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# The effect of cold exposure on circulating transcript levels of immune genes in Dutch South Asian and Dutch Europid men

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ABSTRACT

*Objectives:* Although cold exposure is commonly believed to be causally related to acute viral respiratory infections, its effect on the immune system is largely unexplored. In this study, we determined transcript levels of a large panel of immune genes in blood before and after cold exposure. We included both Dutch Europid and Dutch South Asian men to address whether the immune system is differently regulated in the metabolically vulnerable South Asian population.

*Methods:* Fasted blood samples were obtained from nonobese Dutch Europid (n = 11; mean age 26 ± 3 y) and Dutch South Asian (n = 12; mean age 28 ± 3 y) men before and directly after short-term (~2.5 h) mild cold exposure. Transcript levels of 144 immune genes were measured using a dual-color reverse transcriptase multiplex ligation-dependent probe amplification (dcRT-MLPA) assay.

*Results:* Cold exposure acutely upregulated mRNA levels of *GNLY* (+35%, $P < 0.001$) and *PRF1* (+45%, $P < 0.001$), which encode cytotoxic proteins, and *CCL4* (+8%, $P < 0.01$) and *CCL5* (+5%, $P < 0.05$), both pro-inflammatory chemokines. At thermoneutrality, mRNA levels of four markers of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR)-family, involved in inflammasomes, were lower in Dutch South Asians compared to Dutch Europids, namely *NLRP2* (−57%, $P < 0.05$), *NLRP7* (−17%, $P < 0.05$), *NLRP10* (−21%, $P < 0.05$), and *NLRC4* (−23%, $P < 0.05$).

*Conclusions:* Mild cold exposure acutely increases mRNA levels of genes involved in cytotoxicity of immune cells in blood. In addition, Dutch South Asians display lower circulating mRNA levels of inflammasome genes compared to Dutch Europids.

## 1. Introduction

Inflammation and temperature changes are tightly connected. Already in the first century *A.D.* the classical signs of inflammation were described with the words *calor* (heat), *dolor* (pain), *rubor* (redness), *tumor* (swelling) and *function laesa* (loss of function). The heat production during inflammation that increases body temperature, *e.g.* fever, is mediated by release of cytokines as a result of activation of the innate immune system and facilitates the body to combat infection or disease (Evans et al., 2015). On the other hand, cold temperatures are generally believed to be causally related to acute viral respiratory infections, also called "the common cold". For example, flu epidemics typically occur in the winter season when temperatures are low (Moriyama et al., 2020; Tamerius et al., 2011). Cold temperatures affect viral viability, viral transmission and human behavior, but also host susceptibility via an impaired innate immune defense (Moriyama et al., 2020).

Acute cold exposure results in activation of the sympathetic nervous system (SNS) and release of the catecholamine neurotransmitters

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epinephrine and norepinephrine from the adrenal medulla and sympathetic nerve endings (Madden and Morrison, 2019). These catecholamines bind to α- and β-adrenergic receptors (ARs), which are present on the surface of numerous cells and tissues, including immune cells (Quatrini et al., 2018; Scanzano and Cosentino, 2015). Via the ARs the catecholamines are able to modulate both the innate immune response, mainly via monocytes, dendritic cells and natural killer (NK) cells, and the adaptive immune response, via T lymphocytes and B lymphocytes. They do so in either an anti- or pro-inflammatory manner, dependent on concurrent stimuli (Bellinger et al., 2008; Marino and Cosentino, 2013; Scanzano and Cosentino, 2015). Next to stimulation of the SNS, the hypothalamic-pituitary-adrenal (HPA) axis is activated upon cold exposure, resulting in increased circulating levels of the stress hormone cortisol. Leukocytes as well as lymphoid tissues express glucocorticoid receptors, but the extent and type of receptors differs amongst the different subpopulations of leukocytes. The SNS and HPA axes facilitate an excellent neuroendocrine regulation of the immune system, to promote revival of the body after a stressful challenge such as cold exposure (Axelrod and Reisine, 1984; Bailey et al., 2003).

Only a limited number of clinical studies have addressed the effect of cold exposure on the immune system in humans to date. Short-term cold exposure, either by using cold water immersion (14 °C) (Brazaitis et al., 2014; Janský et al., 1996) or exposure to cold air (4–5 °C) (Brenner et al., 1999; Hennig et al., 1993; Lackovic et al., 1988), was shown to increase the number of circulating leukocytes and lymphocytes. More specifically, several studies have reported a higher presence of circulating neutrophils (Brazaitis et al., 2014) and natural killer (NK) cells (Brenner et al., 1999; Lackovic et al., 1988), and levels of the pro-inflammatory cytokines interleukin-6 (IL-6) (Brenner et al., 1999) and tumor necrosis factor-alpha (TNF-α) (Janský et al., 1996) following short-term cold exposure in healthy individuals. Furthermore, short-term cold exposure was shown to decrease levels of $CD4^+$ cells, without affecting levels of $CD8^+$ cells (Hennig et al., 1993). However, most of these studies focused on only a limited subset of immune cells.

The use of ambient temperature as a modulator of the immune system is of interest due to its possible therapeutic use in several diseases where inflammation plays a key role in pathogenesis, such as infections and auto-immune diseases. Also cardiometabolic diseases including type 2 diabetes and cardiovascular diseases are now increasingly acknowledged to be linked to a chronic low-grade inflammatory state, and therapeutics targeting the immune system, such as inhibiting the pro-inflammatory cytokine IL-1β, are promising with respect to reduction of cardiovascular events (Lumeng and Saltiel, 2011; Ridker et al., 2017; Williams et al., 2019).

To further establish the effect of cold exposure on the immune system, the aim of the current study was to investigate transcript levels of a large panel of immune cell markers in blood of healthy nonobese men before and after short-term mild cold exposure. Since migrant South Asians, a metabolically vulnerable population more prone to develop cardiometabolic diseases, tend to have high levels of circulating pro-inflammatory cytokines, such as TNF-α and IL-6 (Bakker et al., 2013), we included both Dutch Europid and Dutch South Asian participants to address whether the immune system and the effect of cold exposure thereon is differently regulated between these ethnicities.

## 2. Material and methods

### 2.1. Participants

In this study 11 Dutch Europid and 12 Dutch South Asian men were included. Participants were nonobese (body mass index (BMI) 18–27 kg/$m^2$) and young (18–36 years old). Dutch South Asian subjects were included if they were born and raised in the Netherlands and had four grandparents from South Asian descent. Exclusion criteria included smoking, recent participation in a weight loss or exercise program, any significant chronic disease, use of medication known to influence glucose or lipid metabolism, and participation in another study including a medical product.

