantly up- or down-regulated in hibernation, which is concurrent with a switch from food ingestion to lipid oxidation. A hibernating 60 kg American black bear loses ~80 g fat per day (33). Adipose tissue releases free fatty acids and glycerol, and the resulting shrinking of adipocyte membranes supported by dehydration are probably the main sources of increased phospholipid and cholesterol concentrations in the bloodstream during hibernation (Supplemental Tables S2, S3). The similar concentrations of sphingomyelins in winter and summer plasma were 2- to 5-fold greater than the concentrations in human plasma (Supplemental Table S2), and may protect bear cell membranes against degradation and rupture. This might explain the absence of intracellular components in our plasma data.

Chauhan et al. (34) separated serum cholesterols and some phospholipids by thin layer chromatography from four adult female American black bears, and found significant increases in free and esterified cholesterol, and in total SM, and total PC levels, which resemble the trends in our comprehensive data. Recently, Sommer et al. (21) analyzed metabolites by mass spectrometry in serum from 12-15 wild brown bears, and confirmed
the presence of significantly increased levels of free cholesterol, cholesteryl esters, and triglycerides in hibernation, and also determined the levels of various bile salts, which decreased in hibernation. The study also found a general increase in hibernation in the levels of even chain acylcarnitines only. In contrast, we find a general increase in nearly all acylcarnitines (Supplemental Table S2), and our absolute concentrations are significantly higher than those reported by Sommer et al. (21).

Triglycerides are synthesized in the liver after uptake of free fatty acids and glycerol and provide energy to other tissues. The concentration of most lipids in plasma increased or was unchanged in hibernation (Supplemental Table S2). Major transporter proteins, ALB (which transports free fatty acids), APOB (which transports cholesterol, triglycerides and phospholipids in LDL and VLDL particles), as well as the predominant broad-specificity CEL lipase (increased by 32-fold), and total lipase activity were significantly up-regulated in hibernation. In contrast, the levels of a number of proteins interacting with HDL particles were decreased. APOA4 was extremely reduced to 0.04 times its summer levels, which is consistent with the function of the protein in signaling intestinal lipid uptake. The observations that only broad-specificity carriers of multiple lipid classes, APOB and ALB, and the generalist CEL lipase were increased in hibernation support our hypothesis of a common strategy of keeping generalist proteins at increased or normal activity levels, and let more specific ones decrease in hibernation.

Arinell et al. (14) reported increases in the levels of total cholesterol, LDL and triglycerides in hibernating brown bears and showed that none of 12 examined bears exhibited signs of atherosclerosis, unlike humans with similarly elevated plasma lipid levels. Our data support this important finding. We found 1.3-fold higher levels of APOB, the core protein in LDL and VLDL particles, in winter than in summer.

Nitrogen Metabolism and Protein Half-Lives in Hibernation—Absence of urination and the recycling of amino acids for protein synthesis during hibernation require that nitrogen be retained in a non-toxic form. Our analysis of amino acids and biogenic amines disclosed increased levels of molecules containing two or more nitrogen atoms (Supplemental Table S2), a strategy that has not been noticed before.

Intracellular proteins have short half-lives, minutes to days, whereas circulating ALB and IGG can last for months in humans (35). In hibernating bears all plasma protein half-lives will increase markedly, and reduce the need for costly protein synthesis for three reasons. (i) The increased levels of A2M, discussed above, will decrease protein turn-over by reducing all protease activity. (ii) Reaction rates, including protein degradation, will decrease with temperature. A generalization of Arrhenius’ equation implies that the rates of chemical reactions at room temperature will double for every 10 °C increase in temperature. The temperature in hibernating non-pregnant bears is lowered by 6 °C on average (5-7). (iii) Cardiac output in free-ranging hibernating brown bears is reduced to 24% from 3.5 to 0.86 L min⁻¹ (8), i.e. similar to the 25% level of oxygen consumption or metabolic rate in hibernating American black bear (5). Jørgensen et al. (8) also observed echo-dense structures in the hearts of all the hibernating bears caused by aggregation of red blood cells and plasma proteins. Therefore, rates of productive encounters of biochemical partners will decrease, due to reduced mixing rates in blood, as well as increased plasma viscosity and, consequently, decrease protein modification rates and turn-over in hibernation.

Protein Levels and Energy Conservation—The increased hematocrit in hibernation demonstrates significant dehydration. In hibernating bears water comes from fat metabolism exclusively. The complete reaction of 1 mol of triglyceride (0.89 kg triostearylglycerol C₃₇H₇₀O₆) and oxygen yields 55 mol of water, 0.99 kg or 1 L. Since ~80 g fat is burned per day, it takes 11 days to produce 1 L water. Water loss is mainly through the breathing of saturated water vapor at the body temperature of hibernation at ~32 °C, whereas the air in the den at subzero temperatures gives little water vapor back. We conclude that dehydration is substantial and contributes to the maintenance of plasma protein concentrations in hibernation, thereby saving much novel protein synthesis and energy. Moreover, ALB constitutes ~50% of total plasma protein and has a very long half-life, which alone contributes to significant energy savings in hibernation.

Energy conservation is a key issue in all organisms and vital in hibernation. Protein
synthesis at unstressed conditions consumes more than half of organismal energy. Furthermore, it can be strictly regulated for energy savings in various ways. Li et al. (36) quantified absolute protein synthesis rates by deep sequencing of ribosome-protected mRNA fragments in *E. coli* grown on limited and rich media. They concluded that subunits of multiprotein complexes are synthesized in equal proportion regulated by translation initiation and that this rule also applies to yeast, whereas protein synthesis in multicellular organisms might be fine-tuned by additional rules. Pannevis and Houlihan (37) found that isolated hepatocytes of rainbow trout used 70-90% of their total oxygen consumption for cycloheximide-sensitive protein synthesis, and that the energy costs were lowered at decreased rates of protein synthesis, i.e. subject to regulation. This relates to hibernating bears, as liver is the principal tissue for plasma protein synthesis, and the rate of protein synthesis is low.

