

1 **Cold exposure distinctively modulates parathyroid and thyroid hormones in cold-**  
2 **acclimatized and non-acclimatized humans**

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23  
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41

42 **Abstract**

43 **Context:** Cold-induced activation of thermogenesis modulates energy metabolism, but the  
44 role of humoral mediators is not completely understood.

45 **Objective:** To investigate the role of parathyroid and thyroid hormones in acute and adaptive  
46 response to cold in humans.

47 **Design:** Cross-sectional study examining acute response to ice-water swimming and to  
48 experimental non-shivering thermogenesis (NST) induction in individuals acclimatized and  
49 non-acclimatized to cold. Seasonal variation in energy metabolism of ice-water swimmers and  
50 associations between circulating PTH and molecular components of thermogenic program in  
51 brown adipose tissue (BAT) of neck-surgery patients were evaluated.

52 **Setting:** Clinical Research Center.

53 **Patients, Participants:** Ice-water swimmers (winter swim n=15, NST-induction n=6), non-  
54 acclimatized volunteers (NST-induction, n=11, elective neck surgery n = 36).

55 **Main Outcomes and Results:** In ice-water swimmers, PTH and TSH increased in response  
56 to 15min winter swim, while activation of NST failed to regulate PTH and lowered TSH. In  
57 non-acclimatized men, NST-induction decreased PTH and TSH. Positive correlation between  
58 systemic levels of PTH and whole-body metabolic preference for lipids as well as BAT 18F-  
59 FDG uptake was found across the two populations. Moreover, NST-cooling protocol-induced  
60 changes in metabolic preference for lipids correlated positively with changes in PTH. Finally,  
61 variability in circulating PTH correlated positively with *UCP1/UCP1*, *PPARGC1A* and *DIO2*  
62 in BAT from neck surgery patients.

63 **Conclusions:** Regulation of PTH and thyroid hormones during cold exposure in humans  
64 depends on the cold acclimatization level and/or cold stimulus intensity. Role of PTH in NST  
65 is substantiated by its positive relationships with whole-body metabolic preference for lipids,  
66 BAT volume and UCP1 content.

## 67 **Introduction**

68 During evolution, humans have developed various cold-coping mechanisms, including  
69 shivering and non-shivering thermogenesis (NST) in skeletal muscle and/or brown adipose  
70 tissue (BAT) <sup>1</sup>, with the principal thermogenic machinery in BAT represented by  
71 mitochondrial uncoupling protein 1 (UCP1). Importantly, human BAT could have similar  
72 thermogenic functionality to rodents relative to mitochondrial content <sup>2</sup> and several studies  
73 have observed metabolic effects associated with cold-induced BAT activation in humans,  
74 including increased energy expenditure, insulin sensitivity, lipolysis and fatty acid oxidation <sup>2-</sup>  
75 <sup>5</sup>. Cold-induced metabolic activation of thermogenic processes could therefore potentially  
76 help control the energy imbalance and pathophysiological consequences of the nutrient  
77 overload present in obesity and metabolic disease <sup>6</sup>.

78 Adipose organ plasticity allows for substantial morphological and functional  
79 transformation in adaptive response to repeated cold exposure or regular physical activity.  
80 This includes adipose tissue “browning” - the formation of multilocular adipocytes within  
81 white adipose tissue (WAT), indicating enhanced capacity for energy dissipation <sup>7</sup>. It has been  
82 demonstrated that repeated cold exposure can induce BAT activity in lean adults <sup>8</sup>, healthy  
83 individuals with obesity <sup>9</sup> or patients with type 2 diabetes <sup>10</sup>. Therefore, regular cold exposure  
84 could represent an efficient means of the adipose tissue thermogenic program activation in  
85 most individuals, independently of age or adiposity, which are usually negatively associated  
86 with the amount of BAT <sup>11-12</sup>.

87 We studied systemic humoral mediators of thermogenesis in humans well acclimatized  
88 to cold. We hypothesized that this specific population is more likely to possess efficient  
89 thermogenic mechanisms, including thermogenically active BAT, and that systemic mediators  
90 of the thermogenic process could be altered following acute exposure to cold. We therefore  
91 explored the effects of acute and seasonal severe cold exposure on circulating hormones and

92 metabolites that might be involved in the development of cold-coping mechanisms that enable  
93 efficient acute and chronic cold acclimation in individuals that regularly engage in outdoor  
94 ice-cold water swimming (<5°C). We focused (i) on thyroid hormone axis due to its role in  
95 energy homeostasis and its potential to induce adipose tissue browning in both animals and  
96 humans<sup>13-14</sup> and (ii) on parathyroid hormone (PTH) that has been recently shown to induce  
97 adipose tissue browning in rodents and in cultured human adipocytes<sup>15-16</sup>. We also  
98 investigated seasonal differences in baseline (unstimulated) levels of key molecules regulating  
99 or reflecting metabolic preference, insulin sensitivity and thermogenic response to ice-water  
100 swimming together with anthropometric and biochemical characteristics (Cohort 1).

101 Next, we compared effects of ice-cold water swimming (cold stress) and controlled mild  
102 cold exposure (NST activation) in cold acclimatized individuals (Cohort 2) on systemic levels  
103 of PTH and thyroid hormones. We explored the relationships of PTH with BAT volume and  
104 glucose uptake, whole-body energy metabolism and metabolic substrate preference. In  
105 parallel we also studied effects of NST activation in healthy young lean non-acclimatized men  
106 (Cohort 3). The relationships of PTH and thyroid hormones with molecular hallmarks of BAT  
107 thermogenic potential, i.e. UCP1 mRNA and protein, was explored in deep-neck BAT  
108 samples from patients undergoing elective neck surgery, who were neither acclimatized nor  
109 acutely exposed to cold (Cohort 4).

110

## 111 **Methods**

### 112 **Study population and protocol**

113 All participants were fully informed about the study protocol and signed a written  
114 informed consent prior entering the study. The study was approved by the Ethics Committee  
115 of the Faculty of Medicine Comenius University & University Hospital Bratislava (Cohort 1  
116 and 3), by the ethics committees of the Medical University of Vienna (Cohort 2) and the

117 canton of Zürich (Cohort 3). The Study conforms to Declaration of Helsinki, as amended in  
118 2013, and with the standards of the International Conference on Harmonization (ICH) &  
119 Good Clinical Practice (GCP).

120 **Cohort 1:** We recruited 15 middle-aged ice-water swimmers who had engaged in  
121 regular outdoor cold-water swimming for at least past 6 months (Table 1). At the time of the  
122 study, all participants lived in continental European climate and were adequately trained and  
123 acclimatized to endure  $\geq 10$ min of swimming in  $< 5^{\circ}\text{C}$  cold water. The winter ice-water  
124 swimming event, organized by a local ice-water swimming club  
125 (<http://www.ladovemedvede.sk/>), began with approximately 30min acclimation to outdoor  
126 temperature ( $0^{\circ}\text{C}$ ) in light clothing (swimsuit, T-shirt), followed by swimming in  $2.6^{\circ}\text{C}$  water  
127 (15min on average) in the Danube river in Bratislava, Slovakia (February), wearing only a  
128 swimsuit (no thermal protection) and a head cover. Blood was collected indoors ( $25^{\circ}\text{C}$ ) from  
129 a cubital vein approximately 1h before and 10-30min after swimming in the river. Food intake  
130 prior to the event was not restricted, but participants were asked to refrain from food  
131 consumption before the blood collection after swimming. In the following month (average  
132 daily air temperature of sampling period:  $7.2^{\circ}\text{C}$ ), volunteers visited our clinical unit after an  
133 overnight fast for additional blood sampling and assessment of body composition  
134 (bioelectrical impedance, Omron BF511, Japan), blood pressure and pulse (Omron 907,  
135 Japan) and cold-hardening habits (questionnaire). Seven months later, at the end of summer  
136 (average daily air temperature of sampling period:  $18.3^{\circ}\text{C}$ ), phenotyping and blood collection  
137 were repeated (Table 1). Design of the study is detailed in Figure 1. Self-reported weight and  
138 height values from one volunteer who did not attend clinical phenotyping were used in the  
139 analyses. One participant was treated for hypertension (monotherapy with beta-blockers).

