Metabolic hormones and ethnic aspects in obesity

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CHAPTER 5

COLD EXPOSURE INCREASES CIRCULATING FIBROBLAST GROWTH FACTOR 21 IN THE EVENING IN MALES AND FEMALES

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ABSTRACT

Objectives

Cold exposure is linked to cardiometabolic benefits. Cold activates brown adipose tissue (BAT), increases energy expenditure, and induces secretion of the hormones fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15). The cold-induced increase in energy expenditure exhibits a diurnal rhythm in men. Therefore, we aimed to investigate the effect of cold exposure on serum FGF21 and GDF15 levels in humans and whether cold-induced changes in FGF21 and GDF15 levels differ between morning and evening in males and females.

Method

In this randomized cross-over study, serum FGF21 and GDF15 levels were measured in healthy lean males (n=12) and females (n=12) before, during, and after 90 minutes of stable cold exposure in the morning (7:45 am) and evening (7:45 pm) with a one-day washout period in between.

Results

Cold exposure increased FGF21 levels in the evening compared to the morning both in males (+61% *vs.* -13%; $P < 0.001$) and in females (+58% *vs.* +8%; $P < 0.001$). In contrast, cold exposure did not significantly modify serum GDF15 levels, and no diurnal variation was found. Changes in FGF21 and GDF15 levels did not correlate with changes in cold-induced energy expenditure in the morning and evening.

Conclusion

Cold exposure increased serum FGF21 levels in the evening, but not in the morning, in both males and females. GDF15 levels were not affected by cold exposure. Thus, this study suggests that the timing of cold exposure may influence cold-induced changes in FGF21 levels but not GDF15 levels and seems to be independent of changes in energy expenditure.

INTRODUCTION

Over the past decade, the popularity of ice baths and cold showers has been on the rise (1). Interestingly, the beneficial effects of cold exposure have already been described in the Edwin Smith Surgical Papyrus, the oldest known medical document (2, 3). Recent studies have shown the potential benefits of cold exposure, including its ability to reduce anxiety and improve insulin sensitivity in patients with type 2 diabetes (T2D) (2, 4, 5). The exact mechanism(s) underlying the metabolic improvement associated with cold exposure remain unknown.

A possible mechanism by which cold exposure improves metabolic health may be activating brown adipose tissue (BAT). Cold exposure stimulates the release of norepinephrine that binds to beta-adrenergic receptors located on BAT, of which the beta-2-adrenergic receptor seems to be the dominant receptor in humans (6, 7). Binding of norepinephrine triggers the oxidation of fatty acids (FA) and glucose to generate heat, a process facilitated by uncoupling protein 1 (UCP1) (8). Given its capacity to burn energy, activation of BAT holds promise as a potential tool for improving cardiometabolic diseases (9). We previously showed that metabolic BAT activity follows a diurnal rhythm in mice, with the highest uptake of triglyceride (TG)-derived FA by BAT at the onset of the active period, independent of environmental temperature (10). In humans, previous research has shown that glucose uptake by BAT might be higher in the morning (11). In addition, we recently showed that cold-induced thermogenesis, assessed by cold-induced energy expenditure and supraclavicular skin temperature, was higher in the morning than in the evening in humans (12). This difference was observed in males but not in females, suggesting a potential influence of a diurnal rhythm on BAT, which may be sex-dependent. Indeed, sex differences in thermoregulation and the release of various metabolic hormones following a stimulus have previously been reported and could potentially influence BAT activation and metabolic outcomes between males and females (13, 14).

It is known that BAT can release regulatory factors known as batokines with autocrine, paracrine, and endocrine actions that could enhance energy metabolism (15). Preclinical experiments have identified two batokines that have a prominent effect on energy balance: fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15) (16, 17). FGF21, when secreted by activated brown adipocytes, promotes thermogenesis through both UCP1-dependent and UCP1-independent mechanisms (18, 19). Additionally, FGF21 is secreted by the liver in a process dependent on the action of peroxisome proliferator-activated receptor α (PPARα) during fasting conditions (20). An increase in FGF21 levels leads to higher hepatic lipid oxidation, ketogenesis, and

gluconeogenesis (20). Similarly, GDF15 is secreted by brown adipocytes upon activation and targets macrophages to downregulate proinflammatory signals (21). GDF15 also induces satiety by acting on the glial cell line-derived neurotrophic factor (GDNF) family receptor alpha-like (GFRAL) located in the area postrema and nucleus solitary tract of the hindbrain (22-24).

Previous studies, conducted at room temperature, have shown a diurnal variation in FGF21 and GDF15 levels in humans, with the peak early in the morning and nadir in the afternoon (25, 26). Both FGF21 and GDF15 play important roles in metabolic health and therefore are potential tools in the treatment of metabolic diseases such as obesity and atherosclerosis (27-29). Understanding the diurnal influence on cold-stimulated FGF21 and GDF15 levels and potential sex differences may provide valuable insight into the most effective timing for BAT activation in both males and females.

Therefore, in the current study we aimed to investigate 1) the effect of cold exposure on serum FGF21 and GDF15 levels and 2) whether cold-induced changes in FGF21 and GDF15 levels differ between morning and evening in both males and females.

METHODS

Participants and study design

Participants

This study is a secondary analysis of a previously performed randomized cross-over study conducted at the Leiden University Medical Center (12). The study aimed to assess whether cold-induced thermogenesis varies between the morning and the evening in healthy lean males and females (12). A total of 24 participants, 12 males, and 12 females, were included. To be eligible for inclusion, participants had to be aged 18-31 years, with a body mass index (BMI) ranging from 18 to 26 kg/m^2. Participants were excluded if they had any active endocrine, renal, or hepatic disease, were taking medication that could affect glucose and/or lipid metabolism or BAT activity, were smokers, substance abusers, were pregnant, had recent weight changes, or had a disturbed day-night rhythm. The study was performed between December 2019 and December 2020.

Study approval

The study was undertaken in accordance with the principles of the Declaration of Helsinki (30) and approved by the local ethics committee of the Leiden University Medical Center, Leiden, the Netherlands. All participants provided written informed

consent before participation. The trial is registered at ClinicalTrials.gov (registration no. NCT04406922).

