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Agrimonia eupatoria tea intake has the potential to change oxidative and inflammatory response of human pbmc to *ex vivo* lps stimulation – an example of phenotypic flexibility modulation

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Agrimonia eupatoria L. (AE) is an herb widely used in the Bulgarian traditional medicine. Current study aims to assess the anti-inflammatory and antioxidant potential of the herb in an intervention study involving healthy volunteers. A model of bacterial lipopolysaccharide (LPS) *ex vivo* stimulation of peripheral blood mononuclear cells (PBMC) was used to analyse changes in expression of two pro-inflammatory (IL-1β and IL-6) and two antioxidant genes (GCLc and SOD1) after supplementation with agrimony tea for 25 days. The effect of BMI was also taken into consideration. LPS stimulation before intervention (Day 0) significantly stimulated IL-1β and IL-6 both in normal weight (NW) (3.7- and 14-fold, respectively, $p<0.001$) and overweight (OW) (2.8- and 2.5-fold, respectively, $p<0.05$) groups and of GCLc in NW (5 fold, $p<0.001$). After the AE intervention (Day 25) LPS stimulation significantly increased IL-6 (3-fold, $p<0.05$) and IL-1β (3-fold, $p<0.001$) mRNA levels only in OW, while in the NW such effect was not observed. GCLc and SOD1 mRNA levels were not elevated at Day 25 both in NW and OW groups. We established that AE consumption resulted in significant decrease in LPS stimulated expression of IL-6 (7.6-fold, $p<0.001$), IL-1β (3.8-fold, $p<0.001$) and GCLc (3-fold, $p<0.05$) gene expression only in NW.

All these results confirm the anti-inflammatory potential of the herb. They also highlight the capability of NW subjects for a better adaptation after the agrimony intake since their PBMC manifested a better phenotypic flexibility in comparison to the OW subjects in *ex vivo* inflammatory conditions.

Keywords: PBMC, LPS, cytokines, antioxidant enzymes, agrimony

INTRODUCTION

Realization that one's nutrition-related health status is a result of the interaction of individual's genome and life-long dietary exposure has led to the estimation of nutrition as a gene-environment interaction science [1]. A complex regulatory system, affected by environmental parameters, main constituent of which is nutrition, controls expression at all levels [2]. A healthy subject is known to be more adaptable to the constantly changing living conditions, which is a modern explanation of the concept for 'health'. This adaptation process is described as phenotypic flexibility of the individual [3]. Experts in nutrigenomics define the phenotypic flexibility as interaction between all processes involved in the metabolic adaptation. Therefore, the main goal is to select a wide range of biomarkers from genetics, transcriptomics, proteomics, metabolomics fields, behavioral changes and others to evaluate individual's adaptation capacity and by that – his/her health status [4–6].

One of the main goals of recent nutrition studies is to identify and develop standardized methods and techniques to study the changes in the phenotypic flexibility in response to nutrition, lifestyle, physical activity, obesity and other factors. The ability of the organism to regain homeostasis, after its balance has been disturbed by external factors, can be used as an indicator for metabolic health. Stress tests are constructed with the aim to temporarily disturb the homeostasis of the body. In response to such stress tests, the system will aim to restore the balance usually within hours [7].

Bacterial lipopolysaccharides (LPS) have been widely used in models studying inflammation *in vitro* and *in vivo* [8–11] or the mechanisms of anti-inflammatory action of a variety of compounds or plants [12–15]. Treating with LPS can modulate the gene expression by raising the cytosolic protein levels of cytokines (such as IL-1β and IL-6) and pro-inflammatory enzymes (e.g. iNOS) *via* activation of NF-κB transcription factor [13, 16–18]. Thus, application of LPS stimuli appears to be a useful tool to trigger inflammatory response in cell culture and *in vivo*. White blood cells circulate over the whole body and respond to various endogenous and exogenous stimuli. Recent studies, especially transcriptome analyses, show that peripheral blood mononuclear cells (PBMC) are a

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valuable source of data and representative target tissue in intervention studies. Priorities in applying PBMC are: accessibility of the blood samples, easy isolation of PBMC from whole blood, potent complex tissue in studying challenges and complex responses to different stimuli [9–11, 15].