140 **Cohort 2:** This study was performed at the Medical University of Vienna. We used  
141 plasma samples and measurements from 6 participants (characteristics - Table 2) of the

142 clinical study NCT02381483, who were all members of the ice-water swimming club  
143 (<http://www.ladovemedvede.sk/>) and two of them participated in Cohort 1. Study participants  
144 were examined at two separate study visits in the morning after an overnight fast (>10h). At  
145 study visit one, a blood draw was performed followed by an indirect calorimetry using a  
146 computerized open circuit indirect calorimetry (Quark RMR, Cosmed, Rome, Italy) in order  
147 to determine the resting energy expenditure at thermoneutrality (room temperature).  
148 Afterwards subjects received 2,5 MBq/kg<sup>-1</sup> BW [<sup>18</sup>F]-FDG (max 350 MBq) and underwent  
149 the first [<sup>18</sup>F]-FDG PET/CT scan at room temperature (24°C) to detect any basal BAT activity  
150 using a Siemens Biograph 64 True Point PET/CT scanner (Siemens Healthcare Sector,  
151 Erlangen, Germany). The PET and CT images were acquired from the base of the skull until  
152 mid-thigh. On study day two a blood draw was performed, followed by an indirect  
153 calorimetry at thermoneutrality (room temperature). Then, a personalized cooling protocol  
154 was applied for a total of 150 min using a water-perfused cooling vest (CoolShirt Systems,  
155 Stockbridge, Georgia, USA). The water temperature was kept slightly above the shivering  
156 temperature (6,5 ± 3,4°C) and muscle activity was monitored by electromyography (OT  
157 Bioelettronica, Torino, Italy). After 90 min of cold exposure a second indirect calorimetry  
158 was performed followed by the administration of 2,5 MBq/kg<sup>-1</sup> BW [<sup>18</sup>F]-FDG (max 350  
159 MBq) and cold exposure continued for another 60 min until the PET/CT scan followed by  
160 blood sampling.

161 **Cohort 3:** Here we report on the samples and measurements taken at the baseline visit  
162 (before exposure to Fluvastatin) from 11 healthy young men (characteristics - Table 2)  
163 participating in the clinical study NCT03189511, more detailed information can be found  
164 elsewhere<sup>17</sup>. Maximal induction of NST was aimed to achieve by combining cold exposure  
165 with β<sub>3</sub>-adrenergic receptor (AR) agonist (Mirabegron). The volunteers arrived in a fasted  
166 state on two separate days with identical schedule to be first screened by indirect calorimetry

167 for an increase in cold-induced thermogenesis of at least 5% of resting energy expenditure.  
168 Briefly, volunteers were orally administered 200mg Mirabegron (Betmiga, Astellas Pharma,  
169 Switzerland). After 90min rest, standardized cold stimulation with water cooling pads  
170 (Hilotherm Clinic; 10°C setting) was commenced lasting another 120min. Afterwards, blood  
171 samples were drawn and participants received 75 MBq of <sup>18</sup>F-FDG intravenously immediately  
172 followed PET/MR scan for 50±10 minutes (SIGNA PET/MR, GE Healthcare, Waukesha, WI,  
173 USA). Skin and room temperatures were monitored with surface temperature probes. No  
174 shivering was reported by any of the participants. At the end of the cooling period, blood  
175 samples were drawn from antecubital vein and the participant was transferred to the PET/MR  
176 table (SIGNA PET/MR, GE Healthcare, Waukesha, WI, USA). The PET/MR images were  
177 acquired from vertex to mid-torso. All scans were performed in the afternoon (between 2-4  
178 pm).

179 **Cohort 4:** Paired samples of deep neck BAT and adjacent subcutaneous WAT were  
180 obtained from patients undergoing neck surgery under general anesthesia, as described in <sup>18-19</sup>.  
181 We analyzed tissues from 36 patients [gender 5M/31F, age 43.5±14.5 years, body mass index  
182 25.9±4.2 kg.m<sup>-2</sup>, waist 87.6±12.8 cm, body fat 32.4±9.1 % (bioelectrical impedance, Omron  
183 BF511, Japan), fasting plasma glucose: 4.78±1.00 mmol.l<sup>-1</sup>, triglycerides: 1.35±0.44 mmol.l<sup>-1</sup>,  
184 HDL cholesterol: 1.43±0.41 mmol.l<sup>-1</sup>, PTH: 74.15±38.50 pg.ml<sup>-1</sup>]. Anthropometric data are  
185 missing from 2 patients. Patients were diagnosed with nodular goiter (n=15), lateral cervical  
186 cyst (n=5), hyper functional goiter (n=3), Grave's disease (n=3), hypothyroidism and nodular  
187 goiter (n=2), multi-nodal and diffuse goiter (n=1), hypo functional goiter (n=1), papillary  
188 microcarcinoma and Hashimoto's thyroiditis (n=1), papillary carcinoma (n=1), follicular  
189 thyroid adenoma (n=1), parathyroid adenoma (n=1), hyper functional parathyroid adenoma  
190 and nodular goiter (n=1), benign vascular tumor (n=1). Patients were on pharmacotherapy  
191 with Euthyrox (n=11), L-Thyroxin (n=2), Thyrozol (n=4), Euthyrox + Thyrozol (n=2) or



192 progestin (n=1) and/or with antihypertensives (n=10), vitamin D3/calcium/phosphate  
193 supplements (n=6), iron supplements (n=1), hypolipidemics (n=2), insulin pump (type 1  
194 diabetes, n=1). For the analysis of the BAT transcriptome, we only included volunteers with  
195 higher relative BAT *UCPI* gene expression than the median *UCPI* expression calculated by  
196 combining expression levels in all the corresponding subcutaneous WAT samples (Figure  
197 5A). Plasma samples were collected (n=25) and serum levels of free thyroxine (n=30), free T3  
198 (n=16) and thyroid stimulating hormone (TSH, n=30) were measured.

199

## 200 **Biochemical analysis**

201 To generate serum, collected blood was left at room temperature for 30min, centrifuged  
202 (4°C/20min/1200xg) and stored at -80°C. For EDTA/heparin plasma, blood was collected into  
203 pre-cooled tubes, centrifuged immediately (4°C/10min/1200xg) and stored at -80°C.  
204 Circulating parameters were determined in a certified biochemical laboratory (Alpha Medical,  
205 Bratislava, Slovakia) by standardized methods using ADVIACentaur® Immunoassay and  
206 ADVIA®Chemistry systems (Siemens, Germany). Reported intact PTH assay specificity:  
207 <0.1% cross-reactivity with PTH-related peptide.

208

## 209 **Succinate and lactate analysis**

210 Polar metabolites of plasma were extracted by mixing 20 µl of plasma with 180 µl of  
211 80% methanol. Upon 1 hour incubation at 4 °C, clear extracts were obtained by  
212 centrifugation. Non-targeted metabolomics analysis of extracts was performed by flow-  
213 injection – time-of-flight mass spectrometry on an Agilent 6550 QTOF system in negative  
214 mode<sup>20</sup>. Metabolite ions were annotated by accurate mass matching using a m/z tolerance of  
215 0.001. Processing and statistical analysis was done in Matlab (The Mathworks, Natick).