Schwanhäusser et al. (38) quantified mRNA and protein levels, and their half-lives globally in pulse labelled mouse fibroblast cultures, and derived a model predicting protein abundance in cells. Genes with stable mRNAs and stable proteins were enriched in constitutive cellular processes maintaining basic metabolism, suggesting an evolutionary pressure for energy saving. Moreover, they found that protein synthesis consumes more than 90% and transcription less than 10% of cell energy. Thus it is plausible that bears can save expensive translational energy and stay alert, just by having increased levels of mRNA ready for the needed proteins and the translational machinery, at a low energy cost. Indeed, Fedorov et al. (11, 12) in micro array studies of gene expression in American black bear liver, heart, and muscle tissues showed a marked up-regulation of mRNA from genes participating in translation in these tissues. Liver also showed up-regulation of fatty acid beta-oxidation in hibernation as expected. In contrast, amino acid catabolism, cholesterol metabolism, and cellular respiration were significantly down-regulated in hibernation.

In hibernating American black bears protein synthesis and break-down were balanced in skeletal muscle and were 60-70% decreased in winter compared to summer (10). Thus even maintaining the levels of all muscle proteins lead to energy savings approaching the required 75% in hibernation. Therefore, we conclude that decreased levels of the majority of blood plasma proteins and the maintenance or increase of levels of just a few key proteins in blood plasma by liver protein synthesis is the principal energy saver in bear hibernation adaptation, due to dehydration, a lowered temperature, a general down-regulation of translation, and a balanced translation of effector and regulator proteins in the complement and blood clotting pathways.

**Systemic Regulation**—It remains obscure how hibernation is initiated, maintained and completed, although the 45-fold increased levels of SHBG in hibernation (confirmed by immunoblotting Fig. 1D) provides a clue to the maintenance of hibernation physiology. SHBG is generally considered the primary regulator of androgen bioavailability, as seen in the seasonal changes in SHBG concentration in the hibernating little brown bat, having low baseline SHBG levels during the October-April hibernation period, and dramatically peaking during the sexually active June-July period (39). In contrast, testosterone levels in American black bears are high March through mid-July, and low mid-July through December, independent of nutritional and social factors (40). Palmer et al. (41) confirmed and extended the black bear testosterone data, which indicated 2-fold higher levels in January-March than in July-November. We suggest this difference may compensate for hormones bound to the high levels of SHBP in this condition, thus keeping free hormone levels constant. The hibernating European hedgehog appears to be more similar to bear with increased SHBG levels in September-January with a sharp decline in February, reverse of testosterone levels (42). We conclude that bear SHBG primarily acts sex hormone-independently and can activate the SHBG receptor directly. In target cells the SHBG receptor increases cAMP production, which activates protein kinase A (43), thus activating signaling pathways different from sex hormone activated pathways. In fact, high circulating SHBG levels in humans protect against metabolic syndrome, and increases insulin sensitivity independent of sex-hormone concentrations (44). In addition, high SHBG levels have been found to correlate with lower cardiovascular disease risk factors in young men (45). Recently, SHBG deficiency was found in a man suffering from muscle weakness and fatigue, suggesting a direct impact of SHBG also on muscle
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maintenance (46). We conclude that high SHBP levels appear to be beneficial to health via presently unknown mechanisms.

Limitations and Prospects—Quantification of signaling proteins and hormones present at a very low concentration or of small size was outside the capability of our methodology. The bear protein database was effective, but had shortcomings, with some expected proteins missing and others with inaccurate translation.

We have determined and interpreted the changed levels of blood and plasma constituents in free-ranging subadult brown bears on a global scale in winter and summer with emphasis on conservation of health and energy in hibernation. Our results reveal a number of unexpected changes in protein concentration in hibernation, some expanding fundamental physiologic understanding derived from human and non-hibernating animal models. Study of the function and regulation of these proteins and their genes, in the light of bear physiology, will add to and refine present interpretations of complex mammalian biochemical networks and might encourage the development of novel clinical therapies.

Experimental Procedures

Animals—The Scandinavian Brown Bear Research Project (http://www.bearproject.info) collects blood samples twice a year from free-ranging (wild) bears in Dalarna, Central Sweden, during hibernation and activity. All captures were approved by the Swedish Ethical Committee on Animal Research (application numbers C212/9 and C47/9) and the Swedish Environmental Protection Agency. Bears were fitted with GPS collars and VHF transmitters on their first capture at one year of age, when still with the mother. The collared bears were immobilized from a helicopter in summer by a combination of tiletamine-zolazepam and medetomidine, and in the dens in winter giving also ketamine. Anesthesia was antagonized by atipamezol, and the hibernating bears were placed back into the dens. Details on the methods of bear capture, anesthesia and blood sample collection have been published previously (16). Seven subadult bears were analyzed in the present study, B1 through B7 (alias bear ID 0824M, 0910M, 0825F, 0820F, 0819F, 0818F, and 0812M, respectively), comprised four 3-year old nulliparous females, and two 3-year old and one 2-year old males.

Blood and Plasma Samples—Arterial blood was collected in EDTA-tubes (K2E 10.8 mg, 6 mL, BD ref 365900, Becton, Dickinson and Company, NJ USA) during hibernation in February 2010 and again from the same animals when active in June 2010. The blood was kept on ice until centrifugation at 200 g for 15 min, immediately upon returning from the field after 1-2 h. The yield of plasma from winter blood was notably lower. Plasma was frozen and kept on dry ice during shipping. Plasma samples were thawed only once, flash frozen in ca. 25 µL pearls in liquid nitrogen, and stored at -80 °C. A pooled normal human plasma reference sample, K2 EDTA lot 23-40651A (Innovative Research, Mi USA), was treated similarly. Similar paired samples collected in 2012 and 2013 were used for specific validation tests.

Hematology—Blood cell counts and standard biochemical analyses were carried out at the accredited Clinical Chemical Laboratory at Örebro University Hospital, Sweden, as described previously (6, 14). Blood cell analyses were carried out by an automated hematology analyzer (XE-5000, Sysmex Corporation, Kobe Japan); and biochemical analyses in the integrated Vitros instrument (Ortho-Clinical Diagnostics, Rochester, NY USA). Coagulation analyses were carried out at the accredited Clinical Chemical Laboratory at Linköping University Hospital, Sweden, utilizing an automated coagulation analyzer ACL TOP (Instrumentation Laboratory, Bedford, Massachusetts, USA). Factors F2 and F7 were determined using a one-stage clotting assay with deficiency plasmas (Precision BioLogic, Dartmouth, Canada); Fibrinogen was measured by the Clauss method determining the time of clot formation; F8 and PROC were measured by proteolytic cleavage of specific chromogenic peptides, and SERPINC1 indirectly by inhibition of F10 activity in a chromogenic assay. VWF was quantified in an immunoassay.