### 2.2. Study approval

This study was performed in accordance with the principles of the revised declaration of Helsinki (World Medical Association, 2014) and approved by the medical ethical committee of the Leiden University Medical Center (LUMC). All participants provided written informed consent prior to participation (Clinicaltrials.gov NCT03002675).

### 2.3. Study design

This study is a sub-analysis of a single-arm prospective study which was originally designed to assess the effect of extended-release exenatide treatment on brown adipose tissue in non-diabetic individuals (Janssen et al., 2020). This study was performed between September 2016 and February 2018 at the LUMC, the Netherlands. The data used for the current sub-analyses was obtained before participants started their exenatide treatment. Participants arrived at the research unit after a 10-h overnight fast. They were instructed to eat a standardized meal the evening before the study day and were not allowed to perform physical exercise 48 h before the start of the study day. After arrival, body composition was determined using bio-impedance analysis (Bodystat 1500, Bodystat, Douglas, Isle of Man, UK). An intravenous cannula was placed in the antecubital vein. Participants underwent a personalized cooling protocol for optimal visualization of brown adipose tissue. To this end, participants were lying down between two water-perfused mattresses (Blanketrol® III, Cincinnati Sub-Zero Products, Inc, Cincinnati, Ohio, USA) with an initial water temperature of 32 °C, which is considered thermoneutral. After 15 min, resting energy expenditure was measured by indirect calorimetry (JAEGER™ Vyntus™ CPX, Carefusion, Hochberg, Germany). After 45 min of thermoneutrality, venipuncture blood was drawn to obtain plasma and serum, and whole blood for RNA isolation, and the cooling procedure started. In the next hour temperature was steadily decreased to a minimum temperature of 9 °C or until shivering occurred, and then increased by 2–3 °C to prevent shivering. Shivering was defined as an unstoppable contraction of the muscles as reported by the participants and was visually checked by the researchers. After 60 min with the mattresses kept at this personalized temperature, cold-induced resting energy expenditure was measured for 30 min where after the final blood samples were drawn to again obtain plasma and serum, and whole blood for RNA isolation. Plasma and serum were aliquoted and stored at −80 °C until further analyses. Blood samples for RNA isolation were collected in PAXgene® Blood RNA tubes (BD Biosciences) and were handled following instructions from the manufacturer.

### 2.4. RNA isolation and dual-color reverse transcriptase multiplex ligation-dependent probe amplification assay

Automated purification of total RNA extracted from blood collected in PAXgene® tubes was performed using a PAXgene® Blood miRNA Kit (PreAnalitiX, Hombrechtikon, Switzerland), including on-column DNase digestion, according to manufacturer's protocol. RNA yield was determined using a Qubit fluorometer and the Qubit RNA Broad Range Assay (ThermoFisher, Waltham, MA, United States). Dual-color reverse transcriptase multiplex ligation-dependent probe amplification (dcRT-MLPA) assay was performed as described previously (Joosten et al., 2012). Briefly, target-specific RT primers were designed directly downstream of the probe target sequence. Reverse transcription of 125 ng RNA was performed using a mixture of RT primers, M-MLV reverse transcriptase (Promega, Leiden, The Netherlands) and dNTPs and incubating for 15 min at 37 °C, followed by an enzyme inactivation step at 98 °C for 2 min. Following heat denaturing for 1 min at 95 °C, left and right hand half-probes were hybridized to the cDNA at 60 °C overnight

and ligated at 54 °C for 15 min, followed by enzyme inactivation at 98 °C for 5 min. Ligated half-probes were PCR-amplified (35 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s, followed by one cycle at 72 °C for 20 min). PCR-amplified products were 1:10 diluted in HiDi formamide (ThermoFisher) containing 400HD ROX size standard (Applied Biosystems, Foster City, CA, United States) and analyzed on an Applied Biosystems 3730 capillary sequencer in GeneScan mode (BaseClear, Leiden, The Netherlands). MLPA reagents were obtained from MRC Holland (Amsterdam, The Netherlands). Primers and probes were designed by Leiden University Medical Center (Leiden, The Netherlands) and synthesized by Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Pre-selected genes comprised 4 housekeeping genes and 144 genes to profile innate, adaptive and inflammatory immune responses (Table S1). Trace data was analyzed using GeneMapper software 5 (Applied Biosystems). Peak areas of assigned peaks (arbitrary units) were exported for analysis, normalized to housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and log2-transformed. Signals below the threshold value for noise cut-off in Genemapper (log2 transformed peak area ≤7.64) were assigned the threshold value and then divided by 2 to estimate their true value.

### 2.5. Reverse transcription-quantitative polymerase chain reaction analyses

For determination of mRNA levels of caspase 1 (*CASP1*) and interleukin-1 receptor antagonist (*IL1RN*) in blood, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed on a CFX96 PCR machine (Bio-Rad) using IQ SYBR-Green Supermix (Promega). mRNA levels were normalized to the reference gene low-density lipoprotein receptor-related protein 10 *(LRP10)* and expressed as fold difference compared to the Europid group using the ΔΔCT method. Gene-specific primer pairs were used for *CASP1* (forward primer: GCCTGTTCCTGTGATGTGGAG – reverse primer: TGCCCACAGACATTCATACAGTTTC) and *IL1RN* (QuantiTect Primer Assay, Qiagen).

### 2.6. Laboratory measures

With the use of enzymatic kits that are commercially available, serum levels of triglycerides, total cholesterol and high-density lipoprotein cholesterol (HDL-C) (all Roche Diagnostics, Woerden, the Netherlands), free fatty acids (FFA; Wako chemicals, Nuess, Germany), insulin (Meso Scale Diagnostics LLC, Rockville, MD, USA), and plasma glucose (Instruchemie, Delfzijl, the Netherlands) were determined. The Friedewald equation was used to calculate low-density lipoprotein cholesterol (LDL-C) (Friedewald et al., 1972). Plasma cortisol was measured using liquid chromatography-tandem mass spectrometry using an assay that was calibrated against the IFCC ERM-DA451 reference panel (Waters TQS, Waters, Etten-Leur, The Netherlands). Insulin resistance was estimated using the homeostasis model assessment (HOMA) (Matthews et al., 1985) index. The delta (Δ) between cold and thermoneutral temperatures was calculated by subtracting the thermoneutral values from the cold values.