216



## 217 **Gene expression**

218 Total RNA was isolated from whole liquid nitrogen-frozen adipose tissue by Trizol  
219 extraction, treated by Dnase I and re-precipitated by sodium acetate - ethanol solution, washed  
220 in 70% ethanol and dissolved in nuclease-free water for spectrophotometric analysis  
221 (Nanodrop 2000). Total RNA was converted to cDNA using High-Capacity cDNA Reverse  
222 Transcription Kit or miScript II RT Kit. Fast SYBR™ Green Master Mix and specific primer  
223 pairs (5' – 3' forward, reverse *UCPI*: TGTGCCCAACTGTGCAATG,  
224 GAAGGTACCAACCCCTTGAAGA; *RPL13A1*: GGACCGTGCGAGGTATGCT,  
225 ATGCCGTCAAACACCTTGAGA; *PTH1R*: GCCAACTACAGCGAGTGTGTCA,  
226 GGTCAAACACCTCCCGTTCA; *PTH2R*: CTCAACCATAAAGGAGTTGCTTTC,  
227 GTGCATAAAATCCCATGTTCCA; *PPARGCIA*:  
228 TTACAAGCCAAACCAACAACCTTTATC, CACACTTAAGGTGCGTTCAATAGTC;  
229 *DIO2*: TCGATGCCTACAAACAGGTG, CATGTGGCTCCCTCAGCTA) designed in  
230 Primer Express 3.0 (Thermo Fischer Scientific) were used in quantitative real-time PCR  
231 reaction (QuantStudio 5 Real-Time PCR System, Thermo Fischer Scientific). Gene  
232 expression was normalized to a housekeeping gene *RPL13A* and to the average relative  
233 expression of a gene of interest within all the BAT and WAT samples of a dataset (ddCt).  
234 Gene expressions from 4 samples of WAT are unavailable due to being used up for previous  
235 experiments (unpaired BAT sample expression values are included in graphs).

236

## 237 **Protein extraction and western blot**

238 Tissue was stored at -80°C and lysate was prepared by homogenizing tissue sample in  
239 RIPA buffer (0.5% Sodium Deoxycholate, 2mM EDTA, 150mM NaCl, 1% Triton X100;  
240 Sigma-Aldrich) containing protease and phosphatase inhibitors (Sigma-Aldrich) using a mixer  
241 mill (Retsch 400MM) and kept at 4°C until lipid and cell debris were removed by

242 centrifugation (15min/12000rpm/4°C). Protein concentrations were measured (BCA assay,  
243 Thermo Fisher Scientific). Proteins (30µg) were incubated with loading buffer at 95°C/10min,  
244 separated on SDS polyacrylamide gel (12%) and transferred to a PVDF membrane (Merck).  
245 After 1 h blocking (Odyssey blocking buffer with 0.1% Tween20), membranes were  
246 incubated with primary antibodies against UCP1 (1:500, PA1-24894 Invitrogen),  
247 overnight/4°C and against HSP90 beta (1:3000, ab53497 Abcam) and Actin (1:21000, CP01  
248 Calbiochem) for 2h/room temperature. Next, secondary goat anti-rabbit IRDye 680RD or  
249 anti-mouse IRDye 800RD antibodies (1:10000, LI-COR) were used to visualize protein of  
250 interest (Odyssey Infrared Imaging System, LI-COR). Signal for UCP1 was normalized to the  
251 average of the signals for HSP90 and Actin.

252

### 253 **Statistical analysis**

254 All data sets were tested with Shapiro-Wilk normality test. In case of normal  
255 distribution ( $p>0.05$ ), paired Student's t-test was used and in case of not normal distribution,  
256 Wilcoxon matched-pairs signed rank test was used to evaluate the statistical effects of paired  
257 samples. Associations between two variables were analyzed by linear regression and  
258 Pearson's correlation coefficient ( $r$ ) was calculated. Data without normal distribution were  
259 logarithmically transformed before correlation analysis. Statistical analyses were performed in  
260 GraphPad Prism 6 Software Inc and JMP 4.0.4 (SAS). Data with normal distribution are  
261 expressed as mean±SD and data without normal distribution as median (interquartile range -  
262 IQR). In cases of wide distribution (TSH, PTH, ddCt UCP1, ddCt PTH2R), graphs were  
263 constructed with log Y-scale; negative or zero values are not shown in some graphs (Figure  
264 5A: n=6, Figure 5G: n=2). Outlier values outside the mean±3 x SD range were omitted from  
265 analysis in Figure 5F (n=1) and Figure 5G (n=1). Non-physiological values of respiratory  
266 quotient ( $RQ>1$ ) were excluded (Cohort 3, n=1) Cold-induced changes in circulating lactate

267 and succinate were analyzed by Student's paired t-test and false discovery rate-adjusted p-  
268 value was calculated by Benjamini-Hochberg correction.

269

## 270 **Results**

271

### 272 **Acute effects of ice-water swimming on levels of parathyroid and thyroid hormones in** 273 **cold-acclimatized individuals (Cohort 1)**

274 In order to identify changes in hormones and metabolic substrates induced by acute cold  
275 exposure in cold-acclimatized volunteers, we analyzed blood samples taken before and within  
276 30 min after completion of ice-water swimming. Ice-water swimming acutely increased serum  
277 thyroid stimulating hormone (TSH, Figure 2A) and total thyroxine (T4) was also slightly  
278 elevated (Figure 2B), while free T4 (fT4) was decreased (Figure 2C). These data indicate that  
279 ice-water swimming is a powerful stimulus affecting the hypothalamic-pituitary-thyroid axis.  
280 Furthermore, higher weekly ice-water swimming frequency was associated with a larger cold-  
281 swimming-induced decrease in fT4 (Figure 2D), suggesting a faster or more effective fT4  
282 tissue uptake during cold exposure in individuals who are better acclimatized to cold.

283 Serum PTH was markedly elevated after ice-water swimming, on average by more than  
284 78% (Figure 2E). Furthermore, the cold-water swimming-induced increase in PTH was  
285 negatively associated with the number of years dedicated to ice-water swimming habit (Figure  
286 2F), indicating that the response of parathyroid gland to acute cold exposure is less  
287 pronounced after several years of cold acclimatization. In addition, PTH levels induced by  
288 acute cold exposure were negatively associated with visceral adiposity (Figure 2G) and  
289 positively with concomitantly regulated TSH levels (Figure 2H).

290

291 **Acute effects of ice-water swimming on insulinemia and circulating metabolites in cold-**  
292 **acclimatized individuals (Cohort 1)**

293 Ice-water swimming acutely increased serum glycemia in 9 from 11 individuals (Figure  
294 3A), while decreasing insulinemia (Figure 3D). Ice-water swimming also induced an acute  
295 unanimous increase of lactate (Figure 3B) and succinate (Figure 3C). More importantly,  
296 levels of both metabolites correlated positively with PTH (Figures 3E-F).

297  
298 **Seasonal variations in anthropometric, cardiovascular, metabolic and hormonal**  
299 **markers in cold-acclimatized individuals (Cohort 1)**

300 There were no statistically significant differences in cardiovascular and anthropometric  
301 parameters of ice-water swimmers obtained in winter and summer season (Table 1). From all  
302 the measured parameters, only PTH was significantly higher in winter (Table 1). Figure 2I  
303 shows that this was true for majority of examined individuals. It is noteworthy that  
304 overweight, obesity and/or increased abdominal adiposity in most of the examined ice-water  
305 swimmers were not associated with metabolic disease (Table 1).