Study design

An extensive description of the design of the study has been published elsewhere (12). In short, each participant underwent a personal cooling protocol twice, once in the morning (7:45 am) and once in the evening (7:45 pm), with a one-day interval between the two study days. At the start of the study day, height, and waist- and hip circumference were measured. This was followed by the measurement of body weight and body composition using bioelectrical impedance analysis (digital balance; E1200, August Sauter GmBH, Albstadt, Germany and InBody720, InBody Co., Ltd., CA, USA). Afterward, an intravenous cannula was inserted into the antecubital vein to obtain blood samples during the study day. Finally, the participants lay down between two blankets filled with water (Blanketroll III, Cincinnati Sub-Zero Products, Inc, Cincinnati, Ohio, USA) at a thermoneutral temperature of 32°C for 45 minutes. Thereafter, the temperature was gradually decreased until either the participant began shivering or the minimal temperature of 9°C was reached. The temperature was then increased by 2-3°C and maintained at this level for 90 minutes. During the personalized cooling protocol energy expenditure was measured twice using indirect calorimetry (Vyntus CPX, Carefusion, Hochberg, Germany). In addition, blood pressure and heart rate were measured at different times during the personalized cooling protocol (t = 45, 65, and 180 minutes) using a cuff connected to a digital blood pressure device (Welch Allyn, Skaneateles, New York, USA).

Randomization

Participants were randomized to decide in which order the participant underwent the two study days, either morning-evening or evening-morning. The unequal distribution between the two randomization groups (with 10 and 14 participants, respectively) resulted from the randomization of newly recruited participants who replaced those unable to participate due to COVID-19-related restrictions.

Blood collection

For this study we used blood samples collected at three time points: after 45 minutes of thermoneutral conditions, 15 minutes after starting the cooling-down period, and after 90 minutes of stable cold exposure. Fasted venous blood samples were collected using Vacutainer SST II Advanced tubes, and serum was obtained after centrifugation and stored at -80°C until analysis. Serum FGF21 levels were measured using an immunoassay of the U-PLEX Human FGF21 assay platform (Meso Scale Discovery, Rockville, Maryland, USA). Serum GDF15 levels were measured with a custom-built

Luminex Screening assay (R&D Systems, Minneapolis, Minnesota, USA) was used in combination with the Bio-Plex Multiplex system (Bio-Rad, Hercules, California, USA). Serum cortisol levels were measured with the commercially available enzymatic kit (Roch Diagnostics, Woerden, the Netherlands).

Supraclavicular skin temperature measurement

The supraclavicular skin temperature was measured at 1-minute intervals using wireless iButton temperature loggers (31) and through infrared thermographic (IRT) images (FLIR T450sc, FLIR systems Inc., Wilsonville, OR, USA) focused on the upper thorax/neck region at the start of the study visit and after cold exposure. Analysis of the data recorded by the iButton temperature loggers was analyzed using Temperatus software (http://profith.ugr.es/temperatus) (31), while the IRT images were processed via an open-source IRT toolbox software. Further details of the methods were described previously (12).

Energy expenditure

A metabolic cart equipped with a ventilated hood system (Vyntus CPX, Carefusion, Hochberg, Germany) was used to collect data on total carbon dioxide production and oxygen consumption. At each study visit, energy expenditure (EE) was measured twice using indirect calorimetry: first at the end of the thermoneutral period when a stable cold temperature was reached and second during the last 30 minutes of stable cold exposure. The methods were described in detail previously (12).

Statistical analysis

Data are expressed as means ± standard error of the mean. Data normality was confirmed using the Shapiro-Wilk test, visual histograms, and Q-Q plots. All statistical analyses were done separately for males and females. For this clinical study, the statistical analysis of the baseline characteristics of this cohort has been extensively described elsewhere (12). To study the influence of circadian rhythm on cold-induced changes in serum GDF15 and FGF21 levels, a general linear model with repeated measures was used, with 2 within-subject factors. First, the timing during the day (morning *vs*. evening), and second, the cooling phase (thermoneutral, cooling down, and end of cooling). As the serum FGF21 levels, GDF15 levels, and cortisol levels were not normally distributed at all time points, we log10-transformed all serum FGF21 and GDF15 outcomes at the different time points. To compare the change of serum FGF21 and GDF15 levels between the morning and the evening, a delta was created (Δ_{cold}; values at the end of cooling phase *minus* values at thermoneutral period and $\Delta_{cooling}$; cooling-down *minus* thermoneutral) and two-tailed paired Student's t-tests were used. Moreover, to compare the serum FGF21 levels at thermoneutral conditions between

morning and evening as well as the serum GDF15 levels at thermoneutral conditions between morning and evening, paired Student's t-tests were used. To study the association between the change in energy expenditure with the change in serum FGF21 and GDF15 levels, deltas (Δ_{cold} and $\Delta_{cooling\ down}$) were created for all outcomes. Thereafter, nonparametric Spearman-rank correlations (rho) were applied to the raw data as the data were not normally distributed. All statistical analyses were performed using the Statistical Package for the Social Sciences v.25.0 (IBM Corp. Released 2018. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.), whereas all graphs were created with GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, San Diego, California, USA). Significance was set at $P < 0.05$.

RESULTS

Baseline characteristics

As previously reported (12), there were no significant differences in age, weight, and BMI between the males and females included in this study. However, the females were shorter with a higher body surface area (BSA), waist circumference, waist-to-hip ratio, fat mass, and fat percentage compared to males. Cold-induced thermogenesis, as measured by cold-induced energy expenditure and supraclavicular skin temperature, was higher in the morning compared to the evening for males only. In females, there was no diurnal variation in cold-induced thermogenesis, though they displayed an extended time to shivering and had a lower shivering temperature in the morning compared to the evening (12).

Cold exposure in the evening, but not in the morning, increases serum FGF21 in both sexes

At thermoneutral conditions, there were no significant differences in serum FGF21 levels in the morning compared to the evening in both males (332 ± 72 pg/mL *vs.* 288 ± 69 pg/mL; P = 0.088, **Fig. 1A**) and females (285 ± 50 pg/mL *vs.* 242 ± 39 pg/mL; P = 0.403, **Fig. 1D**). Cold exposure significantly increased serum FGF21 levels in the evening compared to the morning in both males ($P_{Interaction} < 0.001$; morning: -13 ± 10% *vs.* evening: +61 ± 21%; P < 0.001, **Fig. 1B** and **C**) and in females ($P_{Interaction} = 0.001$; morning: +8 ± 10% *vs.* evening: +58 ± 13%; P < 0.001, **Fig. 1E** and **F**).