Immunoblotting—Plasma (0.25 µL) was subjected to 4-20% reducing SDS-PAGE. A prestained protein ladder 10-170 kDa (0.5 µL) was included (SM0671, Fermentas). Proteins were blotted to a nitrocellulose membrane and incubated in sequence with 2 mL rabbit polyclonal anti-SHBG primary antibody (ab119436; a 100 residues linear human polypeptide, with segments identical
in amino acid sequence to bear and different from rabbit SHBG, was used as immunogen), Abcam, Cambridge UK) in TBS-1% BSA (1:200), and 2 mL donkey anti-rabbit/Alexa Fluor 647 conjugated secondary antibody (ab150075) in TBS-0.1% Tween20 (1:2000) applying TBS-Tween20 washes in an iBind Western System (Life Technologies). Proteins were visualized in a ChemiDoc MP imaging system (Bio-Rad, CA USA).

**Plasma Protein and Hemoglobin Concentration**—Ultraviolet and ultraviolet-visible absorption spectra were recorded directly on 2 µL undiluted plasma samples using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, De USA). The instrument automatically used a 0.2 mm path length for protein measurement at 220-350 nm and reported the values for 1 cm path length. Owing to a visible content of hemoglobin, the automatic normalizing A340 nm = 0 was turned off. Spectral data were transferred to an Excel spreadsheet (Microsoft Office 2013) and normalized to identical A340 nm values. A280 nm was then used as a measure of protein concentration in g L⁻¹, however corrected by our estimated E0.1%cm⁻¹ = 0.87 for bear plasma (Table S5).

Hemoglobin (HB) spectra were recorded at 220-750 nm at 1 mm light path length. The spectra were normalized in Excel by setting the measured mean values 650-750 nm = 0 and adjusting to 1 cm path length. The spectra showed that HB was fully oxygenated with the Soret peak at 415 nm. The extinction coefficient for tetrameric human oxyhemoglobin at 415 nm, 524280 M⁻¹cm⁻³, was applied.

**Protein Digestion and Peptide Desalting**—For proteomic analyses the tryptic digestion, peptide desalting and MS analyses were performed in two batches of samples. Batch 1 included winter and summer pairs of bear samples B1, B2, a sample of recombinant human insulin-growth-factor-like (IGF1) in complex with IGF-binding-protein 3 "paired" with a water blank, and winter and summer pairs of bear samples B3, B4, B5, B6, and B7, i.e. a total of 16 samples run in the sequence shown, each measured in three consecutive technical replicates. Batch 2 included the commercially available human plasma reference samples (Hs) paired by itself, the B2 pair for a second time (which was due to first round MS instrument failure), and ProteoMiner-enriched bear mixed plasma samples pairs from winter and summer, i.e. six samples prepared and measured in parallel each in three technical replicates.

Protein was digested with trypsin essentially as described by Wiśniewski et al. (47). Plasma (3 µL) protein was unfolded and reduced at 95 °C for 10 min after mixing with 4% SDS, 100 mM DTT, 5 mM EDTA in 0.1 M TrisCl pH 8.5 (17 µL). A volume containing 100 µg protein was transferred to the filter unit (reactor) of a 30 k Amicon ultra cell containing 200 µL 8 M urea in 0.1 M TrisCl pH 8.5. Centrifugations between steps were carried out at 13,500 g for 15 min. An extra wash was carried out before alkylation, which was carried out in the same solvent containing 25 mM iodoacetic acid for 20 min at room temperature. After three washes using 100 µL 8 M urea in 0.1 M TrisCl pH 8.0, urea concentration was reduced by washing with 200 µL 2 M urea in 0.1 M TrisCl pH 8.0. Collection tubes below the filter reactors were replaced with clean ones before sequencing-grade modified porcine trypsin (Promega, Wi USA) 1 µg in 2 µL and then 50 µL 2 M urea in 0.1 M TrisCl pH 8.0 were added to the filter reactor and mixed by tapping. Digestion at room temperature was continued for 20 h. Peptides were spun down into the collection tube and further washed down by 60 µL 2 M urea in 0.1 M TrisCl pH 8.0, and at last by 200 µL water. Peptides were adsorbed to 1 mg washed R3 Poros beads (Applied Biosystems, Ca USA) in 20 µL suspension of 5% formic acid added to the collection tube, together with 5 pmol human [Glu₁]-Fibrinopeptide B for internal MS standard (Sigma-Aldrich, Mi USA) just before the final peptide extraction by water. Beads were collected by centrifugation at 4,000 g for 1 min and washed twice with 200 µL 1% formic acid. Supernatants were removed carefully by micro pipetting. Beads with bound peptides were stored in 50 µL A solvent (0.1% trifluoroacetic acid in 2% acetonitrile) at 4 °C. The pH values were confirmed at all critical steps by applying a fine drop on pH paper.

Columns of 200 µL pipette tips were plugged with a disc of polystyrenedivinylbenzene (Empore SDB-XC; 3M company, Mn USA) punched out by a metal syringe needle and washed with 5 µL B solvent (0.1% trifluoroacetic acid in 90% acetonitrile) followed by 10 µL A solvent. A volume of suspended R3 beads with peptides were transferred to the column and allowed to drain, then washed with 20 µL A solvent, and eluted into an MS-sample plate, twice with 5 µL B solvent and
finally with 10 µL A solvent to elute remaining dissolved peptides. The acetonitrile content was reduced by evaporation, and the wet peptide sample (approximately 25 µg) was diluted by 30 µL 0.1% formic acid, 0.005% heptafluorobutyric acid.

Test experiments prior to final digestions had protein and peptide yields monitored by UV absorption spectroscopy (NanoDrop) as above. Peptide yield was > 50% of original protein content. Initial peptide binding by C18 filters for desalting (3M, Mn USA) was insufficient in our hands. Comparison of adsorption and release of peptides by R2 versus R3 Poros beads demonstrated a higher binding capacity and complete elution from the R3 beads.