306

307 **Effects of cold-inducing non-shivering thermogenesis on metabolic preference and**  
308 **circulating levels of thyroid and parathyroid hormones in cold-acclimatized (Cohort 2)**  
309 **and non-acclimatized (Cohort 3) individuals**

310 The induction of non-shivering thermogenesis (NST) was confirmed by increased  
311 resting metabolic rate (RMR) in both studied populations (Figure 4A). It is important to note,  
312 that while cold-acclimatized ice-water swimmers responded to NST-inducing cold by  
313 increasing whole body metabolic preference for lipids (lowering RQ), non-acclimatized men  
314 increased metabolic preference for glucose (increased RQ) in response to NST-inducing cold  
315 and  $\beta_3$ AR agonist treatment (Figure 4B). Power of this observation is limited by the fact that  
316  $\beta_3$ AR agonist was used in combination with cold to stimulate thermogenic response in non-

317 acclimatized individuals. Cold exposure aimed at inducing NST decreased TSH in both  
318 studied populations (Table 2), and fT4 was not significantly regulated (Table 2). Induction of  
319 NST failed to produce any significant PTH response in ice-water swimmers, but the combined  
320 cold and  $\beta_3$ AR-induced stimulation of NST in non-acclimatized individuals decreased  
321 circulating PTH levels (Figure 4C).

322       Next, we explored correlations between circulating PTH, TSH and fT4 and (i) CIT  
323 (cold-induced change in RMR), (ii) whole-body metabolic preference for lipids and (iii) BAT  
324 glucose uptake and volume measured before and after the cold exposure aimed at inducing  
325 NST (cohorts 2 and 3). Most importantly, by including both cold-acclimatized and non-  
326 acclimatized individuals we showed that NST-associated change in PTH correlated with the  
327 change in whole-body metabolic preference for lipids (Figure 4D). Furthermore, combining  
328 levels of PTH detected in warm and cold environment in both studied populations allowed us  
329 to evaluate the association between circulating PTH and the whole-body metabolic preference  
330 for lipids, confirming presence of the relationship in combined cohorts, as well as in non-  
331 acclimatized individuals (Figure 4E). In addition, both baseline (n=11, r=0.71, p=0.01) and  
332 cold-regulated levels of TSH (n=11, r=0.82, p=0.002) in non-acclimatized individuals  
333 correlated positively with the CIT and cold-induced change in fT4 in non-acclimatized  
334 individuals was negatively associated with baseline RMR (n=11, r=-0.73, p=0.01).

335

#### 336 **Relationship of PTH and thyroid hormones with thermogenic capacity markers in** 337 **brown and white fat of non-acclimatized individuals (Cohort 4)**

338       We also explored the links between PTH and brown adipocyte gene/protein markers in  
339 deep-neck BAT and adjacent subcutaneous WAT from non-acclimatized patients undergoing  
340 elective neck surgery, who were not acutely challenged with cold (Cohort 4). Expression of  
341 *UCP1* mRNA in BAT showed large variability (Figure 5A) but observed variability was

342 paralleled by corresponding variability in PTH. Most importantly, fasting plasma PTH levels  
343 positively correlated with deep-neck BAT gene expression of *UCP1* (Figure 5B), *PPARGCIA*  
344 (Figure 5D) and *DIO2* (n=27, p=0.03, r=0.43) as well as with UCP1 protein levels in BAT  
345 (Figure 5C). The importance of paracrine thyroid activity on adipose tissue was indicated by  
346 positive association between deep-neck BAT expression of *DIO2* and circulating levels of fT4  
347 (Figure 5E).

348 Furthermore, we found that both PTH receptors (*PTH1R*, *PTH2R*) were expressed in  
349 brown and white fat (Cohort 4). While *PTH1R* gene expression was significantly higher in  
350 WAT, *PTH2R* showed higher expression in BAT (Figure 5F-G). Expression levels of *PTH1R*  
351 paralleled those of *PTH2R* in BAT (n=36, p=0.03, r=0.37), but the relationship was not  
352 present in WAT, indicating depot-specific differences in PTH signaling in humans.

## 353 Discussion

354 The mechanisms of cold-induced metabolic and thermogenic activation of BAT have  
355 been extensively studied in recent years due to the promising therapeutic potential in the  
356 prevention and treatment of obesity-related metabolic diseases. In our study, we have  
357 observed distinct cold-induced changes in circulating humoral factors, which are potentially  
358 implicated in the regulation of adaptive thermogenesis.

359 This work clearly shows that while cold-stress-associated ice-water swimming induces  
360 profound increase in circulating PTH in cold acclimatized individuals, it remains unchanged  
361 when milder cold stimulus aimed at inducing NST is applied to similarly acclimatized  
362 individuals and that PTH decreases under similar NST-inducing conditions in individuals not  
363 acclimatized to cold. To our best knowledge, this is the first evidence showing acute cold  
364 exposure-related changes of PTH. Our data suggest that PTH response might be modulated by  
365 both the intensity of cold stimulus, the level of cold acclimatization as well as by the  
366 swimming exercise. Adaptive character of this response is indicated by the relationships

367 between the magnitude of ice-water-induced change in PTH and fT4 and the duration of this  
368 habit and frequency of ice-water swimming sessions, respectively. It could be speculated that  
369 distinct and perhaps more efficient adaptive thermogenic mechanisms develop in experienced  
370 (better acclimatized) ice-water swimmers. This notion is supported by the evidence of altered  
371 thermoregulatory responses present in ice-water swimmers during cold water immersion  
372 compared to controls<sup>21</sup>. We observed that cold-induced thermogenesis seemed to be lower in  
373 cold-acclimatized individuals, which is in line with the previous reports<sup>21</sup>, however BAT  
374 volume defined by the <sup>18</sup>FDG uptake seemed to be similar. We also observed that whole-body  
375 metabolic preference for lipids increases upon NST-stimulating cold exposure in ice-water  
376 swimmers while non-acclimatized individuals increase their preference for glucose under  
377 similar conditions. This is in line with the observation that non-shivering thermogenesis is in  
378 young healthy BAT positive individuals associated with similar increase of respiratory  
379 quotient<sup>3</sup>. This may suggest that regular ice-water swimming is associated with an adaptive  
380 change in the cold-induced whole-body metabolic preference for lipids.

381 The differential regulation of circulating PTH in cold-acclimatized individuals subjected  
382 to ice-water swimming and to non-shivering cold exposure could stem from the intensity of  
383 the cold stimulus, from the exercise component of ice-water swimming<sup>22</sup>, from the post-  
384 swimming shivering thermogenesis and from the difference in the body area exposed to cold  
385 (full-body cold water immersion vs. cooling pads). Meanwhile, the difference in cold-induced  
386 PTH regulation between cold acclimatized and non-acclimatized individuals is most likely  
387 related to the adaptive response to repeated cold water immersion, although we cannot rule  
388 out the effect of  $\beta_3$ AR agonist and the fact that the shivering threshold in cold acclimatized  
389 individuals lies at much lower core body temperature<sup>21</sup>.  
390 Present evidence indicates that PTH or PTH-related protein (PTHrP) drive the thermogenic  
391 program in primary mouse brown, beige and white adipocyte cultures, as well as in brown,