### 2.7. Statistical analysis

Statistical analyses were conducted in SPSS Statistics version 25 (IBM, Armonk, NY, USA) and *R* (version 3.6.2) (Team, 2019). Baseline characteristics between ethnicities were compared using a two-tailed Student's t-test in *R*. mRNA levels were normalized using logarithmic transformation with a base of 2 (log2). Mann-Whitney *U* test was performed in *R* to compare values of mRNA levels at thermoneutrality between ethnicities. The interaction between ethnicity and the effect of cold exposure on mRNA levels were analyzed using a mixed model in SPSS, with ethnicity and temperature as fixed effects. Since there was no interaction effect (all $P > 0.05$), data from both ethnicities were pooled for the analysis of the effect of cold exposure. Comparisons between thermoneutrality and cold were analyzed using Wilcoxon signed rank test in *R*. For the calculation of the log2 fold change (log2FC), the log2 mRNA level after cold exposure was subtracted from log2 mRNA level under thermoneutral temperature (cold-thermoneutral). Pearson correlation was performed using the packages *Hmisc* (Harrell and Dupont, 2020) and *corrplot* (Simko, 2017), and linear regression analysis was conducted in *R* with mRNA level as dependent outcome. Figures were produced using the package *ggplot2* (Wickham, 2016). Percental changes given in text are calculated on raw data and indicate median percental changes. All reported p-values are uncorrected for multiple comparison. For the dcRT-MLPA data, correction for multiple comparison was done with Benjamini-Hochberg False Discovery Rate (FDR) in *R* and results are reported as q-values where applicable. A p-value of <0.05 was considered statistically significant.

## 3. Results

### 3.1. Clinical characteristics

Initially, in this study 24 nonobese males were included, of whom 12 of Dutch Europid and 12 of Dutch South Asian descent. Measurement of mRNA levels from one Dutch Europid participant failed both before and after cold exposure, leaving 23 men to be included in this study. From one Dutch Europid and one Dutch South Asian participant blood draw for the assessment of mRNA levels in blood failed after cold exposure, therefore data from those participants were only used for baseline comparisons, leaving 21 participants for post-cooling measurements. In the total study population, mean age was 27 ± 3 years and mean BMI was 24.3 ± 2.6 kg/m$^2$ (Table 1). The Dutch South Asian participants had a significantly higher fat mass percentage compared to the Dutch Europid participants (18.9 ± 3.2% vs. 14.5 ± 4.9%; $P < 0.05$, Table 1). Fasting glucose, total cholesterol, HDL-C and LDL-C did not differ between ethnicities (all $P \geq 0.05$, Table 1).

### 3.2. Short-term mild cold exposure enhances energy expenditure

Cold exposure activates thermogenic organs to preserve core body temperature, which is reflected by an increase in metabolic rate. We therefore first assessed the effects of cold exposure on resting energy expenditure. Since there was no interaction between ethnicity and the effect of cold exposure on the metabolic parameters of interest or on mRNA levels, we pooled the data from the Dutch Europid and Dutch South Asian subjects. As expected, cold exposure increased resting energy expenditure (+10%, $P < 0.001$) and lipid oxidation (+43%, $P < 0.001$), whereas it decreased glucose oxidation (−28%, $P < 0.01$) as we previously published (Janssen et al., 2020) (data not shown). This

**Table 1**
Baseline characteristics.

| Characteristic | Dutch Europid (n = 11) | Dutch South Asian (n = 12) | Total (n = 23) |
|---|---|---|---|
| Age (years) | 26 ± 3 | 28 ± 3 | 27 ± 3 |
| Weight (kg) | 82.4 ± 9.1 | 78.7 ± 11.3 | 80.5 ± 10.2 |
| Height (cm) | 185.5 ± 5.3 | 178.3 ± 6.1 ** | 182.8 ± 6.7 |
| BMI (kg/m$^2$) | 23.9 ± 2.5 | 24.7 ± 2.7 | 24.3 ± 2.6 |
| Body fat percentage (%) | 14.5 ± 4.9 | 18.9 ± 3.2 * | 16.8 ± 4.6 |
| Fasting glucose (mmol/L) | 4.6 ± 0.2 | 4.8 ± 0.3 | 4.7 ± 0.3 |
| Total cholesterol (mmol/L) | 4.3 ± 0.4 | 4.8 ± 0.8 | 4.6 ± 0.7 |
| HDL-C (mmol/L) | 1.2 ± 0.2 | 1.2 ± 0.3 | 1.2 ± 0.3 |
| LDL-C (mmol/L) | 2.7 ± 0.5 | 3.3 ± 0.9 | 3.0 ± 0.8 |

Values are expressed as mean ± SD. BMI: body mass index; HDL-C: high-density lipoprotein-cholesterol, LDL-C: low-density lipoprotein-cholesterol. *$P < 0.05$, **$P < 0.01$; Dutch South Asian *vs.* Dutch Europid.

coincided with increased serum FFA levels (+37%, $P < 0.01$) and increased plasma cortisol levels (+48%, $P < 0.001$; Fig. 1).