**LC-MS/MS Data Acquisition**—The LC-MS/MS analysis was done using a Dionex Ultimate 3000 nanoLC system connected to a quadrupole Orbitrap (Q Exactive) mass spectrometer equipped with a NanoSpray Flex ion source (Thermo Fisher Scientific, Ma USA). The flow settings were 8 µL per min for the sample loading onto a 2 cm Acclaim PepMap100 C18 100 µm ID pre-column with particle size of 5 µm. The nanoflow was set to 300 nL per min for the peptide separation on the analytical column. The analytical column was a 50 cm Acclaim PepmapRSLC C18 column with 75 µm ID and 2 µm particle size (Thermo Fisher Scientific). The nano-electrospray was done using a Picotip ‘Silicatip’ emitter (New Objectives Inc., Ma USA). The LC solvents were A: 0.1% formic acid, 0.005% heptafluorobutyric acid for the aqueous phase, and B: 90% acetonitrile, 0.1% formic acid, 0.005% heptafluorobutyric acid for the organic phase. The applied gradient was from 12% to 20% solvent B over 60 min, followed by 20% to 40% solvent B over 60 min. Initial tests showed that this gradient gave the best distribution of plasma protein tryptic peptides over the entire gradient.

The Q Exactive mass spectrometer was operated in a data-dependent acquisition mode. A full MS scan in the mass range of 350-1,850 m/z was acquired at a resolution of 70,000 with an automatic gain control (AGC) target of 3×10^6 and maximum fill time of 60 ms. The precursor ions were isolated using a quadrupole isolation window of 3 m/z, and then fragmented in the higher-energy collisional dissociation (HCD) trap with a normalized collision energy set to 30. The under-fill ratio was set to 3.5% with the intensity threshold at 1.2×10^4. Apex triggering was 3 s to 10 s with charge and exclude isotopes exclusion on, and dynamic exclusion set to 30 s. The mass spectrometry proteomics data are available via ProteomeXchange with identifier PXD003946 at http://www.ebi.ac.uk/pride.

**Quantitative Data Analysis Using Progenesis LC-MS Software**—Three technical replicates for each plasma sample were generated using LC-MS/MS as described above. The six raw data files (winter and summer) for each bear and for controls were analyzed separately in Progenesis LC-MS v4.1 (Nonlinear Dynamics, Newcastle Upon Tyne, UK). The raw file features were automatically aligned to an assigned reference file among the six raw files, and peak picking was also done in automatic mode. Peak filtering was carried out with respect to charge state (up to 7 charges) and retention time features within 30 min to 140 min were kept. Protein identifications were obtained using Mascot Server v2.3 (Matrix Sciences, London UK). The database search parameters were precursor and fragment tolerances of 6 ppm and 20 ppm, respectively. One missed cleavage was allowed, cysteine carboxymethylation was fixed, whereas methionine oxidation was set as a variable. False discovery rates (FDR) were 0.3-0.95% with an average of 0.71%. The protein database (Uma db, Supplemental Bear-protein-db-Febr2013.txt) was translated from predicted polar bear Ursus maritimus coding sequences (17). The genome sequences of polar bear and brown bear are similar, showing, on average, two substitutions per 1,000 bp (48). 93% could be annotated by homology using a BLASTP search against the human refseq database (E-value ≤ 1×10^-5). This Uma db contained 21,143 entries; 42% showed ≥ 90% identity to the human homolog. The Uma db yielded approximately 12% more identified proteins than our initial db based on a combination of translated giant panda Ailuropoda melanoleuca genes and American black bear Ursus americanus EST sequences (49, 50).

The Progenesis LC-MS data output was an Excel sheet containing normalized ion counts (summed using unique peptides) for each protein.
(Supplemental Table S5). All data from the seven bears were merged using the mean values of the three technical replicates for each sample. The data was then scaled to normalize for the differences in the Progenesis LC-MS output for each bear. The normalized mean values were used to calculate W/S ratios (fold changes) and p-values (paired t-test). W/S ratios of proteins identified by two unique peptides and seen in at least four of seven bears were saved in Proteins.xlsx (Supplemental Table S1) using the gene names of human homologs, together with their Uma and Uniprot ID, fold change and log2 fold change, paired t-test p-values, and Uniprot function descriptions. Principal component analysis and Volcano plots were done in R version 3.1.1 using the ggplot2 package (51, 52).

Identification and Quantification of Metabolites—The same seven paired sets of winter and summer bear, and the human reference plasma samples were subjected to metabolite concentration determination by the AbsoluteIDQ p180 kit (Biocrates Life Sciences, Innsbruck, Austria). Nineteen biogenic amines, 19 amino acids, 1 hexose, 40 acylcarnitines, 76 phosphatidylcholines, 14 lysophosphatidylcholines, and 15 sphingomyelins were analyzed in a 96-well plate format essentially as described by the manufacturer. Each plasma sample (10 µL) was treated with phenylisothiocyanate to modify amines. Analytes were quantified relative to internal standards of isotopic labelled homologs by multi reaction monitoring QTRAP mass spectrometry and electrospray ionization using an ABI Sciex API5500Q-TRAP instrument (AB SCIEX, Ma USA). Each sample was subjected to LC-MS analyses of biogenic amines and amino acids, and to flow injection analysis-MS/MS of the remainder. Both measurements were performed in duplicate and in positive and negative ionization mode. The dedicated MetIDQ software (Biocrates Life Sciences) provided automated calculation of metabolite concentrations.

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This article contains Supplemental Tables S1 to S5, and the database Bear-protein-db-Feb2013.txt.


Hibernation Survival Strategies in Brown Bear


TABLE 1
Bear coagulation factor quantifications by mass spectrometric and functional analyses

MS data are from seven bears sampled in winter (W) and summer (S) 2010 (Table S1). Functional data includes subadult bears sampled in 2010, 2011, 2012 (Table S4). Mean W/S ratios and p-values from t-tests are paired, i.e. the same bear sampled in W and S. SERPINC1 is antithrombin; PROC, protein C; VWF, von Willebrand factor; FGA, FGB, FGG are A, B and G chains of fibrinogen. VWF was verified only by an immunoassay.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MS data Mean W/S</th>
<th>Functional data Mean W/S</th>
<th>p-value</th>
</tr>
</thead>
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<tr>
<td>SERPINC1</td>
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<td>0.49</td>
<td>0.001</td>
</tr>
<tr>
<td>PROC</td>
<td>0.80</td>
<td>0.77</td>
<td>0.002</td>
</tr>
<tr>
<td>F2 (FII)</td>
<td>1.28</td>
<td>1.18</td>
<td>0.013</td>
</tr>
<tr>
<td>F7 (FVII)</td>
<td>0.65</td>
<td>0.65</td>
<td>0.022</td>
</tr>
<tr>
<td>F8 (FVIII)</td>
<td>0.30</td>
<td>0.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>F10 (FX)</td>
<td>1.24</td>
<td>0.75</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VWF</td>
<td>0.73</td>
<td>0.75</td>
<td>&lt;0.001</td>
</tr>
<tr>
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<td>1.14</td>
<td>0.20</td>
</tr>
<tr>
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<td>1.23</td>
<td>0.12</td>
</tr>
<tr>
<td>FGG</td>
<td>1.21</td>
<td>1.21</td>
<td>0.13</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>1.2</td>
<td>1.2</td>
<td>0.24</td>
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Figure Legends