392 inguinal and epididymal white adipose tissue depots of mice <sup>16, 23-24</sup>. This raises the question  
393 whether PTH could be involved in regulation of adaptive thermogenic process in humans. A  
394 recent study has shown the browning, thermogenic and lipolytic effects of PTH in primary  
395 cultures of human subcutaneous white adipocytes <sup>15</sup>. Here, we provide several pieces of  
396 evidence that PTH might specifically modulate metabolic response to cold stress and/or BAT  
397 thermogenic capacity in cold acclimatized humans. We found that the cold-induced metabolic  
398 preference for lipids, which specifically increased in cold-acclimatized individuals, was  
399 associated with the acute change in PTH. Therefore, we speculate that the ice-water  
400 swimming-induced increase in circulating PTH could potentially be linked with the induction  
401 of whole-body fat utilization. It is important to note that the baseline levels of PTH in cold-  
402 acclimatized ice-water swimmers were more than 18% higher in the winter than in the  
403 summer season. This could reflect presence of adaptive changes that might predispose the  
404 individuals to more effective cold response during the cold water swimming. We also found a  
405 negative association between the ice-water swimming-induced increase of PTH and visceral  
406 adiposity, which might be related to the potential of repeated short-term cold-water-induced  
407 PTH spikes to increase the adipose tissue lipid utilization. Furthermore, the magnitude of the  
408 PTH change associated with the cold-induced NST correlated positively with the BAT  
409 volume and PTH levels before and after ice-water swimming strongly correlated with  
410 circulating lactate and succinate, the key metabolite markers of BAT thermogenesis <sup>25-27</sup>.  
411 Interestingly, acidity associated with systemic lactate accumulation might trigger the release  
412 of PTH <sup>28</sup>. Unlike in ice-water swimming, induction of NST is not associated with systemic  
413 increase of lactate <sup>25</sup>. Therefore, it is plausible to think that lactate release could be an  
414 important factor regulating PTH response to cold although further experiments need to  
415 validate this notion. Lastly, systemic levels of PTH in non-acclimatized individuals who were  
416 not exposed to cold correlated well with BAT markers *UCP1*, *PGC1 $\alpha$*  and *DIO2* mRNA, as

417 well as with UCP1 protein. We propose that this might reflect the natural inter-individual  
418 variability in the thermogenic capacity within the group of patients subjected to elective neck  
419 surgery. Furthermore, primary hyperparathyroidism in humans is associated with higher  
420 prevalence and magnitude of  $^{18}\text{F}$ -FDG uptake into BAT <sup>24</sup> and with elevated expression of  
421 several thermogenic genes in deep neck BAT, but not in subcutaneous WAT <sup>16, 23</sup>. In fact, we  
422 showed that human deep neck BAT and subcutaneous WAT express both types of PTH  
423 receptors, indicating that PTH might in fact directly regulate adipose tissue functional state.  
424 Moreover, PTH1R, a receptor shared by PTH and PTHrP, had significantly higher expression  
425 in WAT, while PTH2R, receptor specific for PTH was enriched in BAT. This observation  
426 replicated our RNAseq data<sup>19</sup>. Collectively, we believe that these are important findings  
427 supporting the physiological role of PTH in cold defense mechanisms, potentially including  
428 the adipose tissue metabolic and/or thermogenic activity.

429       The elevated TSH levels after ice-water swimming and the opposite regulation in  
430 response to cold-induced NST in both non-acclimatized and cold-acclimatized individuals is  
431 in line with the evidence that severe cold (cold water immersion) has the capacity to increase  
432 but mild cold (cold air/cooling pads) results in decreased or unchanged circulating TSH <sup>29-33</sup>.  
433 Similarly, the decrease in ft4 associated with ice-water swimming, which was not found  
434 during cold-induced NST, indicates the sensitivity of thyroid axis to cold exposure intensity  
435 or duration. This is supported by the observation that *DIO2* expression and activity increases  
436 in BAT of healthy men after prolonged mild cold exposure <sup>4</sup> that could promote T4-T3  
437 conversion and T4 tissue uptake. In the group of patients with varying thyroid function  
438 (Cohort 4), fasted ft4 levels positively correlated with brown adipose tissue *DIO2* mRNA,  
439 supporting the sensitivity of human BAT to peripheral thyroid hormones. Furthermore,  
440 individual variability in NST-induced change in ft4 was negatively correlated with energy  
441 expenditure in non-acclimatized men. Our data provide important new evidence on the

442 regulation of thyroid hormones by cold in acclimatized and non-acclimatized humans and on  
443 their role in BAT thermogenesis in humans.

444        Limitations of the study include the imbalanced gender ratio and age variability between  
445 the study cohorts, which reflects the limited capacity to recruit such specific groups (ice-water  
446 swimmers, patients undergoing neck surgery). The smaller number of participants in Cohort 2  
447 and several missing values for plasma parameters of Cohort 4 (blood was not collected at the  
448 pilot stage of the study) certainly limited the statistical power, but in our honest opinion,  
449 presented data provide important evidence on the potential role for PTH in modulating  
450 metabolic and thermogenic response to cold in cold-acclimatized individuals. It is important  
451 to note that the sequence/timing of the post-swimming blood collection was not associated  
452 with any variability in either absolute values or cold-induced changes of PTH, TSH or T4. We  
453 also acknowledge some differences in the NST-inducing protocols (use of  $\beta_3$ -AR agonist,  
454 duration of cold and necessity to lower cooling temperature in cold acclimatized individuals)  
455 between cohorts 2 and 3 that might reduce the ability to compare the effects between the  
456 groups. Complexity of the experimental approach could have been extended by comparing  
457 effects of swimming or comparable physical activity without concomitant cold exposure to  
458 control for exercise-specific changes, as well as by extending the protocol to study time-  
459 dependent dynamics of the cold-induced changes in PTH. However, we believe our study  
460 provides important novel evidence creating the grounds for future experiments exploring the  
461 role of PTH in human metabolic & thermogenic response to cold.

462

## 463 **Conclusion**

464        We report that circulating parathyroid hormone and thyroid axis components were  
465 acutely stimulated by ice-cold water swimming, but not by mild cold exposure inducing non-  
466 shivering thermogenesis in cold acclimatized individuals. The relationships between PTH and

467 cold-induced metabolic substrate preference for lipids and the presence of systemic and  
468 brown adipose tissue markers of thermogenic process provide pilot evidence indicating that  
469 PTH is involved in metabolic and thermogenic response to cold stress in humans.

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472 the study volunteers for participating in this study.

#### 473 **Data Availability**

474 The datasets generated during and/or analyzed during the current study are not publicly  
475 available but are available from the corresponding author on reasonable request.

476

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564

566 **Table 1:** Characteristics of Cohort 1 in winter (all) and seasonal paired comparison on a  
 567 subset of volunteers who underwent clinical examination both in winter (February) and late  
 568 summer (September) period.

characteristic (units)	all volunteers		paired comparisons		
	n (M/F)	winter	n (M/F)	winter	summer
age (y)	13/2	48.7±9.0	10/2	46.1±7.7	46.1±7.7
BMI (kg.m <sup>-2</sup> )	13/2	29.8±4.2	10/2	29.8±4.6	29.5±4.6
waist circumference (cm)	11/2	99.7±10.6	9/2	99.0±11.0	97.5±11.8
body fat (%)	12/2	29.1±6.5	10/2	29.3±6.9	29.2±7.6
skeletal muscle (%)	12/2	32.4±3.7	10/2	32.4±3.9	32.3±4.5
visceral adiposity (rel.u.)	12/2	13.3±5.2	10/2	13.1±5.4	12.8±5.6
systolic BP (mmHg)	8/2	138±19	6/2	133±16	126±18
diastolic BP (mmHg)	8/2	92±7	6/2	90±6	90±9
pulse (n/min)	6/2	63±13	4/2	64±15	66±12
glucose (mmol.l <sup>-1</sup> )	12/2	5.03±0.45	8/2	5.00±0.45	4.77±0.29
insulin (mIU.l <sup>-1</sup> )	12/2	6.89(7.60)	8/2	6.36(5.03)	4.87(2.33)
HOMA-IR	12/2	1.51(1.80)	8/2	1.36(1.54)	1.01(0.58)
AST (μkat.l <sup>-1</sup> )	12/2	0.37±0.08	8/2	0.36±0.06	0.41±0.13
ALT (μkat.l <sup>-1</sup> )	12/2	0.34±0.12	8/2	0.33±0.14	0.32(0.14)
triglycerides (mmol.l <sup>-1</sup> )	12/2	1.03±0.52	8/2	1.01±0.53	0.94(0.78)
total cholesterol (mmol.l <sup>-1</sup> )	12/2	4.79±0.82	8/2	4.76±0.85	5.05±0.91
HDL cholesterol (mmol.l <sup>-1</sup> )	12/2	1.40(0.39)	8/2	1.37(0.30)	1.41(0.19)
hsCRP (mg.l <sup>-1</sup> )	12/2	1.13(1.92)	8/2	1.06(1.43)	1.00(1.30)
TSH (mIU.l <sup>-1</sup> )	12/2	1.81±0.89	8/2	1.98±0.89	1.76±0.79
free T4 (pmol.l <sup>-1</sup> )	12/2	15.21±1.96	8/2	15.92(1.78)	15.64±1.60
total T4 (nmol.l <sup>-1</sup> )	12/2	89.79±10.61	8/2	87.81±11.80	90.65±11.01
PTH (pg.ml <sup>-1</sup> )	12/2	41.65±8.59	8/2	41.78±7.52	35.23±9.49*
duration of IWS (y)	13/2	3.0(5.0)	-	-	-
IWS frequency (n/week)	12/2	2.4±0.8	-	-	-