### 3.3. Short-term mild cold exposure affects transcript levels of several inflammatory markers in blood

We next studied the effects of cold exposure on mRNA levels of cell markers involved in both the innate and adaptive immune response, pattern recognition receptors, and cytokines. Using a dcRT-MLPA assay (Joosten et al., 2012) mRNA levels of 144 inflammatory genes were measured, of which 12 genes were below the detection limit in either the thermoneutral and/or cold condition for all participants and were therefore excluded from analyses (Supplementary Table 1). In the pooled analysis (see above), after cold exposure, 9 genes were significantly upregulated and 8 genes were significantly downregulated. Of note, in Dutch Europids, 9 genes were significantly upregulated, whereas none of the genes was downregulated (Supplementary Fig. 1A). In Dutch South Asians, 4 genes were significantly upregulated and 4 genes were significantly downregulated (Supplementary Fig. 1B). In the pooled analyses, cold exposure increased median mRNA levels of *GNLY* (+35%, $P < 0.001$) and *PRF1* (+45%, $P < 0.001$), both found on cytotoxic T cells and NK cells (Fig. 2A). In addition, the mRNA levels of the chemokines *CCL4* (+8%, $P < 0.01$) and *CCL5* (+5%, $P < 0.05$) both increased after cold exposure. The interferon inducible *GBP5* (+18%, $P < 0.01$) and *IFITM3* (+60%, $P < 0.01$) were also upregulated, whereas *GBP1* (−6%, $P < 0.05$), *IFIT5* (−8%, $P < 0.05$), and *FCGR1A* (−18%, $P < 0.05$) were downregulated. mRNA levels of two ligands for transforming growth factor-β were also affected: levels of *TGFB1* (+5%, $P < 0.05$) increased while that of *BMP6* (−2%, $P < 0.05$) decreased after cold exposure. *PTPRCv1* (+6%, $P < 0.01$), which is located on naive T cells, and *FPR1* (+4%, $P < 0.01$), located on phagocytic cells, increased after cold exposure. mRNA levels of *BCL2* (−10%, $P < 0.05$), coding for a protein that regulates apoptotic death, and *SEC14L1* (−0%, $P < 0.05$), a possible negative regulator of innate antiviral signaling, were both downregulated. Pattern recognition receptors encoded by *NLRP4* (−24%, $P < 0.05$) and *TLR2* (−4%, $P < 0.05$) were decreased after cold exposure. After correction for multiple comparison using FDR-correction only the increased mRNA levels of *GNLY* (FDR adjusted *P*-value < 0.01), *PRF1* (FDR adjusted *P*-value < 0.01) and *CCL4* (FDR adjusted *P*-value < 0.05) after cold exposure remained significant (Fig. 2B).

### 3.4. The changes in serum free fatty acid or cortisol levels do not predict changes in transcript levels

We postulated that an increased sympathetic outflow and an activated HPA-axis after cold exposure would be causally related to cold-induced modulation of the immune system. Therefore, we evaluated the association between the changes in serum FFA, as a reflection of sympathetic activation of white adipose tissue, and cortisol levels with the change in transcript levels of inflammatory markers upon cold exposure. Only the change in *FCGR1A* correlated negatively with the change in plasma cortisol levels ($R^2 = 0.29$, $P = 0.01$; Supplementary Fig. 2A). The remaining genes that were affected by cold exposure did not correlate significantly with the change of serum FFA levels (data not shown) or cortisol levels (Supplementary Figs. 2B–D). There was also no correlation between the change in serum FFA levels and the change in plasma cortisol levels (Supplementary Fig. 2E).

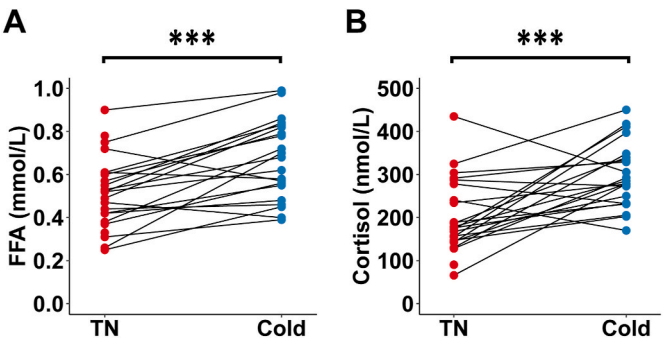


**Fig. 1.** *Short term cold-exposure increases plasma free fatty acid levels (A) and cortisol levels (B). Paired Student t-test was used for statistical comparison. ***P < 0.001. TN: thermoneutral.*

### 3.5. Several inflammatory markers are differently expressed in Dutch South Asians compared to Dutch Europids at thermoneutrality

Since differences in their immune system may at least in part underlie the vulnerable metabolic phenotype of South Asians, we next assessed whether transcript levels of inflammatory markers differed between the Dutch Europid and Dutch South Asian groups at thermoneutrality (Supplementary Table 2 and Fig. 3A). Interestingly, we found that mRNA levels of several genes coding for different classes of pattern recognition receptors were lower in the Dutch South Asians compared to the Dutch Europids (Fig. 3B). More specifically, mRNA levels of four markers of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family were lower in Dutch South Asian compared to Dutch Europid individuals, namely *NLRP2* (−57%, $P < 0.05$), *NLRP7* (−17%, $P < 0.05$), *NLRP10* (−21%, $P < 0.05$) and *NLRC4* (−23%, $P < 0.05$; Fig. 3A). In addition, mRNA levels of *NLRP3*, which encodes the most frequently-studied NLR, tended to be lower in the Dutch South Asians (−10%, $P = 0.06$; Fig. 3A). Furthermore, mRNA level of the pattern recognition receptor *TLR8* was lower in the Dutch South Asian group (−15%, $P < 0.05$; Fig. 3A). Different mRNA levels of other genes in Dutch South Asians were the GTPase-activating protein coding genes *ASAP1* (−21%, $P < 0.05$) and GTPase-activating protein coding gene *TAGAP* (+21%, $P < 0.05$). mRNA levels of the IFN signaling gene *IFITM3* (+152%, $P < 0.05$) and T cell subset marker *PTPRCv1* (+24%, $P < 0.05$) were both higher in the Dutch South Asian group. None of the differences in mRNA levels between ethnicities remained significant after correction for multiple comparison using FDR-correction (all FDR adjusted *P*-value > 0.05).

### 3.6. The transcript level of IL-1 receptor antagonist is lower in Dutch South Asians and correlates positively with body fat percentages

The NLRs are present in a protein complex called the inflammasome. The inflammasome controls the activation of caspase-1, which is essential for the cleavage of pro-IL-1β into the biologically active form IL-1β (Garlanda et al., 2013). IL-1β is regarded to play a causal role in the development of type 2 diabetes mellitus and cardiovascular diseases (Ridker et al., 2017; Stienstra et al., 2011). To further elucidate a possible ethnic difference in the regulation of the inflammasome, we performed an additional qPCR on IL-1 receptor antagonist (*IL1RN*), which is thought to be released in parallel with IL-1β and could thus serve as surrogate marker for circulating endogenous levels of IL-1β, and caspase-1 (*CASP1*). The mRNA level of *IL1RN* was higher in Dutch South Asians versus Dutch Europids (+17%, $P < 0.05$; Fig. 4A), and that of *CASP1* (+23%, $P = 0.12$; Fig. 4C) did not differ between ethnicities. One Dutch South Asian participant showed a high *IL1RN* mRNA level, which appeared an outlier. After exclusion of this outlier, *IL1RN* tended to be higher in Dutch South Asians versus Dutch Europids (+14%, $P = 0.06$). Since the Dutch South Asian group had a higher body fat percentage at baseline, we performed correlation analysis between transcript levels of both these genes and fat percentage. Both *IL1RN* and *CASP1* negatively correlated with fat percentage in Dutch South Asian subjects ($R^2 = 0.72$, $P < 0.001$ and $R^2 = 0.65$, $P < 0.01$, respectively), but not in Dutch Europid subjects ($R^2 = 0.20$, $P = 0.16$ and $R^2 = 0.05$, $P = 0.53$, respectively; Fig. 4B, D).