FIGURE 1. Protein and metabolite changes in blood plasma in free-ranging brown bears during hibernation in winter and in the same bears when active in summer. (A) Absorption spectra of undiluted plasma samples collected in winter (W) and summer (S) from seven brown bears (bear IDs at top right. M, male; F, female. All were 3-year old, except for the 2-year old 0910M). Top: Total protein at 280 nm; bottom: Hemoglobin at 415 nm (Supplemental Table S5). (B) Principal component analyses of plasma proteins, amines and phospholipids showed a clear separation of winter (red) and summer (blue) plasma. (C) Volcano plots of W/S ratios of plasma proteins, amines and phospholipids versus p-values from paired t-tests. Dashed lines at p = 0.05. (D) Immunoblot of sex-hormone-binding-globulin (SHBG) confirming the large increase in hibernation [proteomics detected 45-fold increased levels in winter plasma (p = 0.0006)]. The arrows indicate two forms of glycosylated SHBG [like in human SHBG (28)]. Heavy and light chains of bear IgG were ~52 kDa and 27 kDa. All seven bears analyzed in 2010 showed identical results, which were confirmed by samples collected from subadult bears in 2012 and 2013.

FIGURE 2. Interplay and regulation of plasma protein factors in coagulation, fibrinolysis and complement defense in hibernating brown bears. Protein names come from the corresponding human genes. The mean of winter/summer (W/S) ratios of protein levels for each of seven subadult bears and the p-values from paired t-tests quantified by mass spectrometry have been summarized and illustrated by squared frames. Thick frame, p-value < 0.05. W/S ratio increased to > 1.2-fold in hibernation (red); Moderate W/S ratio changes 0.8 to 1.2-fold (black); W/S ratio decreased to < 0.8-fold in hibernation (blue). Component not detected (dashed). Component absent in the bear protein database (yellow). Coagulation factors quantified by functional assays are shown in space filling color, red increased and blue decreased in hibernation (Table 1). The figure was modified after http://www.reactome.org/PathwayBrowser/.
Fig. 1
Table S2. Metabolites identified and quantified by mass spectrometry in 2010 paired blood samples of seven brown bears, ID 0824M, 0910M, 0825F, 0820F, 0819F, 0818F, 0812M, a human reference plasma sample, and comparable published data.

<table>
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<th>Analyte</th>
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<th>Summer bears mean</th>
<th>Human mean</th>
<th>Bear W/S published</th>
<th>T-test paired</th>
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</thead>
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<td></td>
<td>µM</td>
<td>µM</td>
<td>µM</td>
<td></td>
<td></td>
<td>paired</td>
</tr>
<tr>
<td>Carnitines:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>So-2016</td>
<td></td>
<td></td>
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<tr>
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<td>43.39</td>
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<td>1.65</td>
<td>0.005</td>
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<td>1.32</td>
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*Notes:*, not significant, p > 0.05; nd, not determined.
**Supplemental Tables S2, S3, S4, S5**

Biochemical Foundations of Health and Energy Conservation in Hibernating Free-Ranging Subadult Brown Bear *Ursus arctos*


Table S2. Metabolites identified and quantified by mass spectrometry in 2010 paired blood samples of seven brown bears, ID 0824M, 0910M, 0825F, 0820F, 0819F, 0818F, 0812M, a human reference plasma sample, and comparable published data.  
St-2013 (Stenvinkel et al., 2013 (18)): Median of paired plasma W and S concentrations of 16 bears, including the 7 bears of the present study.  
So-2016 (Sommer et al., 2016 (21)): Mean of serum W and S concentrations of 7 to 15 brown bears.  
NS, not significant, *p*>0.05; nd, not determined.

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<th>Summer bears (\mu\text{M})</th>
<th>Human mean (\mu\text{M})</th>
<th>W/S mean paired</th>
<th>T-test paired W/S</th>
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**Phosphatidylcholines:**

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| lysoPC a C16:0 | 84.61 | 38.68 | 123.00 | 2.34 | 0.000 |
| lysoPC a C17:0 | 3.91 | 2.82 | 2.80 | 1.42 | 0.001 |
| lysoPC a C18:0 | 4.21 | 4.02 | 1.78 | 1.12 | 0.734 |
| lysoPC a C18:1 | 71.64 | 44.55 | 35.70 | 1.76 | 0.008 |
| lysoPC a C18:2 | 45.36 | 30.56 | 22.67 | 1.55 | 0.001 |
| lysoPC a C18:2 | 27.08 | 15.40 | 48.13 | 1.93 | 0.003 |
| lysoPC a C20:0 | 5.93 | 2.83 | 2.96 | 2.15 | 0.000 |
| lysoPC a C20:3 | 14.06 | 9.79 | 8.75 | 2.20 | 0.000 |
| lysoPC a C24:0 | 0.86 | 0.88 | 0.24 | 1.03 | 0.718 |
| lysoPC a C26:0 | 1.55 | 1.10 | 0.43 | 1.42 | 0.038 |
| lysoPC a C26:1 | 0.71 | 0.67 | 0.26 | 1.12 | 0.539 |
| lysoPC a C28:0 | 1.98 | 1.90 | 0.44 | 1.07 | 0.531 |
| lysoPC a C28:1 | 1.52 | 1.84 | 0.45 | 0.87 | 0.073 |
| PC aa C24:0 | 1.02 | 1.05 | 0.14 | 1.12 | 0.756 |
| PC aa C26:0 | 3.01 | 2.20 | 0.94 | 1.34 | 0.043 |
| PC aa C28:0 | 2.19 | 3.49 | 1.77 | 0.64 | 0.004 |
| PC aa C30:0 | 4.70 | 6.88 | 3.18 | 0.74 | 0.040 |
| PC aa C32:0 | 56.33 | 38.82 | 12.50 | 1.55 | 0.010 |
| PC aa C32:1 | 57.89 | 37.43 | 16.77 | 1.60 | 0.000 |
| PC aa C32:2 | 4.96 | 5.70 | 4.28 | 0.93 | 0.355 |
| PC aa C32:3 | 0.97 | 1.33 | 0.51 | 0.76 | 0.025 |
| PC aa C34:0 | 577.86 | 412.79 | 169.67 | 1.45 | 0.002 |
| PC aa C34:2 | 163.71 | 347.14 | 340.67 | 1.59 | 0.007 |
| PC aa C34:3 | 31.71 | 53.94 | 17.43 | 0.67 | 0.036 |
| PC aa C34:4 | 2.97 | 9.25 | 1.57 | 0.34 | 0.001 |
| PC aa C36:0 | 11.92 | 21.84 | 1.22 | 0.58 | 0.006 |
| PC aa C36:1 | 357.50 | 369.43 | 39.67 | 1.01 | 0.724 |
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| PC aa C36:3 | 283.64 | 266.71 | 118.00 | 1.14 | 0.626 |
| PC aa C36:4 | 362.07 | 289.00 | 174.67 | 1.32 | 0.057 |
| PC aa C36:5 | 29.34 | 81.56 | 19.67 | 0.37 | 0.000 |
| PC aa C36:6 | 1.36 | 4.62 | 0.74 | 0.30 | 0.000 |
| PC aa C38:0 | 6.97 | 7.32 | 2.14 | 0.99 | 0.580 |
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| PC aa C38:4 | 492.50 | 432.43 | 94.27 | 1.20 | 0.234 |
| PC aa C38:5 | 208.86 | 266.71 | 45.40 | 0.80 | 0.027 |
| PC aa C38:6 | 78.25 | 71.15 | 53.20 | 1.13 | 0.424 |
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| PC aa C40:2 | 1.56 | 1.99 | 0.94 | 0.80 | 0.010 |
| PC aa C40:3 | 5.47 | 7.67 | 1.13 | 0.77 | 0.036 |
| PC aa C40:4 | 42.76 | 37.96 | 3.71 | 1.17 | 0.292 |
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| PC aa C42:4 | 0.74 | 1.51 | 0.49 | 0.49 | 0.000 |
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**Sphingomyelins**