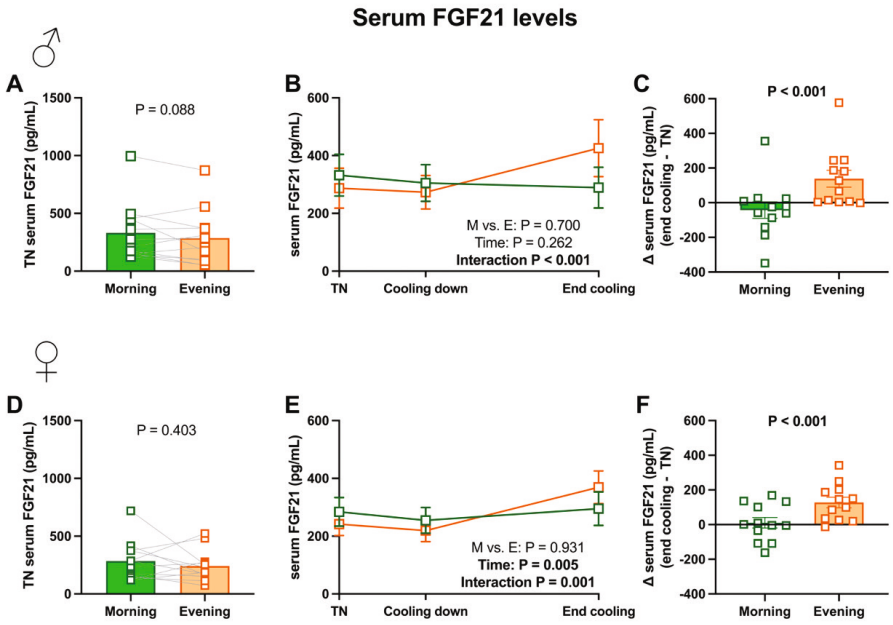

Figure 1. Changes in serum FGF21 levels after cold exposure in the morning compared to the evening in both males and females

Box plots showing serum FGF21 levels at thermoneutral (TN) conditions in the morning and the evening in males (n=12) **(A)** and females (n=12) **(D)**, and the cold-induced changes of serum FGF21 levels (end of cooling minus thermoneutral) during the morning and the evening in males (n=12) **(C)** and females (n=12) **(F)**. Squares represent individual values, boxes represent means, and deviations represent the standard error of the mean. Linear model showing the changes of serum FGF21 levels after cold exposure in the morning and the evening in males (n=12) **(B)** and females (n=12) **(F)**. The changes were assessed during three time points (i.e., thermoneutral, cooling down, and end of cooling). However, for two participants, serum FGF21 levels in the cooling down phase in the evening could not be measured (n=10). Squares represent means, and deviations represent the standard error of the mean. Green boxes, lines, and boxes represent the morning values, and orange boxes, lines, and boxes represent the evening values.

Neither cold exposure in the morning nor in the evening affects serum GDF15 levels in both sexes

At thermoneutrality, we observed similar GDF15 levels in the morning and in the evening in males (297 ± 41 pg/mL *vs.* 295 ± 32 pg/mL; P = 0.674, **Fig. 2A**) and females (315 ± 65 pg/mL *vs.* 289 ± 40 pg/mL; P = 0.865, **Fig. 2D**). Additionally, cold exposure did not modify serum GDF15 levels (P_{time} = 0.126, **Fig. 2B**). Further analyses showed that serum GDF15 levels before and after cold exposure were similar in the morning compared to the evening ($P_{Interaction}$ = 0.257; morning: +31 ± 18% *vs.* evening: +4 ± 7%; P = 0.293 **Fig. 2B** and **C**). Similarly, in females, cold exposure did not modify serum GDF15 levels (P_{time} = 0.929, **Fig. 2E**) and we did not detect significant differences in the GDF15 responses

upon cold exposure in the morning and the evening ($P_{Interaction}$ = 0.402; morning: +3 ± 15% *vs.* evening: +8 ± 6%; P = 0.395, **Fig. 2E** and **F**).

We also performed sensitivity analyses comparing $\Delta_{cooling\ down}$ of serum FGF21 and GDF15 levels during cooling compared to the thermoneutral phase in the morning and the evening. However, we observed no effect of cold exposure and therefore, no significant differences between morning and evening (data not shown).

In addition, we correlated circulating FGF21 levels with serum GDF15 levels, however we did not find a significant correlation (data not shown).

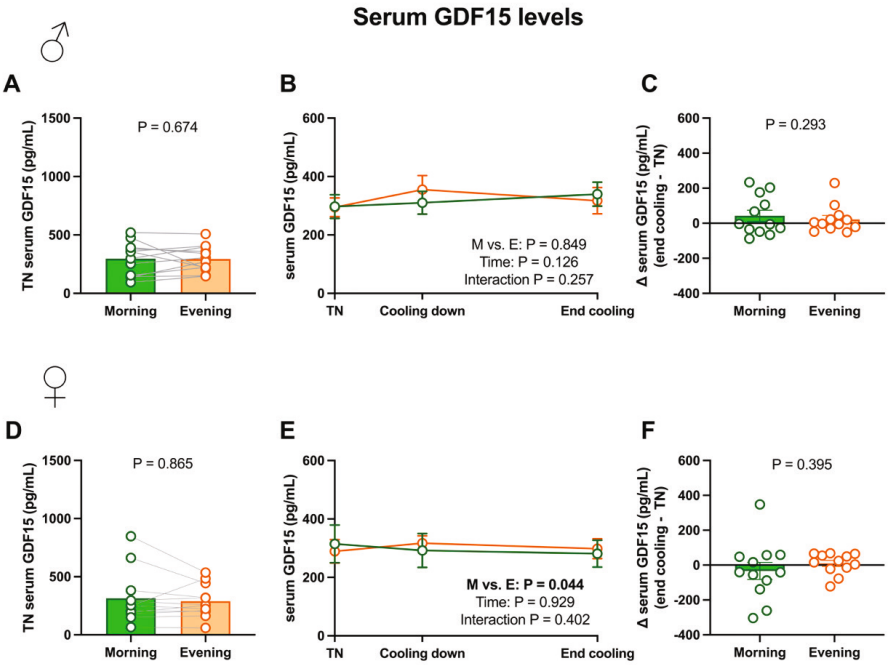

Figure 2. Changes in serum GDF15 levels after cold exposure in the morning compared to the evening in both males and females

Box plots showing serum GDF15 levels at thermoneutral (TN) conditions in the morning and the evening in males (n=12) **(A)** and females (n=12) **(D)**, and the cold-induced changes of serum GDF15 levels (end of cooling minus thermoneutral) in the morning and the evening in males (n=12) **(C)** and females (n=12) **(F)**. Dots represent individual values, boxes represent means, and deviations represent the standard error of the mean. Linear model showing the changes of serum GDF15 levels after cold exposure in the morning and the evening in males (n=12) **(B)** and females (n=12) **(E)**. The changes were assessed during three time points (i.e., thermoneutral, cooling down, and end of cooling). However, for two participants serum GDF15 levels in the cooling-down phase in the evening could not be measured (n=10). Dots represent means, and deviations represent the standard error of the mean. Green circles, lines, and boxes represent the morning, and orange circles, lines, and boxes represent the evening.