In our study, we approached to include application of LPS stimulation that can induce inflammatory and oxidative stress response in PBMC freshly isolated from healthy subjects, which is thought to be representative as an example of stress test. Changes in gene expression in these cells can be informative about their susceptibility to such stimuli and differences in this response after an intervention can be used as an indicator about altered phenotypic response. We included intake of infusion from the herb *Agrimonia eupatoria* (AE) by healthy subjects as a possible source of compounds with a potential for counteraction to the inflammation stimuli. In addition, we checked whether the response will depend on Body Mass Index (BMI). Expression levels of antioxidant defense and inflammation related genes were analysed.

Agrimonia eupatoria L. (AE) is an herbal remedy used in Bulgarian folk medicine. Because of its high polyphenol and flavonoid content, it is established that the herb is a valuable source of antioxidants and possesses anti-inflammatory properties [19–21].

It is usually applied in prevention and treatment of liver, kidney and gall bladder diseases, conditions like mild diarrhea, pulmonary and gastrointestinal inflammatory diseases, ulcers, bleeding gums, rheumatism, hemorrhoids, and even in diabetes or obesity [22]. Enrollment of AE tea in an intervention study with the application of the PBMC LPS stimulation model would provide data about its healing properties based on antioxidant and/or anti-inflammatory activities.

MATERIALS AND METHODS

Plant material and infusion procedure

For implementing the intervention we used dry aerial parts of the plant, readily available in the drugstores. The procedure for preparing the infusion was following the traditional agrimony tea recipe: 2.5 g of the plant material was infused with 200 mL of boiling water for 10 min. The tea was prepared in the Department of biochemistry, molecular medicine and nutrigenomics, Medical University – Varna, Bulgaria and volunteers consumed it on the spot.

Intervention and volunteers

Prior to intervention an approval from the local ethics committee was received (Protocol №27/21.02.2013). Each one of the volunteers who responded to the invitation and joined the intervention was first interviewed about their lifestyle habits, health status and tea consumption frequency and habits. They all signed informed consent prior to the start of the intervention.

The intervention included 40 clinically healthy volunteers, aged between 20 and 60 years. They were divided in two groups regarding their Body Mass Index (BMI) – 23 subjects with BMI<25 – normal weight (NW), and 17 with BMI≥25 overweight subjects (OW). They consumed 200 mL/day of agrimony tea prepared as described above for a period of 25 days.

PBMC collection and separation

Fasting blood samples were collected before start of the intervention (Day 0) and at the end of the intervention period (Day 25) using lithium heparin vacutainer tubes. Whole blood was used to continue further with the isolation of PBMC. For that purpose we used LeucoSep™ centrifuge separation tubes (by GreinerBioOne, Austria) containing a porous barrier which enables cell separation by means of density gradient centrifugation following the manufacturer's instruction.

Cell cultivation and experimental design

Cell yield was determined using standard trypan blue staining method and seeded in density of 1×10^6 cells/well in RPMI 1640 (Sigma-Aldrich, Germany), supplemented with 0.01M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2 mM α-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS (Sigma-Aldrich, Germany).

Stimulation of the cells was performed with bacterial LPS (*Escherichia coli* 026:B6, Sigma-Aldrich, USA), diluted in saline solution (0.9 % NaCl). Final concentration used in PBMC treatment was 100 ng/mL.

Both at Day 0 and Day 25 there were two treatment groups – control, cultivated only with RPMI medium and the test group, cultivated in culture medium containing 100 ng/mL LPS. Flasks were incubated for 4 h at 37°C in a humidified chamber with 5% CO_2 atmosphere. Each treatment was performed in duplicate.

RNA isolation and cDNA synthesis

After the incubation period, total RNA was extracted from the cells with Tri reagent (Ambion®, Life Technologies, USA). RNA was subsequently DNase treated (RiboPure™ – Blood Kit; Sigma-Aldrich, USA). First strand cDNA synthesis was performed with 0.8 μg of total RNA using Thermo Scientific M-MuLV reverse transcriptase (USA) following the steps of manufacturer's instructions.

Real-Time PCR

Quantitative gene expression analysis was performed using two-step real-time qPCR. Each reaction was amplified in a reaction mix containing SYBR Green qPCR 1 × Master Mix with ROX (KAPA SYBR FAST qPCR Kit, KAPA BIOSYSTEMS, USA) and 0.3 μM of each primer.