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| **Published data** (Stenvinkel et al., 2013 (ref 18):)

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Glc = 1.14
Published data are shown in Italics. Values for blood cells were based on the mean of 12 bears, including the seven bears of the present study (6).

Triglycerides and cholesterol-total were based on the median of 16 bears, including the seven bears of the present study (18).

Triglycerides, Cholesterol-total, -HDL, and -LDL for the seven bears have been published previously (14).

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Abbreviations: RDW (Red blood cell distribution width); nd, not determined.
Table S4. Functional analyses of coagulation factors antithrombin (AT=SERPINC1), protein C (PC=PROC), F2 (FII=prothrombin), F8 (FVIII), F7 (FVII), fibrinogen (Fib); and immunochemical analysis of von Willebrand factor (VWF)

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W/S paired mean: 0.49 0.78 1.18 0.30 0.65 1.29 0.75
SD: 0.07 0.21 0.23 0.10 0.19 0.41 0.12
T-test paired: 8.5E-10 0.002 0.013 2.0E-06 0.022 0.24 7.4E-05

*Diluted and repeated
Table S5. Protein and hemoglobin concentration in bear blood plasma.

UV spectra were normalized to identical values at A340 = mean of winter or summer, respectively. Corrections were mean Winter A340 = 3.03 cm⁻¹; mean Summer A340 = 2.84 cm⁻¹ for 2010 samples.

UV-VIS spectral values were recorded at 1 mm light path. The A415 values were corrected to 10 mm light path, and for mean = 0 for A 650-750 nm.

Human HbO₂ molar extinction coefficient at 415nm is 524280 M cm⁻¹ and was applied to bear tetrameric hemoglobin Hbα₂Hbβ₂ heme₄ Mr = 2×(15110+16009) + 4×616.5 = 64704, calculated from amino acid sequences Uma_R010673 and Uma_R019543 (Bear-protein-db-Febr2013.txt). (Mr for human Hb = 64500).

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<td>2.79</td>
<td>5.32</td>
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<tr>
<td>0820-F</td>
<td>52.26</td>
<td>53.58</td>
<td>1.89</td>
<td>3.60</td>
<td>0.233</td>
<td>7.21</td>
</tr>
<tr>
<td>0819-F</td>
<td>53.36</td>
<td>51.29</td>
<td>3.30</td>
<td>6.29</td>
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</tr>
<tr>
<td>0818-F</td>
<td>50.46</td>
<td>51.81</td>
<td>2.15</td>
<td>4.09</td>
<td>0.265</td>
<td>8.19</td>
</tr>
<tr>
<td>0812-M</td>
<td>59.28</td>
<td>59.70</td>
<td>5.37</td>
<td>3.73</td>
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</tr>
<tr>
<td>Mean</td>
<td>54.79</td>
<td>54.79</td>
<td>2.84</td>
<td>5.42</td>
<td>0.351</td>
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<tr>
<td>SD</td>
<td>3.56</td>
<td>3.97</td>
<td>1.20</td>
<td>2.29</td>
<td>0.148</td>
<td>4.59</td>
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Experimental mixtures:

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<tr>
<th>Bear ID</th>
<th>Protein W/S</th>
<th>Protein increase in W %</th>
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<tr>
<td>W-mix</td>
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</tr>
<tr>
<td>S-mix</td>
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Table continued
<table>
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<tr>
<th>Bear ID</th>
<th>Protein g/L normalized</th>
<th>Protein increase in W/S</th>
<th>Hb A415 normalized</th>
<th>Hb conc µM</th>
<th>Hb conc g/L</th>
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<tbody>
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<td>1105-F</td>
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<td>1104-F</td>
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<td>1.191</td>
<td>2.63</td>
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<tr>
<td>1204-M</td>
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<tr>
<td>1202-F</td>
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<td>1.247</td>
<td>2.31</td>
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<tr>
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<td><strong>2.39</strong></td>
<td><strong>3.66</strong></td>
<td><strong>4.55</strong></td>
</tr>
<tr>
<td><strong>SD</strong></td>
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<td>0.038</td>
<td>3.5</td>
<td>1.58</td>
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subject disrupted the study for medical reasons. We analyzed period followed by 21 days of bed rest in head-down tilt position. Ten healthy men were restrained to the metabolic ward of the study for medical reasons. We analyzed period followed by 21 days of bed rest in head-down tilt position. Ten healthy men were restrained to the metabolic ward of the study for medical reasons. We analyzed period followed by 21 days of bed rest in head-down tilt position. Ten healthy men were restrained to the metabolic ward of the study for medical reasons. We analyzed period followed by 21 days of bed rest in head-down tilt position. Ten healthy men were restrained to the metabolic ward of the study for medical reasons. We analyzed period followed by 21 days of bed rest in head-down tilt position. Ten healthy men were restrained to the metabolic ward of the study for medical reasons. We analyzed period followed by 21 days of bed rest in head-down tilt position. 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SHORT COMMUNICATION