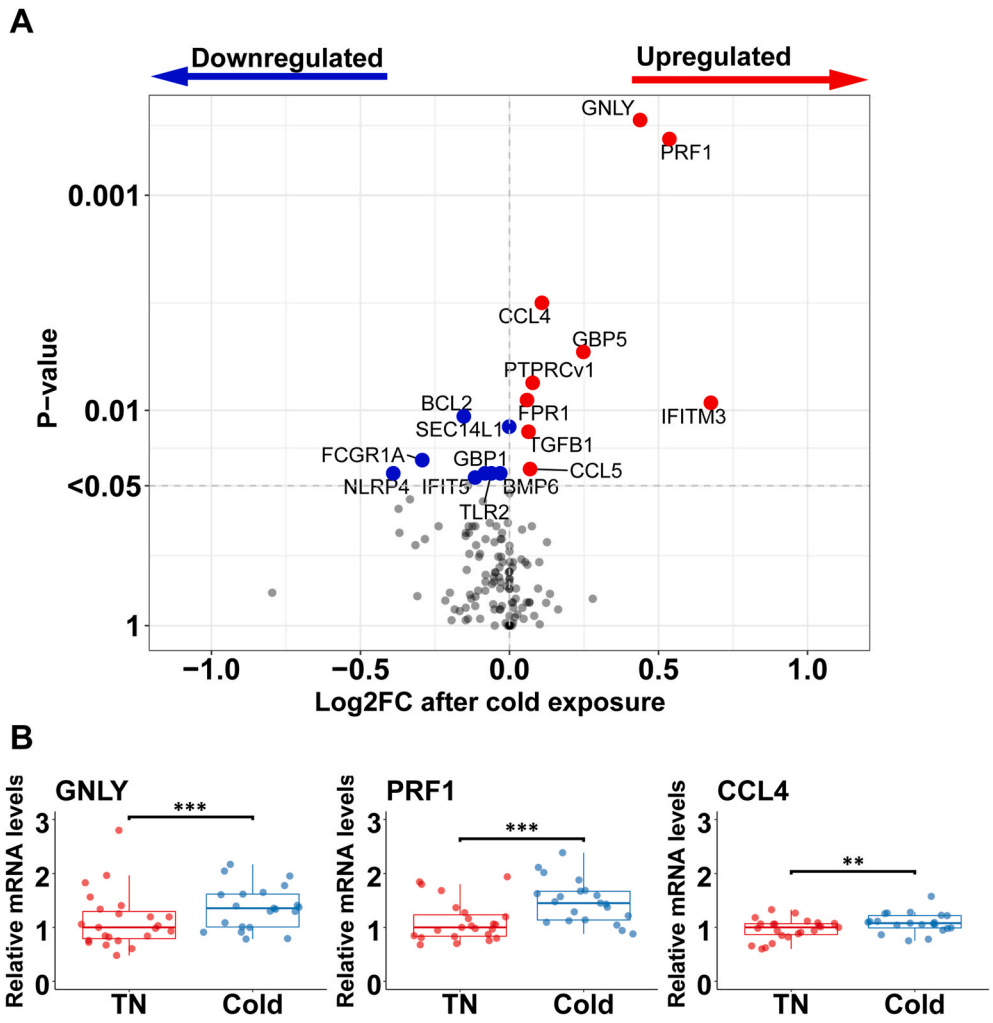


**Fig. 2.** Short term cold-exposure affects mRNA levels of several inflammatory markers in blood. (A) Overview of the effect of cold exposure on mRNA levels of immune genes. The p-value is presented on the y-axis on a logarithmic scale, the x-axis presents the Log2 fold change of mRNA levels after cold exposure [median levels after cold exposure (in log2) minus median levels at thermoneutral condition (in log2)]. (B) The effect of cold exposure on mRNA levels of GNLY, PRF1 and CCL4. mRNA levels are normalized to those of the housekeeping gene GAPDH and calculated as $2^{-\Delta CT}$. Data are expressed as relative to thermoneutral (TN) and presented as median and interquartile range (IQR). Wilcoxon signed rank-test was used for statistical comparison. ***P < 0.001, **P < 0.01.

## 4. Discussion

The effect of short-term mild cold exposure on the immune system is largely unexplored. Therefore, in the current study, we investigated transcript levels of a large panel of immune genes in blood of healthy men before and after short-term mild cold exposure. To determine whether the immune system and the effect of cold exposure thereon is differently regulated in the metabolically vulnerable South Asian population, we included both Dutch Europid and Dutch South Asian participants. We show that short-term mild cold exposure significantly affected transcript levels of 18 immune system related genes, regardless of the ethnicity. After short-term mild cold exposure we particularly showed higher mRNA levels of *GNLY* and *PRF1,* encoding pro-inflammatory proteins involved in cytotoxic T cell and NK cell activation, and higher mRNA levels of *CCL4* and *CCL5,* encoding chemoattractants. In addition, at thermoneutrality circulating mRNA levels of various pro-inflammatory inflammasome components were lower in Dutch South Asian compared to Dutch Europid participants.

To the best of our knowledge, this is the first study that investigated the effect of short-term mild cold exposure on transcript levels of such a large panel of immune system related genes in blood. Previous studies showed that exposure to a cold ambient temperature (4–5 °C) for 30 min up to 120 min increases plasma leukocytes, granulocytes and NK cells, and decreases $CD4^+$ cells in lean men (Brenner et al., 1999; Lackovic et al., 1988). In our study we cannot distinguish whether the changes in mRNA levels reflect changes in cell composition or cellular gene expression. Interestingly, several studies show that catecholamines, either exercise-induced or after infusion, mobilize perforin (*PRF1*)-expressing NK cells to the circulation (Nagao et al., 2000; Pedersen et al., 2016; Søndergaard et al., 1999). Since cold exposure also increases the circulating PRF1 transcript, it is likely that cold exposure, via catecholamines and cortisol, similarly induces demargination of immune cells that is at least partly responsible for the observed changes in circulating transcripts.