Serum cortisol increases after cold exposure in the evening in males only

Since cold exposure induces a stress response, we also compared the effects of cold exposure on circulating cortisol levels between morning and evening. First, as expected and in accordance with its diurnal rhythm, at thermoneutrality serum cortisol levels were higher in the morning compared to the evening in both males (318 ± 20 nmol/L *vs.* 114 ± 25 nmol/L; P < 0.001, **Supplemental Fig. 1A**) and females (526 ± 80 nmol/L *vs.* 270 ± 45 nmol/L; P = 0.002, **Supplemental Fig. 1C**). Additionally, serum cortisol only increased after cold in the morning in males compared to the evening (+20 ± 12% *vs.* -28 ± 8%; P = 0.008, **Supplemental Fig. 1B**). However, in females, circulating cortisol did not change significantly in the morning nor in the evening (-16 ± 11% *vs.* -5 ± 21%; P = 0.953, **Supplemental Fig. 1D**).

In addition, we correlated circulating FGF21 levels with serum cortisol, however, we did not find a significant correlation (data not shown).

The change of cold-induced serum FGF21 levels in the evening is correlated with the change of supraclavicular skin temperature but not with energy expenditure, in only females

We next assessed whether the effects of cold exposure on circulating FGF21 and GDF15 levels were associated with the cold-induced effects on supraclavicular skin temperature and energy expenditure. We found a significant and positive correlation between the change of serum FGF21 levels and the change of supraclavicular skin temperature in the evening in females (rho = 0.836, P < 0.001, **Supplemental Fig. 2B**), but not in males (P = 0.247, **Supplemental Fig. 2A**). We did not find a significant correlation in the morning for any sex (all P ≥ 0.142, **Supplemental Table 1**). Furthermore, the change in GDF15 levels did not correlate with the change in supraclavicular skin temperature at any time point (**Supplemental Table 1**). A lack of significance was also observed in the correlation between the change of serum FGF21 levels and the percental change in cold-induced energy expenditure in the morning and the evening in males (rho = -0.455, P = 0.160 and rho = 0.264, P = 0.433, respectively, **Supplemental Fig. 3A** and **B**) or females (rho = -0.300, P = 0.370 and rho = -0.082, P = 0.811, **Supplemental Fig. 3C** and **D**). Similarly, the change of cold-induced GDF15 levels was not correlated with the cold-induced changes of energy expenditure in the morning and the evening in males (rho = 0.591, P = 0.056 and rho = -0.255, P = 0.450, **Supplemental Fig. 4A** and **B**) or females (rho = -0.145, P = 0.670 and rho = 0.082, P = 0.811, **Supplemental Fig. 4C** and **D**). Finally, we assessed the effect of cold exposure on blood pressure and heart rate and found that cold exposure increased systolic (SBP) and diastolic blood pressure (DBP) similarly in the morning and evening in males (SBP: $P_{Interaction}$ = 0.499; morning: + 20 ± 2% *vs.* evening: +17 ± 2%; P = 0.337; **Supplemental Fig. 5A and B;** DBP:

$P_{Interaction}$ = 0.123; morning: +25 ± 4% *vs.* evening: +20 ± 3%; P = 0.150; **Supplemental Fig. 6A and B**) and females (SBP: $P_{Interaction}$ = 0.275; morning: +11 ± 1% *vs.* evening: +9 ± 2%; P = 0.428; **Supplemental Fig. 5C and D**; $P_{Interaction}$= 0.218; DBP: morning: +17 ± 2% *vs.* 13 ± 2%; P = 0.258; **Supplemental Fig. 6C and D**), without affecting heart rate (males: $P_{Interaction}$=0.426; morning: -9 ± 4% *vs.* evening: -7 ± 4%**;** P = 0.652; **Supplemental Fig. 7A and B**; females: $P_{Interaction}$ = 0.157; morning: +0 ± 4% *vs.* evening: -4 ± 5%; P = 0.289; **Supplemental Fig. 7C and D**).

The change in cold-induced serum FGF21 levels is negatively correlated with the change in serum triglycerides

Here, we investigated whether the changes in FGF21 and GDF15 levels are correlated with changes in circulating lipids. We observed that the change of serum FGF21 levels was negatively and significantly related to the change of serum free fatty acids exclusively in the morning in males (rho = -0.874, P < 0.001, **Supplementary Table 1**). However, this correlation was neither observed in the morning for females (rho = -0.085, P = 0.794) nor in the evening for both sexes (**Supplementary Table 1)**. On the other hand, we found a significant and negative correlation between the change of serum FGF21 levels and the change in circulating triglycerides, but only in the evening for females (rho = -0.580, P = 0.048, **Supplementary Table 1**). No significant correlations were observed in the other analyses (**Supplementary Table 1**). Lastly, the change in serum GDF15 did not correlate with any of the circulating lipids (**Supplementary Table 1**).

DISCUSSION

In this study, we observed that cold exposure increased serum FGF21 levels in the evening but not in the morning in both males and females. However, serum GDF15 levels were not affected by cold exposure, and no diurnal variation was found. Furthermore, no correlations were seen between the changes in serum FGF21 and GDF15 and changes in cold-induced energy expenditure in both sexes. Our findings thus suggest that the timing of cold exposure influences cold-induced changes in FGF21 levels.

Although circulating FGF21 has been reported to exhibit a diurnal rhythm, peaking early in the morning and reaching a nadir in the afternoon (26), we did not find higher thermoneutral FGF21 levels in the morning compared to the evening. This seeming discrepancy may be explained by the fact that the early peak of FGF21 already occurred prior to our FGF21 measurements during the study days in the morning (26). Moreover, the response of this rhythm to cold stimulation has remained unclear so far (26). Our study indicates that FGF21 levels only increase after cold exposure in the evening for

both males and females. The increase of FGF21 in the evening only could be attributed to the natural diurnal effect of FGF21, as the nadir is in the afternoon which increases towards the peak early in the morning. However, previous research showed that the biggest increase in FGF21 levels starts around midnight, which was much later than the completion of our study days (26). In addition, previous research showed that the natural increase of FGF21 levels was about 20% from nadir to peak (26). We found an increase in the evening of 61% in males and 58% in females after 90 minutes of cold exposure, supporting that the increase in FGF21 levels is likely not only attributed to the diurnal variation of FGF21. Nonetheless, this response is still modest compared to the approximately tenfold higher circulating FGF21 levels seen in patients with sepsis compared to healthy individuals (32).