Physical inactivity and platelet aggregation function in humans and brown bears: A comparative study


1Örebro University, Faculty of Health, Department of Cardiology and Acute Internal Medicine, Karlstad, Sweden, 2Université de Strasbourg, CNRS UMR 7178, Institut Pluridisciplinaire Hubert Curien, Strasbourg, France, 3Aalborg University, Department of Chemistry and Bioscience, Aalborg, Denmark, 4Norwegian Institute for Nature Research, Trondheim, Norway, 5Department of Forestry and Wildlife Management, Inland Norway University of Applied Sciences, Koppang, Norway, and 6Örebro University, Faculty of Health, Department of Cardiology, Örebro, Sweden

Abstract

Physical inactivity increases the risk of thromboembolism. However, good standardized human models on inactivity are in short supply and experimental models are few.

Our objective was to investigate how standardized bed rest affects platelet aggregation in humans and to investigate if aggregation is altered in a translational model system – the hibernating brown bear (Ursus arctos). We collected blood from (1) healthy male volunteers participating in a 21-day bed rest study in head-down tilt position (−6°) 24 h a day; (2) free-ranging brown bears captured during winter hibernation and again during active state in summer. We analyzed platelet function using multiple electrode platelet aggregometry. In total, 9 healthy male volunteers (age 31.0 ± 6.4 years) and 13 brown bears (7 females and 6 males, age 2.8 ± 0.6 years) were included. In hibernating bears adenosine diphosphate, arachidonic acid, thrombin receptor activating peptide, and collagen impedance aggregometry tests were all halved compared to summer active state. In human volunteers no statistically significant changes were found between baseline and the end of bed rest. In human male volunteers 3 weeks of bed rest did not affect platelet function. In hibernating brown bears platelet aggregation was halved compared to summer and we hypothesize that this is a protective measure to avoid formation of thrombi under periods of low blood flow.

Keywords

Thrombosis, Platelets, Platelet aggregation, Immobilization

Introduction

Physical inactivity is a risk factor for venous and arterial thrombosis, and sedentary lifestyle increases the risk for cardiovascular disease [1,2]. In this respect the hibernating brown bear, Ursus arctos, could serve as a translational model. Indeed, while inactivity is a thromboembolic risk factor in humans, this is not the case for hibernating bears, which have almost no physical activity for 6 months each year [3–5]. In this study we analyzed how 21 days of bed rest, a long-term model of enforced physical inactivity, affects platelet aggregation in humans and whether platelet aggregation is differentially altered during hibernation compared to active state in brown bears.

Materials and methods

The European Space Agency and the German Aerospace Center conducted a head-down-tilt-bed-rest (−6°) study in 2011–2012. Ten healthy men were restrained to the metabolic ward of the German aerospace Center (DLR) for a 7-day ambulatory control period followed by 21 days of bed rest in head-down tilt position (−6°) 24 h a day followed by a 7-day recovery period. One test subject disrupted the study for medical reasons. We analyzed samples from 9 male volunteers (age 31.0 ± 6.4 years and weight 77.2 ± 5.7 kg).

All volunteers were nonsmokers and free of any clinical/biomedical sicknesses. For inclusion test, subjects were obliged to have a negative thrombophilia screening panel test (antithrombin III, protein C and S, factor V Leiden, prothrombin mutation, and antiphospholipid antibody causing elevation in partial thromboplastin time) because of the long period of inactivity. Fasting (9 h) blood samples were collected in the morning. A detailed description of subject recruitment, screening, the participants, and metabolic ward conditions has been described by Buehlmeier et al [6]. This prospective, cross-over study was conducted under different dietary campaigns, but our study includes data from the conventional diet part of the study only. Blood samples for aggregometry were drawn at baseline and after 19 days of bed rest, and hematological tests were taken at baseline and after 10 days. This bed rest study was approved by the independent ethics committee of the Ärztekammer Nordrhein, Düsseldorf, Germany, and was performed according to the Declaration of Helsinki. All subjects participated after providing signed informed consent.

Blood was drawn from free-ranging brown bears (7 females and 6 males, age 2.8 ± 0.6 years) during winter hibernation in February (weight 48.1 ± 14.0 kg) and from the same bears when active in June (weight 50.9 ± 16.6 kg) 2010–2011 in Dalarna, Sweden, according to a protocol for capture and anesthesia [7]. Brown bears typically reach reproductive maturity before the age...
of 5 years and rarely live the age of 25 [8]. The study of bears was approved by the Swedish Ethical Committee on animal research. Whole blood was analyzed with a platelet function analyzer (Multiplate impedance aggregometer, Dynabyte, Munich, Germany) [4]. Final agonist concentrations were adenosine diphosphate (ADP) 6.4 μM, arachidonic acid (ASPI) 0.5 mM, thrombin receptor activating peptide (TRAP) 32 μM, protease-activated receptor 4 (PAR-4; AYPGKF) 20 nM, or collagen (COL) 1 μg/mL. Aggregometry was performed within 1 h after blood sampling from the anaesthetized bears.

For statistical comparison, a paired t test was used and p < 0.05 was considered statistically significant.

Results

There was a small, but statistically significant increase in hemoglobin during bed rest compared to baseline due to the known hypovolemia resulting from the head-down tilt. Brown bears had statistically significantly higher levels of hemoglobin and lower levels of platelet count in winter compared to summer (Table I).

There were no differences in aggregation in humans between baseline and 19 days of bed rest. In bears, ADP, ASPI, TRAP, and collagen impedance aggregometry tests were all statistically significantly lowered by half during winter compared to summer (Table I). TRAP and PAR-4 are based on human peptide sequences and were less effective in bear than in human platelet activation as could be expected. There were no sex differences in aggregometry or hematocrit in the bear population. Rectal temperature of the bears was 33.4 ± 1.1°C in winter and 39.8 ± 0.8°C in summer which is approximately 2°C higher than normal body temperature in summer, caused by the bear running during the helicopter capture [9]. To test for the effect of temperature, aggregometry was performed in a subset of samples (n = 6) collected during winter, using identical blood samples examined at 33°C as well as 37°C. We could not document any significant differences (Table II).