Short-term mild cold exposure increased transcript levels of *GNLY*, *PRF1, CCL4* and *CCL5*. *CCL4* and *CCL5* encode for the C–C motif chemokine ligands 4 and 5, respectively. Both are chemoattractants and therewith able to attract other immune cells to the site of inflammation, such as T cells, dendritic cells, eosinophils, NK cells, mast cells and basophils. They are secreted by numerous immune cells, which include cytotoxic T cells (Bernardini et al., 2012; Cocchi et al., 1995; Levy, 2009; Mikolajczyk et al., 2016). *GNLY* encodes the protease granulysin and *PRF1* encodes the pore-forming protein perforin. Both granulysin and perforin are present in the granules of cytotoxic T cells and NK cells, which they release after recognition of a target cell that is considered as dangerous. At that point, perforin is able to form transmembrane pores in the target cell wall allowing granulysin, amongst others, to diffuse into the target cell cytosol leading to apoptotic cell death (Belizário et al., 2018; Harari et al., 2009; Tewary et al., 2010; Voskoboinik et al., 2015). Thereby, the cytotoxic granules are involved in the first-line protection against a viral infection. In addition to infectious diseases, the cytotoxic granules are also well-acknowledged to play a role in the control of neoplasms (van den Broek et al., 1995; Voskoboinik et al., 2015). The increased transcript levels of *GNLY*, *PRF1*, *CCL4* and *CCL5* in

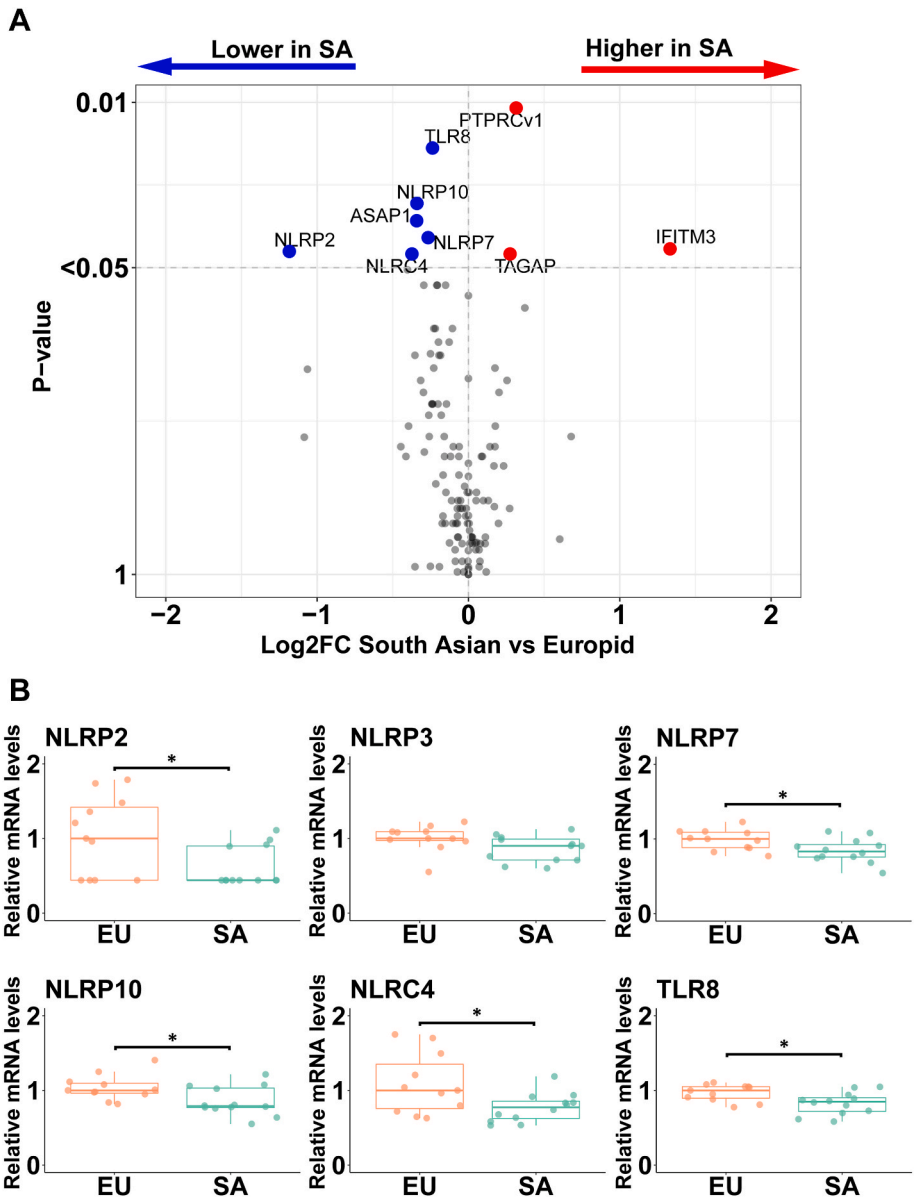


**Fig. 3.** At thermoneutrality, circulating mRNA levels of several markers for pattern recognition receptors are lower in Dutch South Asians compared to Dutch Europids. (A) Overview of the difference in immune mRNA levels at thermoneutrality between Dutch South Asians (SA) and Dutch Europids (EU). The p-value is presented on the y-axis on a logarithmic scale, the x-axis presents the Log2 fold change of mRNA levels between Dutch Europids and Dutch South Asians [median levels Dutch South Asians (in log2) minus median levels Dutch Europids (in log2)]. (B) mRNA levels of NLRP2, NLRP3, NLRP7, NLRP10, NLRC4 and TLR8 in Dutch Europids and Dutch South Asians. mRNA levels are normalized to housekeeping gene GAPDH and calculated as $2^{-\Delta CT}$. Data are expressed relative to Dutch Europids and presented as median and interquartile range (IQR). Mann-Whitney *U* test was used for statistical comparison. *P < 0.05.

our study, in combination with previous reports showing an increase in NK cells, might suggest that cold exposure, already when mild and for a short duration, quickly prepares the human body to fight an unknown visitor by provoking a pro-inflammatory state, in which demargination of immune cells is likely involved.