Fasting is also known to influence FGF21 levels. This is a consequence of enhanced FFA release from white adipose tissue following fasting, resulting in activation of hepatic PPARα and FGF21 release (20). Of note, cold exposure seems to have similar effects on circulating FGF21 levels as fasting (33). A previous study where participants fasted for up to 72 hours resulted in increased FGF21 levels specifically in the evening, similar to our findings where cold exposure only increased serum FGF21 levels in the evening (34). To exclude the effect of fasting on serum FGF21 levels, participants fasted for the same time period before the study day in the morning and evening. In addition, the increase of FG21 in the evening only might have resulted from a stress response of the body to cold exposure. However, we did not find a significant increase in serum cortisol levels after cold exposure, and no significant correlation was found between the change in serum FGF21 levels and serum cortisol levels. This implies that the increase of serum FGF21 levels in response to cold may not be attributed to a higher stress level induced by cold exposure.

Interestingly, we found a positive correlation between the cold-induced change in supraclavicular skin temperature and serum FGF21 levels in the evening, specifically in females. The change in skin temperature may indicate alterations in either BAT activity or blood flow in the supraclavicular area (28). This suggests that FGF21 might play a more prominent role in maintaining normothermia or influencing BAT activity in the evening compared to the morning. The prominent role of FGF21 in cold-induced thermogenesis is supported by a previous study showing that individuals with lower FGF21 secretion shivered more intensely compared to individuals with higher FGF21 secretion and that a higher increase in FGF21 independently predicted a greater increase in cold-induced thermogenesis (35, 36). However, this correlation was not evident during the evening in males, hinting at a potential influence of female sex hormones on cold response. These sex-specific response differences align with our

previous findings, emphasizing the need for further studies to understand the role of sex hormones in cold-induced responses (12).

We previously showed that cold-induced energy expenditure was higher in the morning compared to the evening (12). Since cold increases energy expenditure via the activation of BAT, and FGF21 has a role in increasing cold-induced thermogenesis, we hypothesized that the FGF21 would also increase more in the morning compared to the evening. However, since we also did not find a correlation between cold-induced energy expenditure and cold-induced FGF21 levels, it suggests that the cold-induced increase in FGF21 levels might occur independently of changes in energy expenditure. This increase in FGF21 levels could potentially be a consequence of enhanced release by BAT and the liver following sympathetic stimulation.

In addition, we found a negative correlation between the cold-induced change in serum FGF21 levels and the change in FFA levels in the morning in males only, with the lowering of serum FGF21 levels with an increase in serum FFA. The decrease of FGF21 could have led to a decreased hepatic fat oxidation and thereby an accumulation of FFA in the circulation in the morning in males only.

In females, we found a negative correlation between the cold-induced change in serum FGF21 levels and triglyceride levels in the evening only. This supports the hypothesis of an increased BAT activity in the evening in females only, as indicated by a positive correlation between the cold-induced change in supraclavicular skin temperature and serum FGF21 levels in the evening in females. Increased BAT activity leads to increased skin temperature and the utilization of circulating triglyceride-derived FA, resulting in lower circulating triglycerides (10). The lack of correlations of change in serum FGF21 levels and the changes in skin temperature and triglycerides in males could indicate potential differences in BAT activity or metabolic responses to cold exposure between sexes (13, 14).

Interestingly, and in contrast to our expectation, cold exposure did not significantly increase circulating GDF15 levels, neither in the morning nor in the evening. These findings are in line with results in mice that were subjected to prolonged cold exposure (4°C) for up to 21 days. While prolonged cold exposure increased GDF15 gene expression, particularly in BAT and WAT, there was no corresponding rise in plasma GDF15 levels (21). While it is known that GDF15 is released by BAT in mice, hence it is called a batokine, GDF15 release in humans is more complex. Human GDF15 is expressed by many different tissues specifically after different stressors. GDF15 is expressed particularly in the gastrointestinal system, kidneys, placenta, and prostate,

suggesting that BAT may not be the primary contributor to GDF15 levels and could explain the lack of observed effects in our study (37). Additionally, it is possible that rapid breakdown and/or clearance of GDF15, with its short half-life of 3 hours, contributed to the lack of effect (38). We cannot exclude that other cellular stressors besides cold could have influenced our study as well (39). However, since all participants were healthy young individuals who were not subjected to any other (metabolic) stressors known to influence GDF15 levels this is unlikely (40). Furthermore, the circadian rhythm observed in GDF15 could have influenced our results as well. GDF15 is shown to peak at midnight and show a nadir at noon (25). As our study days were conducted at 7:45 am and 7:45 pm, the serum levels of GDF15 could have been in the decreasing phase during the morning and on the rise at 7:45 pm. Consequently, it could therefore be that we could not find a significant difference in cold-induced responsiveness of serum GDF15 levels between morning and evening. Additionally, previous research observed a considerable variation in the natural diurnal rhythm of GDF15 levels among individuals, with not all participants displaying diurnal fluctuations (25). Therefore, we may have missed the variation in GDF15 levels and/or that cold-induced GDF15 levels are not influenced by the timing of the day in our study.

A strength of this study was the use of a personalized cooling protocol, which accounted for individual differences in shivering time and temperature. Females, for example, had a longer time to reach shivering and a lower shivering temperature in the morning compared to the evening (12). Applying the same temperature to all individuals may have led to a failure to elicit cold-induced changes due to insufficient cold exposure. However, this study is not without limitations. It was not specifically powered to compare males and females, as the focus was on within-subject comparisons. Ideally, we would have incorporated a control group consisting of both males and females who had undergone measurements of FGF21 and GDF15 levels under thermoneutral conditions in both morning and evening. Then we could have accounted for the individual natural diurnal rhythm of FGF21 and GDF15. Additionally, the duration of stable cold exposure was relatively short (90 minutes) compared to previous research that utilized longer cold exposure periods to observe effects on FGF21 and GDF15 (21, 41). While mice have a more pronounced and faster release of FGF21 upon cold exposure compared to humans, studies in humans have shown a significant increase in circulating FGF21 after fasting for 7-10 days and after prolonged exposure to mild cold for 10 days in men (19, 42, 43). Therefore, it is possible that longer cold exposures may reveal effects that were not detected in our study.

In conclusion, we observed that 90 minutes of cold exposure specifically increases serum FGF21 levels in the evening, regardless of sex, while serum GDF15 levels are not affected. Further research is needed to explore the underlying mechanisms.