Primer sequences used for the Real-Time PCR were as follows: RPL37A (Bioneer, USA) Forward ATTGAAATCAGCCAGCACGC; Reverse AGGAACCACAGTGCCAGATCC; IL-1β (Alpha DNA, Canada) Forward TCCCCAGCCCTTTTGTTGA, Reverse TTAGAACCAAATGTGGCCGTG; IL-6 (Bineer, USA) Forward AAACAACCTGAACCTTCCAAAGA, Reverse GCAAGTCTCCTCATTGAATCCA; GCLc (Bioneer, USA) Forward GGAGGAAACCAAGCGCCAT, Reverse CTTGACGGCGTGGTAGATGT; SOD1 (Invitrogen, USA) Forward GTGCAGGTCCTCACTTTAAT, Reverse CTTTGTCAGCAGTCACATTG. Analysis was performed on AppliedBiosystems® 7500 Real-Time PCR instrument (USA). The amount of mRNA of each gene of interest was normalized according to the amount of mRNA encoding RPL37A. Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [23]. Each sample was analyzed at least in triplicate. The results are presented as relative units mRNA±SEM.

Statistical analysis

GraphPad Prism 7.0 software (USA) was used for statistical analysis and graphics. Student's *t* test was used for column srtatistics. A *p* value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

This study was undertaken in order to test wherther an *ex vivo* stimulation of isolated human PBMC with LPS would detect changes in the response, expected after 25 day intake of an herbal remedy – *Agrimonia eupatori*a tea. We examined the effect on expression of four selected genes – antioxidant defense related (glutamate-cysteine ligase, GCLc and superoxide dismutase 1, SOD1) and inflammation related (IL-1β and IL-6). We compared the response to LPS stimulation before Day 0 to the response after Day 25 of the intervention period. We also determined the effect of AE tea intake on respective genes expression in non-stimulated cells again before and after the intervention. In addition we compared the response of PBMC from normal weight (NW) subjects to the response of overweight (OW).

Effect of ex vivo LPS stimulation on PBMC gene expression – LPS Day 0 vs. untreated Day 0 or verification of the model for stimulation of genes expression under LPS treatment

Various studies prove the potential application of PBMC in diagnostics of diseases like myeloid leukemia, atherosclerosis, autoimmune disease, etc. which are characterized with a specific gene expression profile of the PBMC [24–28].

Lipopolysaccharides (LPS) are the most abundant component within the cell wall of Gram-negative bacteria. They can stimulate the release of inflammatory cytokines in various cell types, leading to an acute inflammatory response toward pathogens [29]. Acute inflammation is characterized by increased blood flow and vascular permeability, accumulation of fluid, leukocytes, and inflammatory mediators like cytokines. The cytokines are the main inflammatory mediators which orchestrate the inflammatory response on the level of cell activation and infiltration, as well as the systemic responses to inflammation. Cytokines involved in acute inflammation are IL-1, TNF-α, IL-6, IL-11, IL-8 and others. IL-1 and TNF-α are the primary cytokines that mediate acute inflammation induced in animals by intradermal injection of bacterial LPS [30]. Main source of IL-1α and IL-1β are the mononuclear phagocytes, fibroblasts, keratinocytes and T and B lymphocytes. Both cytokines play a role in the fever induction. They activate cyclooxygenase (COX) and increase the prostaglandins synthesis [29]. They also stimulate the T cell proliferation. There are data from *in vitro* and *in vivo* studies which prove that IL-1α and IL-1β also induce the synthesis of C-reactive protein (CRP) which is a protein from the acute phase of inflammation [29]. In the acute phase of inflammation IL-6 acts as a growth factor for mature B cells and stimulates their transformation into antibody-producing plasma cells. Up-regulation of IL-6 production is established also in a variety of chronic

inflammatory and autoimmune disorders like thyroiditis, type I diabetes, rheumatoid arthritis, etc. [31, 32].

It is known that oxidative stress and production of reactive oxygen species (ROS) is provoking inflammatory processes and production of chemokines and cytokines [33]. On the other hand, bacterial endotoxins that bind to TLR4 and activate NF-κB pathway also stimulate the NOX4 complex, which in turn generates ROS [34, 35]. This in turn raises the question about the effect on the antioxidant enzymes which are responsible to combat the action of ROS after such inflammatory stimuli. Gamma glutamate-cysteine ligase (GCL) is the rate-limiting enzyme in *de novo* biosynthesis of glutathione which is the most abundant endogenous antioxidant in the cell. Superoxide dismutase (SOD) catalyzes the transformation of the superoxide anion to hydrogen peroxide. Compounds or conditions that increase the production of the superoxide anion induce the activity of different SOD isoforms.

According to literature, the peak of the cytokine release after LPS stimulation in cell culture is reached until the 4th hour and this is the reason why we chose this time period for LPS stimulation [10].