It is interesting to speculate about the long-term immune-modulating effects of cold exposure. Similar to after cold exposure, aerobic exercise acutely increases circulating transcripts of NK cells (Contrepois et al., 2020) in addition to the number and activity of NK cells (Millard et al., 2013; Pedersen and Hoffman-Goetz, 2000; Pedersen et al., 1990). Already 15 min post-exercise the NK cell transcripts decline (Contrepois et al., 2020) and NK cell number and activity drop even below baseline level after 2 h (Pedersen and Hoffman-Goetz, 2000; Pedersen et al., 1990). Since both cold exposure and exercise activate the immune system and stimulate the release of catecholamines and cortisol, it is likely that mild cold exposure also only transiently increases transcripts, which should be subject of future investigation. In addition, several studies showed that the initial immune response after short-term mild cold exposure is absent when cold exposure is repeatedly applied such as during cold acclimatization. For example, the increased plasma leukocyte and neutrophil levels observed in lean males after a first immersion into cold water (14 °C) was blunted after 16 days of repeated cold water immersion (Brazaitis et al., 2014). In addition, individuals trained with meditation, breathing techniques and long-term cold exposure, responded in a more anti-inflammatory manner to lipopolysaccharide administration (Kox et al., 2014). Compared to controls, in the trained individuals circulating levels of lipopolysaccharide-induced pro-inflammatory TNF-α, IL-6 and IL-8 were lower, while levels of the anti-inflammatory IL-10 were higher (Kox et al., 2014). However, since this study made use of a combined intervention, it cannot be concluded whether these effects were solely due to chronic cold exposure. More studies are needed to further investigate the differences between the effect of short-term cold exposure in comparison with chronic cold exposure on modulation of the immune system.

The physiological responses after cold exposure are principally regulated by the SNS and HPA axis. To investigate the role of the HPA axis in the effect of short-term mild cold exposure on circulating transcript levels of immune genes, we determined plasma levels of the stress hormone cortisol. We showed that cold exposure increased cortisol levels, which is in line with most previous studies (Hennig et al., 1993),

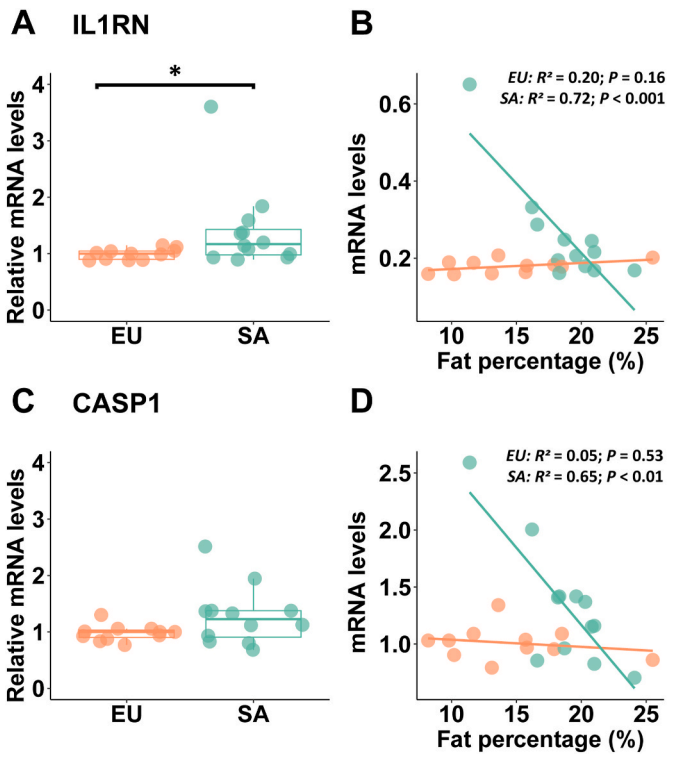


**Fig. 4.** At thermoneutrality, circulating mRNA levels of IL1RN and CASP1 correlate negatively with fat percentage only in Dutch South Asians. A) mRNA level of interleukin-1 receptor antagonist (IL1RN) in Dutch Europids (EU) and Dutch South Asians (SA). C) mRNA level of caspase-1 (CASP1) in Dutch Europids and Dutch South Asians. B + D) Correlation between mRNA levels of IL1RN and CASP1, calculated as $2^{-\Delta CT}$, with body fat percentage in Dutch Europids and Dutch South Asians. mRNA levels are normalized to housekeeping gene low-density lipoprotein receptor-related protein 10 (LRP10). Data in panels A and C are expressed relative to Dutch Europids and presented as median and interquartile range (IQR). Mann-Whitney *U* test was used for statistical comparison between ethnicities. *P < 0.05.

whereas some studies showed no effect of cold exposure on cortisol (Brenner et al., 1999). Cortisol is known to have both anti-inflammatory and pro-inflammatory effects, which seem to be, at least partly, dependent on the duration of cold exposure and on the present state of the immune system (Cruz-Topete and Cidlowski, 2015). More specifically, an acute stress stimulus seems to augment a pro-inflammatory response to enable revival after exposure to a stressor, while chronic stress exposure induces an anti-inflammatory response, likely in order to restore homeostasis (Busillo and Cidlowski, 2013; Dhabhar, 2002, 2014). Interestingly, this pattern coincides with the immune responses reported in several intervention studies after short-term (pro-inflammatory) versus chronic (anti-inflammatory) cold exposure (Brazaitis et al., 2014; Brenner et al., 1999; Kox et al., 2014; Lackovic et al., 1988). However, we and others (Brenner et al., 1999; Hennig et al., 1993) have not been able to demonstrate a link between cortisol levels and modulation of the immune system after cold exposure. A possible explanation could be that the effect of cold exposure on the immune system is merely regulated by the SNS. Indeed, several immune cells express β-adrenergic receptors and increased noradrenaline levels are consistently found after cold exposure in clinical trials (Brazaitis et al., 2014; Hennig et al., 1993; van der Lans et al., 2015). Both Brazaitis et al. (2014) and Hennig et al. (1993) showed an increase in noradrenaline levels after cold exposure, however in both studies no correlation between noradrenaline levels and various immune markers was observed. In contrast, Brenner et al. (1999) did find a relation between total leukocyte count, granulocytes and lymphocytes and increased noradrenaline levels, albeit with low correlation coefficients. The interaction between the SNS and the immune system is furthermore demonstrated in mechanistic studies by the fact that some immune cells are also able to synthesize, store and release catecholamines themselves, to further modulate the inflammatory response (Flierl et al., 2007; Nguyen et al., 2011). The influence of catecholamines on the immune system remains an interesting topic for future research.