569 Values are expressed as mean±SD or median (interquartile range). BMI – body mass index, BP – blood pressure,  
 570 HOMA-IR – homeostatic assessment model of insulin resistance [(fasting glucose x fasting insulin)/22.5], AST  
 571 – aspartate transaminase, ALT – alanine transaminase, hsCRP – high sensitivity C-reactive protein, TSH –  
 572 thyroid stimulating hormone, T4 – thyroxine, PTH –parathyroid hormone, IWS – ice-water swimming. Visceral  
 573 adiposity – relative units (rel.u.) with total 30 levels according to Omron Healthcare (<9: normal, 10-14: high,  
 574 15-30: very high). The paired comparison was performed using Student’s paired t-test or Wilcoxon matched-  
 575 pairs signed rank test (\*p<0.05).

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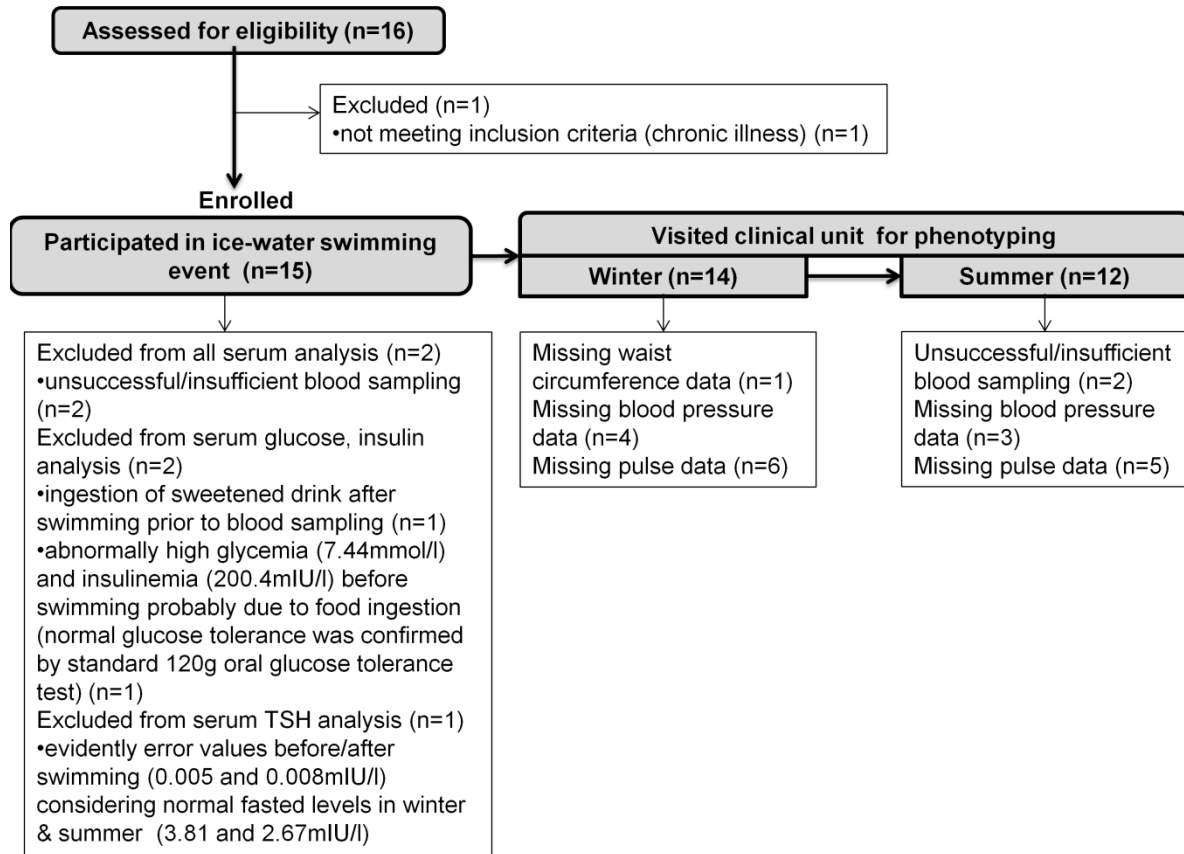


578 **Table 2:** The antropometric, metabolic and hormonal parameters of Cohorts 2 and 3 at warm  
579 (baseline) and non-shivering cold condition.

<b>study group</b>	<b>cold-acclimatized (Cohort 2)</b>		<b>non-acclimatized (Cohort 3)</b>	
<b>n</b>	<b>5 males/1 female</b>		<b>11 males</b>	
<b>condition</b>	<b>warm</b>	<b>cold</b>	<b>warm</b>	<b>cold</b>
<b>age (years)</b>	37.2±4.4	-	22.7±2.6	-
<b>BMI (kg.m<sup>-2</sup>)</b>	25.1±5.0	-	23.4±1.9	-
<b>fat mass (kg)</b>	20.3±11.3	-	16.5±3.5	-
<b>lean body mass (kg)</b>	60.7±10.8	-	60.5±2.6	-
<b>RMR (kcal/day)</b>	1719.5±299.5	1830.3±293.7*	2166.8±277.2	2424.6±320.8**
<b>RQ (VCO<sub>2</sub>/VO<sub>2</sub>)</b>	0.81±0.04	0.77±0.06*	0.78±0.08	0.84±0.07**
<b>CIT (%)</b>	-	6.7±4.7	-	12.2±9.8
<b>BAT volume (cm<sup>2</sup>)</b>	-	137.6±118.2	-	56.8±37.8
<b>SUV mean/LBM (a.u.)</b>	-	2.9±0.54	-	4.0±0.87
<b>PTH (pg.ml<sup>-1</sup>)</b>	24.6±7.5	23.5±7.8	25.5(13.6)	20.5(9.9)**
<b>TSH (mIU.l<sup>-1</sup>)</b>	2.3±1.1	1.7±0.8*	1.2±0.4	1.1±0.3*
<b>free thyroxine (pmol.l<sup>-1</sup>)</b>	16.2±0.9	16.0±0.1.15	18.2±1.7	18.8±1.2