We detected whether the *ex vivo* LPS stimulation is effective and analyzed the response of PBMC to the LPS stimuli by measuring the changes in gene expression levels of IL-6, IL-1β, GCLc and SOD1 in both groups of NW and OW.

Before the intervention period, Day 0, four hour incubation of *ex vivo* cultured PBMC with 100 ng/mL LPS resulted in a significant increase in IL-1β and IL-6 both in NW ($p<0.001$) and OW ($p<0.05$) groups, and also of GCLc in NW group ($p<0.001$) (figure 1).

Treating the PBMC of NW subjects with LPS on Day 0 increased mRNA levels for IL-6 approximately 14 times ($p<0.001$), and in the PBMC of OW group – nearly 2.5 times ($p<0.05$). Similarly, treating with LPS preceding the intervention led to elevation in the levels of IL-1β of both groups – in NW group it was by 3.7 times ($p<0.001$) and in OW – by 2.8 times ($p<0.05$). Notably before the intervention with agrimony intake treating of PBMC with LPS significantly stimulates transcription of GCLc in NW subjects by 5 times ($p<0.001$), while the induction in OW group is visible, but not statistically significant. We established no significant changes for the levels of SOD1 gene expression in both groups (Figure 1).

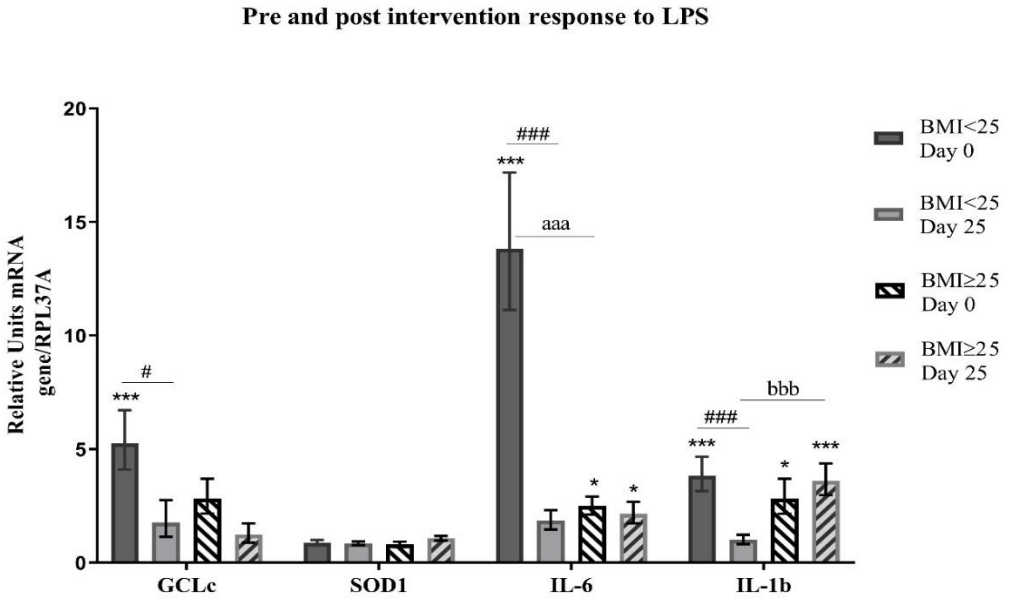

Figure 1. Changes in gene expression levels as a response of PBMC to LPS stimulation vs. untreated cells for both groups of volunteers – NW (BMI<25) and OW (BMI≥25). Gene expression is presented as relative units mRNA ±SEM normalized to RPL37A as endogenous control gene. Legend: *p<0.05, ***p<0.001 vs. untreated cells; #p<0.05, ###p<0.001 Day 25 vs. Day 0; ^{aaa}p<0.001 Day 0 BMI<25 vs. BMI≥25; ^{bbb}p<0.001 Day 25 BMI<25 vs. BMI≥25.

As seen on Figure 1, on Day 0 before the intervention period treatment with bacterial LPS induces several times and statistically significant the mRNA levels of both cytokines, which proves the effectively induced inflammatory response in these cells. It is known that the oxidative stress and free radicals production is linked to inflammation conditions and cytokines production [33]. The

observed induction of the gene for GCLc in the NW group ($p<0.001$) is probably linked to stimulation of glutathione synthesis, presumably evoked due to the need of an antioxidant agent to combat an eventual oxidative stress during the application of inflammatory stimuli.