Table II. Aggregation results in bears at different temperatures.

<table>
<thead>
<tr>
<th></th>
<th>33°C</th>
<th>37°C</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP test AUC (±SD)</td>
<td>78.1 (26.0)</td>
<td>65.8 (23.6)</td>
<td>0.15*</td>
</tr>
<tr>
<td>ASPI test AUC (±SD)</td>
<td>74.4 (26.9)</td>
<td>76.3 (18.2)</td>
<td>0.76*</td>
</tr>
<tr>
<td>TRAP test AUC (±SD)</td>
<td>26.8 (9.0)</td>
<td>25.4 (12.9)</td>
<td>0.72*</td>
</tr>
<tr>
<td>COL test AUC (±SD)</td>
<td>69.2 (24.5)</td>
<td>69.8 (12.6)</td>
<td>0.98*</td>
</tr>
<tr>
<td>PAR-4 test AUC (±SD)</td>
<td>37.0 (13.2)</td>
<td>26.8 (10.9)</td>
<td>0.05*</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation.

ADP, adenosine diphosphate; ASPI, aspirin; AUC, area under curve; COL, collagen; PAR-4, protease-activated receptor 4; TRAP, thrombin receptor activating peptide.

Discussion

Three weeks of bed rest did not affect platelet function in humans. In hibernating brown bears, platelet aggregation was halved compared to active state. We therefore speculate that brown bears, in order to compensate for lying still for 6 months with low circulatory state, have developed reduced platelet aggregation as a protection against thromboses.

In hibernating animals organ damage due to thrombosis has not been found [10], despite increased blood viscosity [11], low blood flow [12], low heart rate, [13] and physical inactivity. Bears had numerically lower platelet aggregation, even in summer, with all agonists except ADP compared to humans. This might be due to a species difference in amino acid sequence of the platelet receptor, enabling only partial activation of the agonists added. ADP and collagen provided the most consistent results between species in an earlier animal study [14]. As the metabolic rate during hibernation is only 25% of the summer activity rate [15], protein synthesis is reduced and central processes appear to be maintained by increased levels of very few key or broad-spectrum proteins. Thus all coagulation factor levels were reduced to <80% except for the three key

Table I. Aggregation and hematometry in humans versus bears.

<table>
<thead>
<tr>
<th>Humans (n = 9)</th>
<th>Bears</th>
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<tbody>
<tr>
<td></td>
<td>Bedrest</td>
</tr>
<tr>
<td>n</td>
<td>Winter</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
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<tr>
<td>Red blood cells (10^9/L)</td>
<td>5 ± 0</td>
</tr>
<tr>
<td>Hemoglobin (Hgb) (g/L)</td>
<td>151 ± 8</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>44.3 ± 2.3</td>
</tr>
<tr>
<td>White blood cells (10^9/L)</td>
<td>7.4 ± 2.3</td>
</tr>
<tr>
<td>Platelets (10^9/L)</td>
<td>245 ± 71</td>
</tr>
<tr>
<td>AUC (Ohm·min)</td>
<td>57 ± 17</td>
</tr>
<tr>
<td>ADP</td>
<td>105 ± 16</td>
</tr>
<tr>
<td>TRAP</td>
<td>114 ± 30</td>
</tr>
<tr>
<td>COL</td>
<td>96 ± 14</td>
</tr>
<tr>
<td>AUC/platelet count</td>
<td>84 ± 16</td>
</tr>
<tr>
<td>ADP</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td>ASPI</td>
<td>0.46 ± 0.11</td>
</tr>
<tr>
<td>TRAP</td>
<td>0.50 ± 0.18</td>
</tr>
<tr>
<td>PAR-4</td>
<td>0.42 ± 0.11</td>
</tr>
<tr>
<td>COL</td>
<td>0.36 ± 0.07</td>
</tr>
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</table>

ADP, adenosine diphosphate; ASPI, aspirin; AUC, area under curve; COL, collagen; PAR-4, protease-activated receptor 4; TRAP, thrombin receptor activating peptide.
components factors II, X, and fibrinogen, which were increased by 20% as shown by quantitative proteomics and verified by functional analyses [16]. This could be the most economical way to preserve a coagulation response during hibernation. Two different European space campaign bed rest studies on healthy volunteers showed no alterations in coagulation factors involved in secondary hemostasis at the end of bed rest compared to baseline [17–19].

A study by Broadley et al. documented that a short period of 45 minutes of supine rest reduced platelet aggregation, and the authors postulate that the reduction partly can be explained by a fall in plasma catecholamines [20]. The reverse, an increase in platelet aggregability and an increase in catecholamine levels, has been observed after assumption of the upright posture [21]. Catecholamines can act on alpha-2 adrenoceptors and promote platelet aggregation [22]. In longer bed rest studies psychological stress has been reported [23], but without changes in catecholamine levels [24].

We observed a reduced platelet count from approximately 260 × 10⁹/L to 170 × 10⁹/L during hibernation. Studies on the influence of platelet count and platelet aggregometry have shown decreased aggregation when platelet count drops below 150 × 10⁹/L [25] – beyond what we observed in this study (174 × 10⁹/L ± 51). We therefore propose that the reduction in platelet aggregation in brown bears is not linked to platelet concentration reduction. In our previous aggregometry study (mid-April 2009 data) approximately 7–10 days after leaving the den, platelet aggregometry was similar to hibernation in the present study although platelet count had a mean value of 207 × 10⁹/L ± 24 [4]. The observed decrease in platelet count corresponds with studies of hibernating hamsters [26,27]. Upon arousal thrombocytopenia was reversed suggesting storage and release, possibly by margination of platelets during hibernation [26]. As hematocrit plays a role in the degree of margination [28], the reduced platelet count may in part be an effect of a higher hematocrit due to dehydratation during hibernation.

In humans, hypothermia enhances agonist-induced platelet aggregation [29]. However, we found hibernating bears to have reduced platelet aggregation during moderate hypothermia and when we studied platelet function at different ex vivo temperatures there were no differences in aggregation.

In conclusion, in human male volunteers, 3 weeks of bed rest did not affect platelet function. In hibernating brown bears, platelet aggregation was halved compared to summer and we hypothesize that this is a protective measure to avoid formation of thrombi under periods of low blood flow.

Declaration of interest

The authors report no conflicts of interest.

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