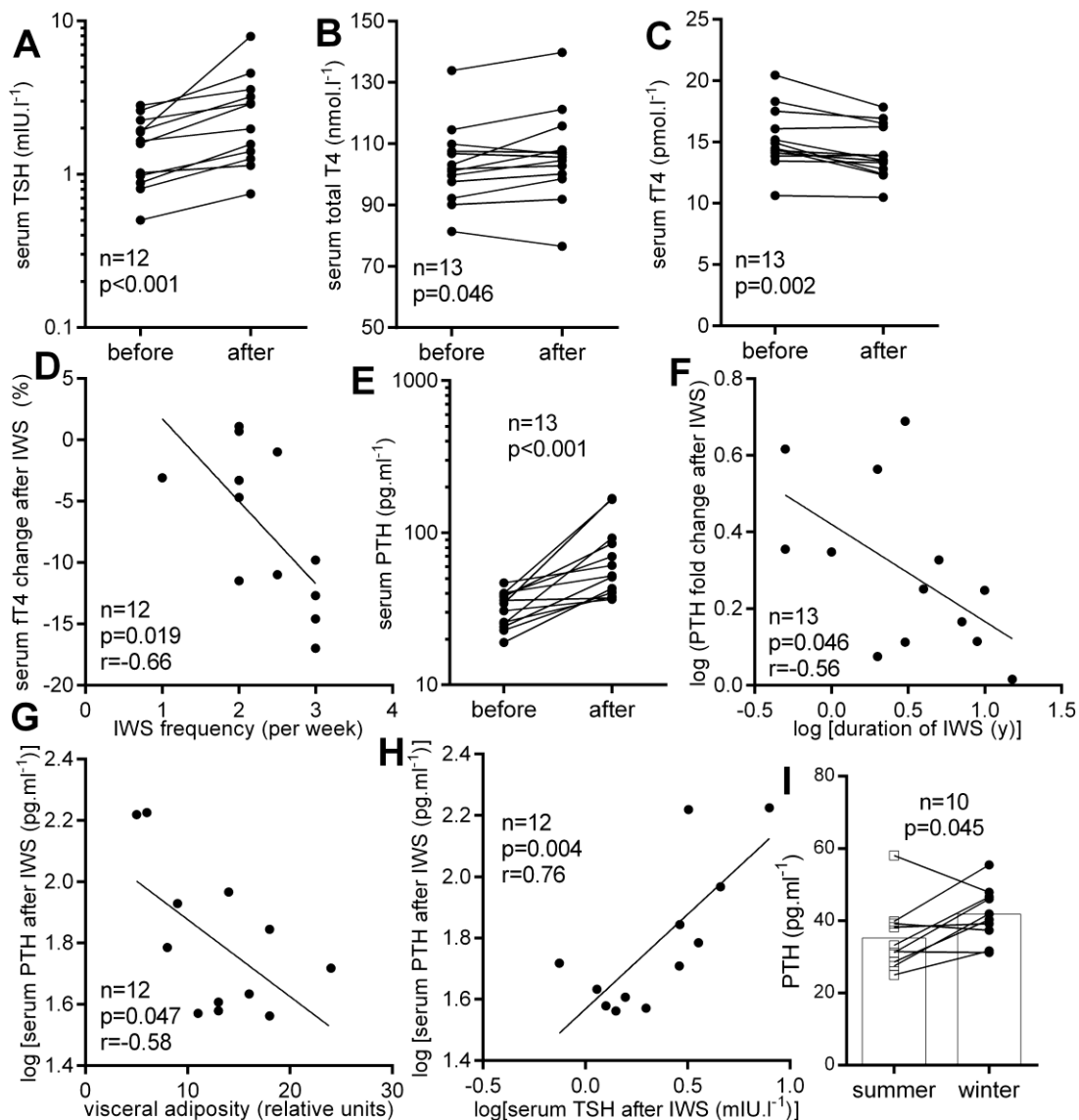
580 RMR – resting metabolic rate, RQ – respiratory quotient, CIT – cold-induced thermogenesis (change in RMR),  
581 BAT – brown adipose tissue, SUV – <sup>18</sup>F-fluorodeoxyglucose standardized uptake value normalized to lean body  
582 mass (LBM), PTH – parathyroid hormone, TSH – thyroid stimulating hormone. Effect of cold exposure was  
583 analyzed by two-tailed paired Student’s t-test. \*p<0.05, \*\*p<0.01  
584

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587

**Figure 1:** Study design for Cohort 1.



588

589

590 **Figure 2: Cold affects circulating parathyroid and thyroid hormones in ice-water**

591 **swimmers (Cohort 1).** Acute effect of ice-water swimming (IWS) on serum (A) thyroid

592 stimulating hormone (TSH), (B) total and (C) free thyroxine (fT4) and (D) its relationship

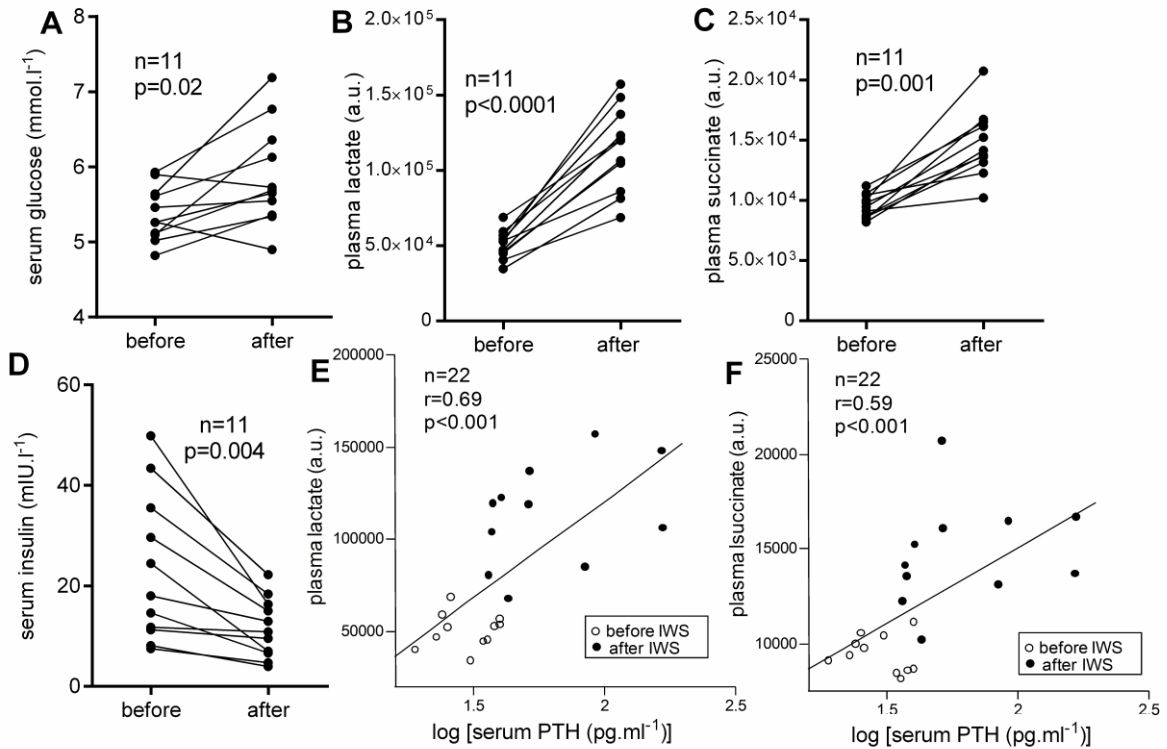
593 with IWS frequency; acute effect of IWS on (E) parathyroid hormone (PTH) levels and (F) its

594 relationship (F) number of years dedicated to IWS habit, (G) visceral fat and (H) with TSH;

595 (I) PTH seasonal difference. Two-tailed Student's paired t-test (or Wilcoxon matched-pairs

596 signed rank test and linear regression were used to statistically analyze data.  $r$  – Pearson's

correlation coefficient.