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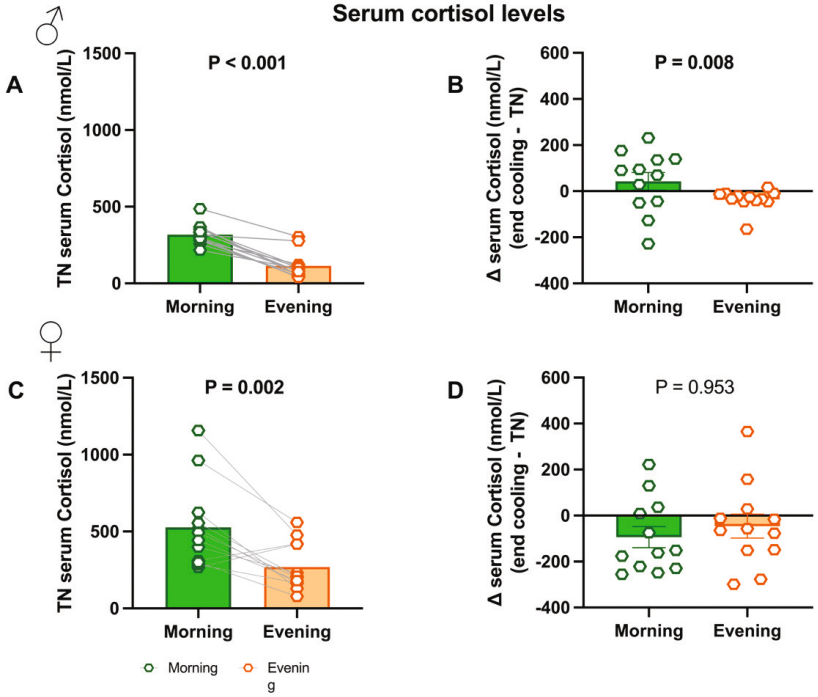
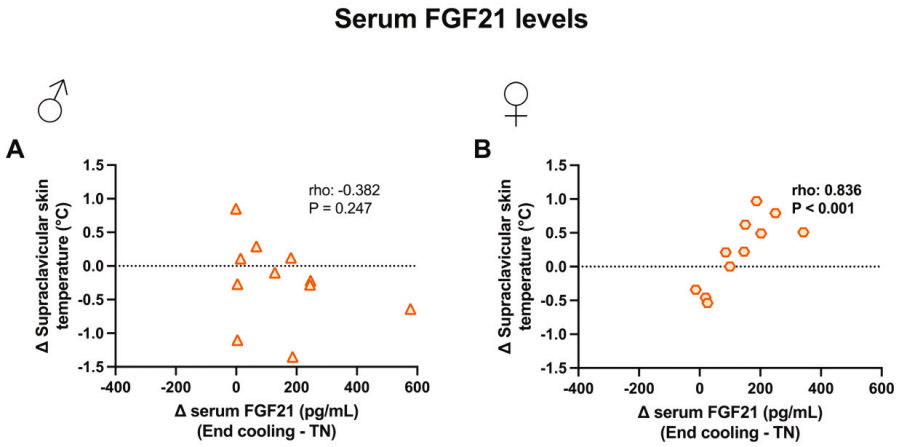
SUPPLEMENTAL DATA



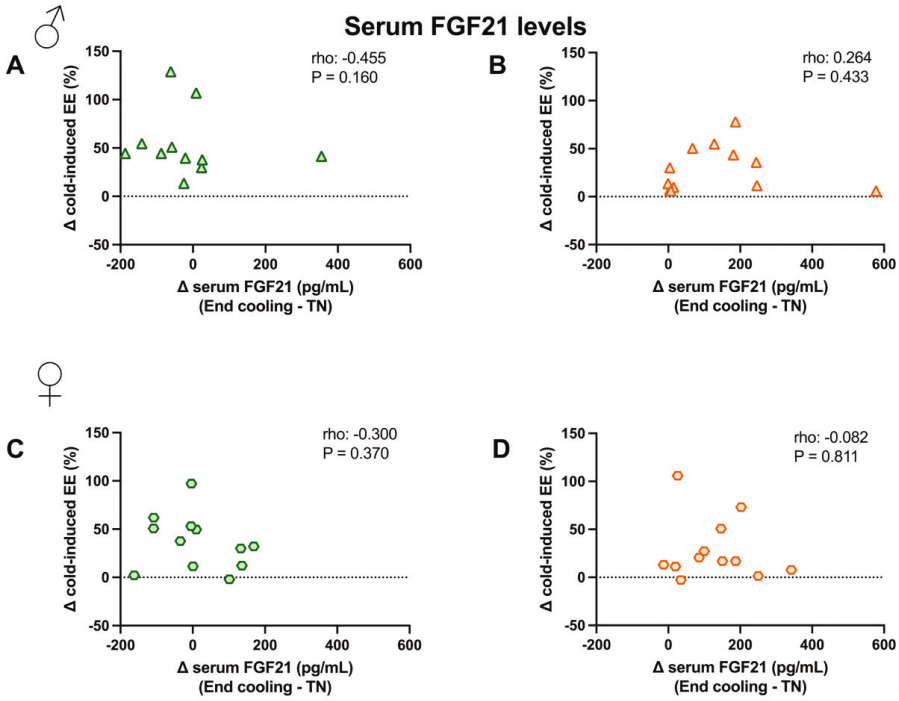
Supplemental Figure 1. Changes in serum cortisol levels after cold exposure in the morning compared to the evening in both males and females

Box plots showing serum cortisol levels at thermoneutral (TN) conditions in the morning and the evening in males (n=12) **(A)** and females (n=12) **(C)**, and the cold-induced changes of serum cortisol levels (end of cooling minus thermoneutral) in the morning and the evening in males (n=12) **(B)** and females (n=12) **(D)**. Hexagons represent individual values, boxes represent means, and deviations represent the standard error of the mean.



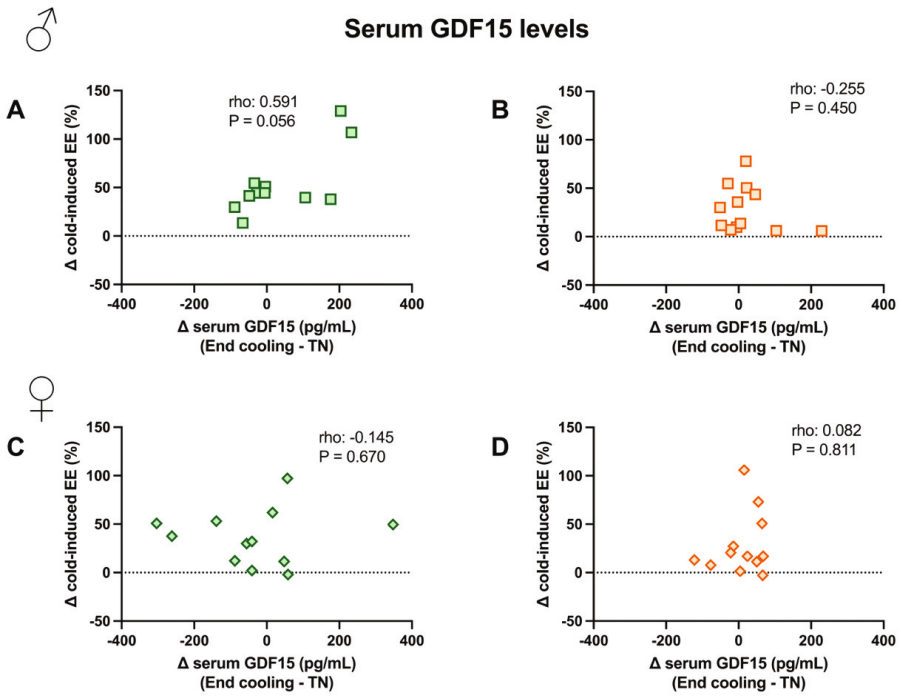
Supplemental Figure 2. Correlations between the change of serum FGF21 levels and the change of supraclavicular skin temperature after cold exposure in the evening