Effect of ex vivo LPS stimulation on PBMC gene expression after the AE tea intake – LPS Day 25 vs. untreated Day 25 or what is PBMC reactivity after the intervention

Intervention with AE tea for a period of 25 days resulted in a lower effect of the same treatment conditions on cultured PBMC from the NW group. On Day 25, treatment with LPS in the NW group did not induce the gene expression of IL-1β anymore, while the OW group still remained sensitive to the inflammatory stimuli by an increase in the expression of this gene by 3 times ($p<0.001$) compared to the non-treated cells (Fig. 1). Similarly, after 25 days of intervention, gene expression of IL-6 in the PBMC of NW group was not affected by LPS stimulation, but the levels in the OW group were approximately 3 times increased ($p<0.05$). No significant changes about the mRNA levels of GCLc and SOD1 were established after application of LPS on Day 25 both in NW and OW groups. This might be interpreted as an increased sustainability of NW subjects' PBMC to inflammatory stimuli after the 25 days agrimony intake period and could be perceived as an improved phenotypic flexibility profile. Considering the BMI as a factor with a high impact on the inflammatory profile outlook, it is known that overweight and obesity are characterized by a higher mass of adipose tissue, which is known to be accompanied by a low-grade inflammation in a different degree [36]. Presumably, this could be a reason for the sensitivity of PBMC of OW subjects to an additional inflammatory stimulation.

Effect of AE tea intake on the response to LPS treatment – LPS Day 25 vs. LPS Day 0 or is there any difference between gene expression levels in LPS provoked cells before and after intervention

Significantly lower mRNA levels of IL-6, IL-1β and GCLc ($p<0.001$, $p<0.001$ and $p<0.05$, respectively) were established after the intervention period in LPS treated cells of the NW group only. There were no significant differences in gene expression of the analyzed cytokines and antioxidant enzymes in LPS treated PBMC of OW group (Fig. 1).

Comparing the levels of IL-1β and IL-6 mRNA in cells treated with LPS on Day 0 and Day 25 we see that there are significantly lower levels (approximately 3.8-fold for IL-1β and 7.6-fold for IL-6, $p<0.001$) after the intervention in the NW group. This confirms the reformed response to LPS stimuli probably manifesting an improved phenotypic flexibility of these cells. However, we did not observe such changes in the OW group as they remain as sensitive to the inflammatory stimulation as they were before the intervention period. Conditions like diabetes, metabolic syndrome, and diseases caused or accompanied by chronic low-grade inflammation are considered to appear in cases where metabolic homeostasis and therefore phenotypic flexibility is disturbed. Our results probably confirm the reduced capacity of PBMC from OW subjects to manifest phenotypic flexibility of some degree, which might be explained by the metabolic changes they probably already bear. Similarly, in the NW group mRNA levels of GCLc are significantly lower (3-fold, $p<0.05$) in conditions of LPS stimuli application after the intervention period. After the 25 days of the intervention period the inflammatory and oxidative response of PBMC from the NW group is significantly weaker when treated with LPS. An intake did not result in a difference in SOD1 mRNA levels in LPS treated cells on Day 0 and Day 25 in both NW and OW groups.

Difference between NW and OW groups in regard to their response to LPS stimulation before and after the AE tea intake – LPS Day 0 NW vs. LPS Day 0 OW and LPS Day 25 NW vs. LPS Day 25 OW or how the BMI affects the LPS response

Gene expression of IL-6 in response to LPS stimuli on Day 0 was significantly lower in the OW group when compared to NW (about 5.5-fold, $p<0.001$), while there was no significant difference for Day 25 (Fig. 1). With reference to IL-1β we observed no significant difference when comparing Day 0 of LPS response of both groups of volunteers whereas a significantly higher level of gene expression for LPS stimualated cells on Day 25 was estimated for OW (3.6-fold, $p<0.001$). There was no significant difference in the changes of antioxidant enzymes gene expression in response to LPS stimulation between both groups.

It is known that adiposity is characterized with increased infiltration of macrophages in the adipose tissue [37]. It is considered that these macrophages are the main source of inflammatory mediators such as TNF-α and IL-6 which disturb the normal function of adipose tissue by inducing the inflammatory profile and suppressing the insulin potency in the tissue [38–40].