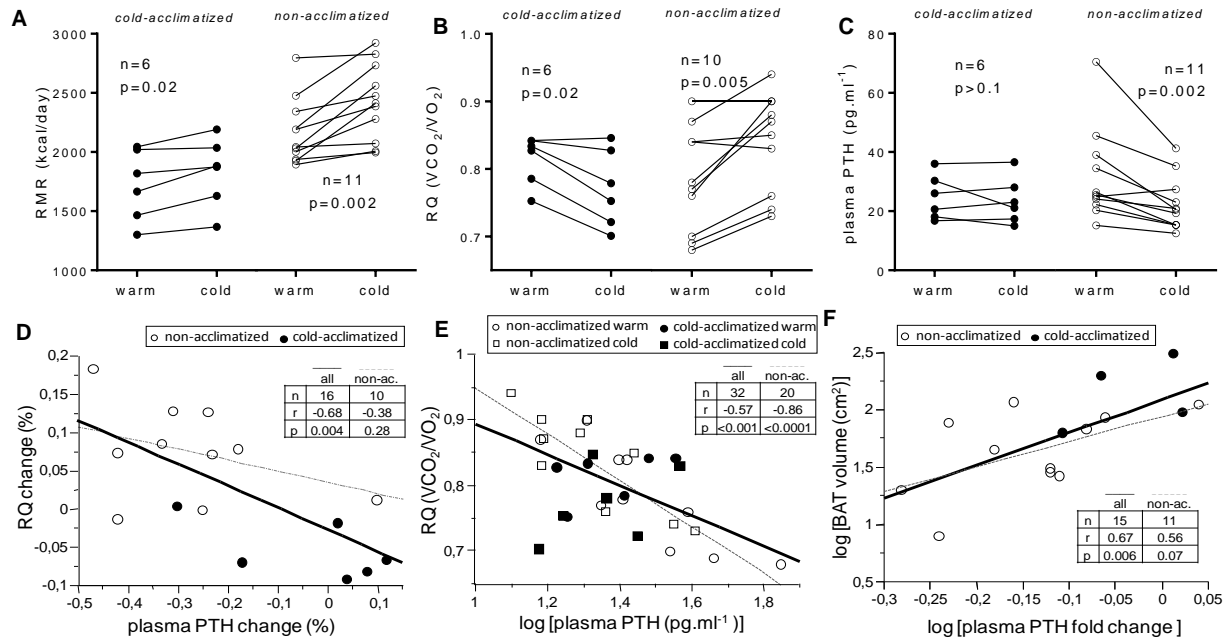


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598 **Figure 3: Ice-water swimming modulates substrate metabolites in ice-water swimmers**  
599 **(Cohort 1).** Acute effects of ice-water swimming (IWS) on peripheral (A) glycemia, (B)  
600 lactate and (C) succinate levels and (D) insulinemia; (E-F) associations of circulating  
601 metabolites with parathyroid hormone (PTH) levels during ice-water swimming. Paired  
602 Student's t-test (A, B, D) Wilcoxon matched-pairs signed rank test (C) and linear regression  
603 (E-F) were used to analyze data; r – Pearson's correlation coefficient.

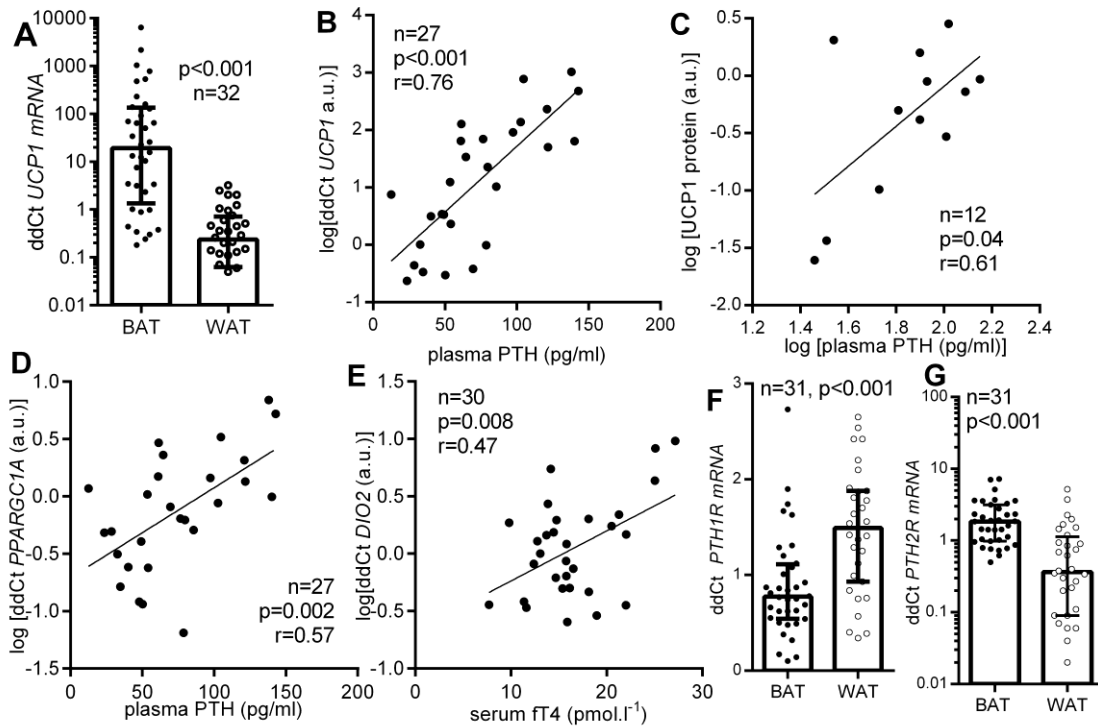
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607 **Figure 4: Cold-induced non-shivering thermogenesis distinctly modulates circulating**  
 608 **parathyroid hormone in cold-acclimatized and non-acclimatized individuals.** Effect of  
 609 non-shivering cold exposure (Cohort 2) and combined cold/ $\beta_3$ -adrenergic stimulation (Cohort  
 610 3) on (A) the resting metabolic rate (RMR) (B), metabolic substrate preference (respiratory  
 611 quotient - RQ) and (C) circulating parathyroid hormone (PTH); Relationships between PTH  
 612 and (D, E) RQ and (F) brown fat volume (the only woman in the group was removed from the  
 613 correlation). (D-F) fit lines distinguish correlations in all individuals (full line) or within non-  
 614 acclimatized (non-ac.) group only (grey dashed line). Two-tailed Student's paired t-test or  
 615 Wilcoxon matched-pairs signed rank test and linear regression were used to statistically  
 616 analyze data.  $r$  – Pearson's correlation coefficient.  
 617



618

619 **Figure 5: Parathyroid hormone levels are related to human brown fat thermogenic**  
620 **program (Cohort 4).** (A) the relative gene expression of uncoupling protein 1 (UCP1) in  
621 deep neck brown (BAT) versus subcutaneous white adipose tissue (WAT), (B, C) the  
622 relationship between circulating parathyroid hormone (PTH) levels with BAT UCP1 mRNA  
623 and protein levels and (D) with the gene expression of peroxisome proliferator-activated  
624 receptor gamma coactivator 1-alpha (*PPARGC1A*); (E) association between circulating free  
625 thyroxine (ft4) and type 2 deiodinase (*DIO2*) gene expression in BAT; (F, G) gene  
626 expression levels of parathyroid hormone receptors (*PTH1R*) in BAT and WAT displayed as  
627 median with interquartile range, analyzed by Wilcoxon matched pairs signed-rank test.  
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