Spearman's correlations between the cold-induced change of serum FGF21 levels and change of supraclavicular skin temperature (Δ_{cold}: end of cooling minus thermoneutral (TN)) in males (n=11; tiangles) **(A)** and females (n=11; hexagons) **(B)**, in the evening. Due to a problem with the data acquisition, data on the supraclavicular skin temperature of 1 male and 1 female were excluded. Symbols represent individual values.



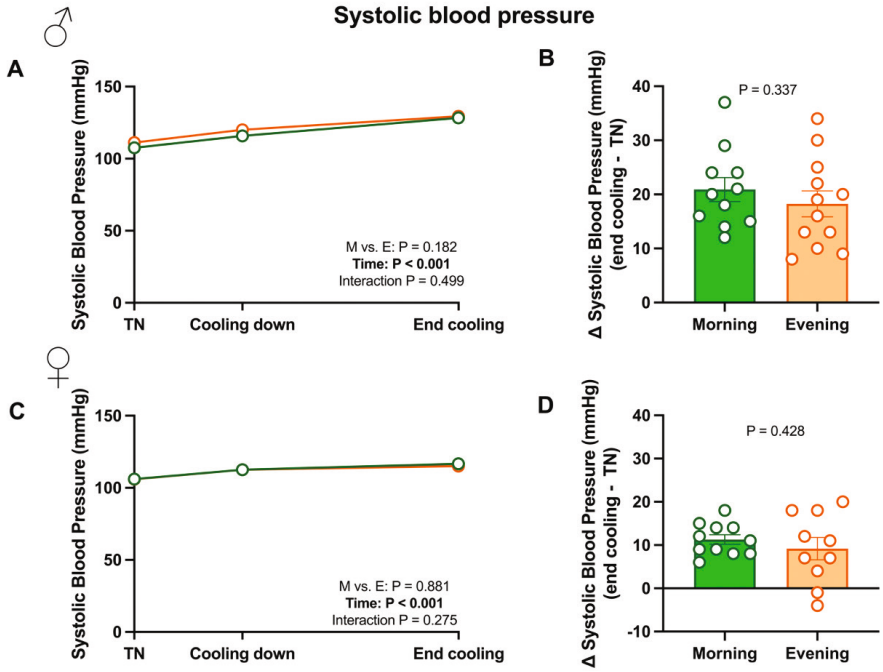
Supplemental Figure 3. Correlations between the change of serum FGF21 levels and the change of energy expenditure after cold exposure in the morning and evening

Spearman's correlation plots between the cold-induced change of serum FGF21 levels and the percentual change in energy expenditure (EE) (Δ_{cold}: end of cooling minus thermoneutral (TN)) in males (triangles) **(A** and **B)** and females (hexagons) **(C** and **D)**, in the morning (n=11; green triangles and hexagons) **(A** and **C)** and evening (n=11; orange triangles and hexagons) **(B** and **D)**. The gas exchange measurement failed for 1 male and 1 female due to technical issues. Symbols represent individual values.



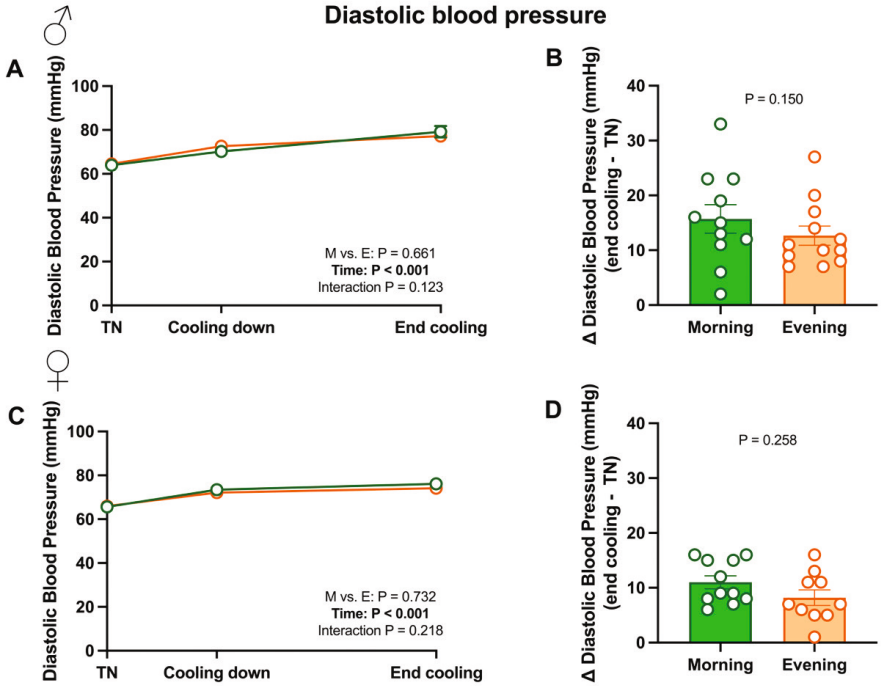
Supplemental Figure 4. Correlations between the change of serum GDF15 levels and the change of energy expenditure after cold exposure in the morning and evening

Spearman's correlation plot between the cold-induced change of serum GDF15 levels and the percentual change in energy expenditure (EE) (Δ_{cold}: end of cooling minus thermoneutral (TN)) in males (squares) **(A** and **B)** and females (diamonds) **(C** and **D)**, in the morning (n=11; green squares and diamonds) **(A** and **C)** and evening (n=11; orange squares and diamonds) **(B** and **D)**. The gas exchange measurement failed for 1 male and 1 female due to technical issues. Symbols represent individual values.