An interesting finding in this study is the lower transcript levels of genes encoding pattern recognition receptors of the inflammasome (*NLRP10*, *NLRP7*, *NLRP2* and *NLRC4*) and the higher transcript level of *IL1RN* in blood of Dutch South Asians compared to Dutch Europids. The inflammasome protein complex is able to activate caspase-1 from pro-caspase-1, which is essential for the cleavage of pro-IL-1β into the pro-inflammatory cytokine IL-1β (Garlanda et al., 2013; Strowig et al., 2012). Increased activation of the inflammasome and production of IL-1β is shown to be related to obesity and insulin resistance (Alexandraki et al., 2006; Stienstra et al., 2011). In addition, therapeutics inhibiting IL-1β, using the monoclonal antibody canakinumab, or the inflammasome, using colchicine, have shown a promising effect in clinical trials with respect to reduction of cardiovascular events (Nidorf et al., 2020; Ridker et al., 2017; Tardif et al., 2019). The lower transcript levels of inflammasome genes in Dutch South Asians in this study is therefore in contrast to our expectation, since South Asians are more prone to develop insulin resistance and, in interventional studies, migrant South Asians seem to have increased levels of circulating pro-inflammatory cytokines such as IL-6 and TNF-α, compared to other ethnic groups (Bakker et al., 2013; Petersen et al., 2006). However, we did find a higher mRNA level of the gene (*IL1RN*) coding for the IL-1 receptor antagonist (IL-1Ra) in Dutch South Asians compared to Dutch Europids. IL-1Ra is an anti-inflammatory competitive ligand of the IL-1 receptor, which blocks the biological activities of IL-1β to maintain homeostasis (Hannum et al., 1990). IL-1Ra is thought to be released in parallel with IL-1β and could thus serve as surrogate marker for circulating endogenous levels of IL-1β (Koenen et al., 2011). The main source for IL-1Ra are hepatocytes, monocytes, macrophages and adipocytes (Gabay et al., 1997; Juge-Aubry et al., 2003). In cohort studies, obese individuals indeed show have higher serum levels of IL-1Ra (Elisia et al., 2020; Meier et al., 2002), which correlate positively to the anorexigenic hormone leptin, insulin resistance and lean body mass (Meier et al., 2002). In patients with Cushing's disease (*i.e.*, chronic cortisol exposure), who display a phenotype characterized by a high proportion of abdominal fat, IL-1Ra is also positively correlated with leptin and in addition with truncal fat mass (Ueland et al., 2003). In addition, monocytes stimulated with leptin *in vitro* increase expression and secretion of IL-1Ra (Gabay et al., 2001). Of note, compared to other ethnicities, at a similar BMI, non-obese migrant South Asian individuals have a higher body fat percentage and higher serum leptin concentrations (Benedetti et al., 2019; Chandalia et al., 2007; Mente et al., 2010). Correspondingly, we show that body fat percentage is higher in Dutch South Asians compared to Dutch Europids, which could thus possibly explain their higher circulating mRNA level of *IL1RN*. However, in contrast to this hypothesis, we showed a negative correlation of *IL1RN* with body fat percentage in the Dutch South Asian individuals. It is known that the inflammasome components are predominantly present in adipose tissue (Koenen et al., 2011). It can thus be speculated that the lower circulating mRNA levels of the inflammasome components and the negative correlation between *IL1RN* and body fat percentage in Dutch South Asians is the result of migration of immune cells towards the adipose tissue in Dutch South Asians, leading to a compensatory downregulation in blood. For future research it would be highly interesting to further explore this hypothesis by obtaining adipose tissue biopsies.

This study has several limitations. mRNA levels of immune cells was measured in whole blood samples, without isolating cells using flow cytometry analyses. Therefore, we cannot relate RNA expression data with immune cell numbers and composition in blood. However, the extensive panel of immune genes measured in the current study allows

the detection of patterns within immune pathways, as illustrated by the consistent up- or downregulation of genes within one pathway. In the current study, we used a personalized cooling protocol, meaning that participants were exposed to different temperatures. This cooling protocol however minimizes shivering and aims to make the metabolic response comparable between all participants. Concerning the ethnic differences, it is important to note an ethnicity is defined by cultural traditions. Hence, apart from biological factors, differences in behavior and lifestyle could influence the results in this study. Nonetheless, there is no evidence that traditional risk factors can fully explain the substantial elevated risk of cardiometabolic diseases in South Asians, underlining the importance of fundamental research in this field (Kanaya et al., 2014; Sattar and Gill, 2015). Lastly, although the sample size of the study is fairly small and our observations warrant validation in larger cohorts that also include women, the consistency in the direction of the mRNA levels within the inflammasome pathway strengthens the importance of found differences in transcript levels between ethnicities.

In summary, we show that short-term mild cold exposure increases transcript levels of genes involved in cytotoxicity. This could reflect that short-term mild cold exposure quickly boosts the immune response towards a pro-inflammatory state, in preparation to fight an infection, at least partly by demargination of immune cells. The possibility to influence the immune system by exposing humans to cold exposure, even at a mild temperature and for a short duration, is highly interesting for treatment, or possibly even prevention, of several conditions markedly associated with inflammation, such as infectious diseases, autoimmune disorders and cancer (Gerard and Rollins, 2001; Golia et al., 2014). In addition, we show that Dutch South Asians, a population prone to develop cardiometabolic diseases, display an altered immune status, including lower mRNA levels of inflammasome genes and higher mRNA levels of *IL1RN*. Further studies are warranted to elucidate whether anti-inflammatory therapies targeting the inflammasome pathway are especially beneficial for the treatment of cardiovascular diseases in the South Asian population.

## Author contributions

Maaike E. Straat: Performed experiments, Analyzed data, Interpreted results of experiments, Prepared figures, Drafted manuscript; Borja Martinez-Tellez: Interpreted results of experiments, Edited and revised manuscript; Laura G.M. Janssen: Performed experiments, Critically reviewed manuscript; Suzanne van Veen: Performed experiments, Critically reviewed manuscript; Robin van Eenige: Analyzed data, Critically reviewed manuscript; Aan V. Kharagjitsing: Critically reviewed manuscript; Sjoerd A.A. van den Berg: Performed experiments, Critically reviewed manuscript; Yolanda B. de Rijke: Performed experiments, Critically reviewed manuscript; Mariëlle C. Haks: Performed experiments, Critically reviewed manuscript; Patrick C.N. Rensen: Conception and design of research, Edited and revised manuscript; Mariëtte R. Boon: Conception and design of research, Interpreted results of experiment, Edited and revised manuscript.

## Declaration of competing interest

The author(s) declare no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2022.103259.

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