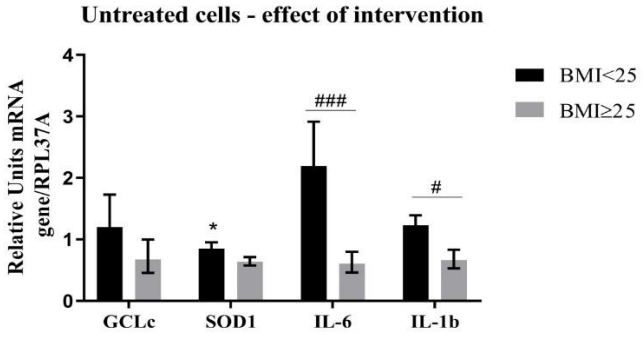

Figure 2. Changes in gene expression levels in untreated PBMC as a result of the 25 days of AE intervention. Gene expression is presented as relative units mRNA ±SEM normalized to RPL37A as endogenous control gene. Legend: *p<0.05, Day 25 vs. Day 0; #p<0.05, ###p<0.001 BMI<25 vs. BMI≥25. Each column represents changes in levels of gene expression in untreated control cells after the AE intervention (Day 25) compared to the expression on Day 0 as a control.

Presumably constant low-grade inflammation and cytokine activity in obese subjects leads to lower susceptibility to inflammatory stimuli represented as significantly lower IL-6 levels at the starting point (Day 0) in comparison to NW, while levels of IL-1β are visibly but not significantly lower.

After the tea intake period (Day 25) we observed a drastic drop in the levels of cytokines expression for the NW subjects since they might be with increased stability towards the LPS stimuli. When comparing the response between them and OW in Day 25 it is visible that IL-1β is expressed siginifcantly higher in OW (3.6-fold, p<0.001) (Fig. 1). The latter group manifests similar levels of both cytokines expression on Day 0 and Day 25 apparently regardless of herbal intervention. This in contrast is not the case in the NW group and they display significantly lower IL-1β levels than the OW group.

Effect of AE tea intake on gene expression in non-stimulated cell – control Day 0 vs. control Day 25 or how AE tea intake changes inflammatory/redox status in non-compromised individuals

Data indicate that AE tea intake does not influence inflammatory/redox status in healthy, non-compromised individuals, as represented by the lack of difference in studied genes expression before (Day 0) and after (Day 25) the intervention. The only statistically significant difference after the 25 days of AE infusion intake is observed in the NW group, where levels of SOD1 gene expression are significantly decreased compared to the levels of mRNA before the intervention (by 15%, p<0.05). Presumably, by being rich in polyphenols and a powerful antioxidant itself, the agrimony herbal infusion is compensating the need for the antioxidant enzyme SOD1 and therefore reducing its gene expression by mechanisms which need further investigation to be established.

When comparing the differences in the expression levels of studied genes between the NW and OW groups we established a stronger change in cytokines expression levels in the NW (approximately 4-fold for IL-6 and 2-fold for IL-1β) (Fig. 2). We may speculate that this is a possible immunostimulatory effect of the herb itself where the OW subjects are expected to already possess higher cytokine expression because of the suggested low-grade inflammation and probably the tea intake doesn't affect them with the similar intensity. This, in turn, is also observed and confirmed by the lower response to LPS stimulation of these subjects (Fig. 1).

In literature, there are *in vitro* studies investigating the biological effect of different herbs on PBMC gene expression and response to inflammatory stimuli [13, 14, 41, 42]. However, intervention studies comparing the *in vivo* effect by *ex vivo* LPS stimulation and response of PBMC from individuals with varying BMI are difficult to find [43, 44]. Yet, interventions applying this approach are limited but still gaining popularity because of its multilateral informative potential [45–47].

CONCLUSION

The main aim in our study was to establish a well-working model for induced inflammation in PBMC isolated from human whole blood by application of bacterial LPS. Changes in the transcription levels of IL-6 and IL-1β in PBMC cells are informative about their susceptibility to LPS stimuli, which can be used as a stress test in studying phenotypic response and assessment of the

phenotypic flexibility under different conditions. Furthermore, this outline can be used for examining anti-inflammatory and antioxidant properties of the tested herbal extracts, fractions or isolated compounds. Our study also demonstrates differences in the metabolic and phenotypic flexibility of subjects varying in their BMI, as well as differences in their potential to counteract to environmental challenges and stimuli. AE tea intake leads to decreased response to LPS stimulation in NW subjects, which could be an explanation of its possible preventive anti-inflammatory properties. In addition to unchanged reactivity to LPS treatment as a result of AE intake, OW individuals demonstrate no ability of their PBMC to respond to agrimony intake with a change in studied genes, with the exception of SOD1.

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