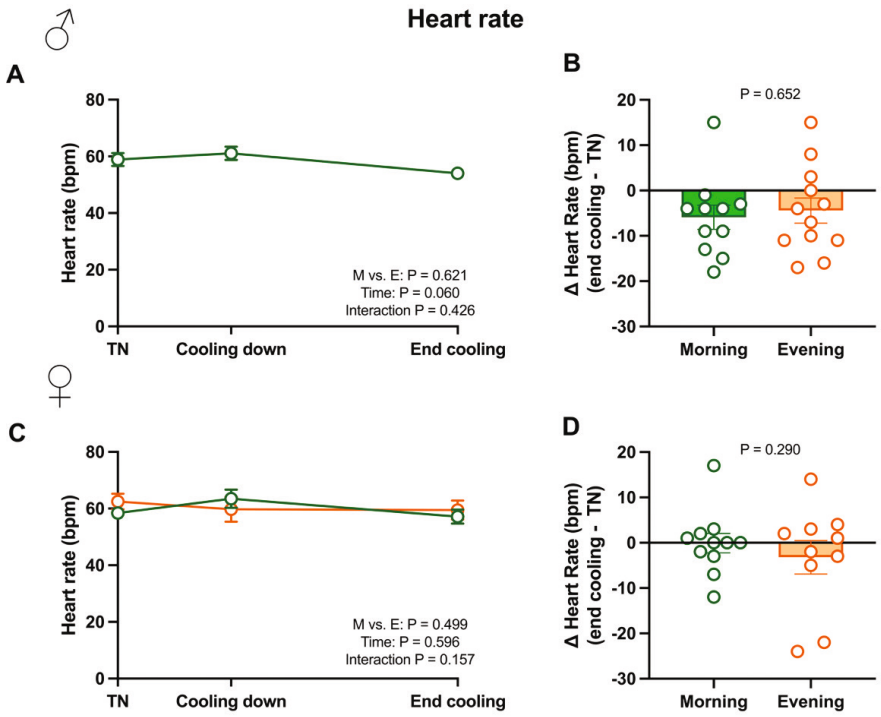
Supplemental Figure 5. Changes in serum systolic blood pressure after cold exposure in the morning compared to the evening in both males and females

Linear model showing the changes of systolic blood pressure after cold exposure in the morning and the evening in males (n=12) **(A)** and females (n=12) **(C)**. Blood pressure was assessed at three time points (i.e., thermoneutral, cooling down, and end of cooling). Box plots showing the cold-induced changes of systolic blood pressure (end of cooling minus thermoneutral) during the morning and the evening in males (n=12) **(C)** and females (n=12) **(D)**. Dots represent individual values, boxes represent means, and deviations represent the standard error of the mean. However, for one male and one female participant, systolic blood pressure could not be measured in the morning at the end of cooling (n=11). In the evening, systolic blood pressure could not be measured for four female participants during cooling (n=8), and for two female participants at the end of cooling (n=10). Dots represent means, and deviations represent the standard error of the mean. Green boxes, lines, and boxes represent the morning values, and orange boxes, lines, and boxes represent the evening values.



Supplemental Figure 6. Changes in serum diastolic blood pressure after cold exposure in the morning compared to the evening in both males and females

Linear model showing the changes of diastolic blood pressure after cold exposure in the morning and the evening in males (n=12) **(A)** and females (n=12) **(C)**. Blood pressure was assessed during three time points (i.e., thermoneutral, cooling down, and end of cooling). Box plots showing the cold-induced changes of diastolic blood pressure (end of cooling minus thermoneutral) during the morning and the evening in males (n=12) **(B)** and females (n=12) **(D)**. Dots represent individual values, boxes represent means, and deviations represent the standard error of the mean. However, for one male and one female participant, diastolic blood pressure could not be measured in the morning at the end of cooling (n=11). In the evening, diastolic blood pressure could not be measured for four female participants during cooling (n=8), and for two female participants at the end of cooling (n=10). Dots represent means, and deviations represent the standard error of the mean. Green boxes, lines, and boxes represent the morning values, and orange boxes, lines, and boxes represent the evening values.



Supplemental Figure 7. Changes in serum heart rate after cold exposure in the morning compared to the evening in both males and females

Linear model showing the changes in heart rate after cold exposure in the morning and the evening in males (n=12) **(A)** and females (n=12) **(C)**. Heart rate was assessed during three time points (i.e., thermoneutral, cooling down, and end of cooling). Box plots showing the cold-induced changes of heart rate (end of cooling minus thermoneutral) during the morning and the evening in males (n=12) **(B)** and females (n=12) **(D)**. Dots represent individual values, boxes represent means, and deviations represent the standard error of the mean.). However, for one male and one female participant, heart rate could not be measured in the morning at the end of cooling (n=11). In the evening, heart rate could not be measured for four female participants during cooling (n=8), and for two female participants at the end of cooling (n=10). Dots represent means, and deviations represent the standard error of the mean. Green boxes, lines, and boxes represent the morning values, and orange boxes, lines, and boxes represent the evening values.

Supplemental Table 1. Correlations between the change of serum FGF21 and GDF15 levels and the change of supraclavicular skin temperature and serum lipids after cold exposure in males and females, in the morning and evening

	Males		**Females**	
	Morning	**Evening**	**Morning**	**Evening**
Supraclavicular skin temperature				
		Δ Serum FGF21 levels (pg/mL)		
	Rho, P	Rho, P	Rho, P	Rho, P
Δ Supraclavicular skin temperature (°C)	-0.473; 0.142	-0.382; 0.247	-0.264; 0.433	0.836; 0.001**
		Δ Serum GDF15 levels (pg/mL)		
Δ Supraclavicular skin temperature (°C)	0.427; 0.190	0.109; 0.750	0.027; 0.937	0.227; 0.502
Lipids				
		Δ Serum FGF21 levels (pg/mL)		
Δ Serum Free fatty acids (mmol/mL)	-0.874; <0.001***	-0.085; 0.794	-0.273; 0.391	0.084; 0.795
Δ Serum Triglycerides (mmol/mL)	-0.294; 0.354	-0.014; 0.966	-0.245; 0.443	-0.580; 0.048*
		Δ Serum GDF15 levels (pg/mL)		
Δ Serum Free fatty Acids (mmol/mL)	-0.168; 0.602	0.269; 0.400	-0.147; 0.649	-0.263; 0.409
Δ Serum Triglycerides (mmol/mL)	0.559; 0.059	0.266; 0.403	-0.329; 0.297	0.119; 0.731

Spearman's correlations between the cold-induced change of serum FGF21 and GDF15 levels and change of supraclavicular skin temperature and serum lipids (Δ_{cold}: end of cooling minus thermoneutral (TN)) in males and females, in the morning and evening. *P < 0.05, **P < 0.01, ***P < 0.001,