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Evaluation of Expression Profiles Due to Garlic Intake in Young Men and Women

Brusgaard Klaus[*], Burton Mark

Department of Clinical Genetics, Odense University Hospital, Denmark

ABSTRACT

Garlic has shown many and diverse beneficial effects on health. Focus has been on which compounds have effect. We analyze the transcriptional consequences of digesting limited amounts of raw garlic and analyze the overall outcome. This study focuses on the reported health related effects of garlic digestion in relation to the observed transcriptional changes to identify and understand the eventual casual outcome. We utilize a non-hypothesis driven global approach to analyze the transcriptional consequences of eating moderate amounts of garlic in a group of young people. Unsupervised principal component´s analysis and heat maps showed changes in gene expression of RNA extracted from peripheral blood using microarrays. Pathway analysis was performed using gene set enrichment analysis with KEGG, Reactome and Gene Ontology defined gene sets as input. A number of significant pathway changes were observed. When correcting for gender difference a possible relation to beneficial effects observed in both men and women were observed. Especially the gender specific immune dimorphism upon garlic digestion Our results support the notion of a link between olfaction, neurology and the immune system and imply that a common mechanism explains the link between garlic ingestion and the previous reported pleiotropic positive effects.

Keywords: Garlic; Gender specific dimorphisms; Olfactory system; Transcriptional regulation

INTRODUCTION

Garlic has throughout history been valued for its healing effects as well as the popular culinary contribution to the taste experience. The chemical complexity of garlic is considered the source of the diverse beneficial effects on thrombosis, heart disease, diabetes, nerve disorders and cancer, as well as being circulatory strengthening. Despite a growing list of scientifically proven effects that support the broad and potent beneficial effects of the herb, the underlying mechanisms are relatively poorly described. Various sulfur compounds are present in garlic, among others these are γ-glutamyl-S-allyl-L-cysteines and S-allyl-L-cysteine sulfoxide, which is organosulfur, compounds (OSCs). A variety of compounds are formed by the processing or metabolizing of these compounds e.g. diallyl sulfide, diallyl disulfide, diallyl trisulfide, diallyl tetrasulfide, ajoene and S-allyl cysteines. In addition to their antioxidant, hypoglycemic, an-

ti-inflammatory, and immunomodulatory effects, these compounds exert cardiovascular benefits/effects [1]. Garlic preparations can be categorized as powders, oils, AGE (Aged Garlic Extract) or raw (not processed). The chemical profile of these preparations may vary with respect to the different compounds they contain. S-allyl cysteine has been identified as AGE's active and stable component. However, as a result of the volatility of allicin, which is a derivative of alliin, the active component in garlic powder is less well established [1-3]. The perennial herb has shown many possible beneficial effects. In a meta-analysis based on the analysis of garlic powders performed by Kwak et al. it was demonstrated that garlic may have a beneficial effect on free blood glucose (FBG) and LDL-C, though for the latter only in males [4]. Likewise, OSCs and flavanoides from Garlic has shown to decrease FBG, intestinal glucosidase inhibition, serum cholesterol, triacylglycerol and LDL-cholesterol, resulting in decreased blood glucose and lipid levels. Likewise, show-

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ing increased GLUT-4 translocation, glucose uptake and insulin action, SOD, GPx and catalase activity [5-9]. Similarly, Garlic has shown anti-hypotensive action when tested using different experimental animal models and when administered to mildly hypertensive patients under controlled clinical trials [10-12]. Performing meta-analysis on studies using Kwai or Allicor; Standard garlic powder; Kyoli; Aged garlic extract; Cardiomax; Garlic oil; Dentou-Ninniku-Ranwo; Egg yolk-enriched garlic powder it was concluded that garlic can lower blood pressure in hypertensive patients. Additionally, using mainly five assays; Ames test, sister chromatid exchange (SCE), chromosomal aberrations (ChAb), micronucleus (MN), and comet assay (CA), garlic have been demonstrated to possess a broad antigenotoxic effect against various mutagenic and/or carcinogenic agents both *in vivo* and *in vitro*. Various modes of action have been proposed considering its antigenotoxic and anticarcinogenic potential [10].

The main 4 mechanisms being: (a) The effect on the metabolizer enzymes of drugs which inactivate toxic substances and facilitate their excretion (b) tumor growth inhibition (c) induction of apoptosis, and (d) the effective stimulation of the immune system [13,14]. Further, "Aged Garlic Extract" (AGE) and one of its active ingredients S-Allyl-L-Cysteine (SAC) have shown therapeutic properties in alzheimer disease (AD) in mouse models [15,16]. Treatment with AGE and SAC did not only lower Aβ concentration in APP-transgenic (Tg) mice, but also improved tau pathology and increased levels of synaptic protein markers. In a study by Numagami et al. SAC, was shown to have therapeutic value in rat focal ischemia due to reduced edema formation, infarction area, motor dysfunction and memory impairment [17]. Despite the overwhelmingly positive effects demonstrated little is known about the underlying pathophysiological mechanisms or the benefit as a potential protective agent. In this study, we describe the global changes in transcriptomic activity from a short period of using a moderate amount of fresh garlic and its potential link to some of the beneficial effects.

METHODS

10 healthy age matched individuals (26.7+/-2.4 years) were invited for the study, 7 women (age 23-29) and 3 men (age 24-29). Each participant was offered a bulb, approximately 3 grams, of garlic per day over a period of one week. On day one and seven 10 ml of peripheral blood was extracted in PaxGene tubes (Qiagene, Denmark). Total RNA was isolated using the RNeasy Micro Kit according to the RNeasyH Micro Handbook (Qiagen). The quantity of RNA was assessed with the NanoDrop spectrophotometer ND-8000 (NanoDrop Technologies), and RNA quality using the Agilent 2100 Bioanalyzer (Agilent Technologies). Transcriptome analysis was performed using Clariom D Pico Assays (ThermoFisher Scientific, Denmark) according to the manufacture's recommendation using an Affymetrix GeneChipTM Fluidics Station and a GeneChipTM Scanner.

Data and Statistical Analyses

Background correction, normalization, and gene expression index calculation of probe intensities were performed using the robust multi-array average (rma) method embedded in the Transcriptome Analysis Console (TAC) Software (version 4.0.1,

Thermo Fischer Scientific, Waltham, MA, USA). Next, all probes defined by TAC as being not expressed, were removed from further analysis. All subsequent calculations were performed using the open-source R-environment (R version 3.6) (http://cran.r-project.org/). First, the normalized probe expression matrix was collapsed by gene symbol using maximal probe intensity, thus providing one gene expression value per sample per gene. We next calculated the variation in gene expression for all genes. Based on the expression of the top 100 most variable genes we calculated the principal components using prcomp function embedded in the stats R-package (version 0.1.0). The associated principal components based sample clustering were then visualized using the plot 3d function embedded in the rgl R-package (version 0.106.8). Gene expression levels very visualized by heatmaps using the ComplexHeatmap R-package (version 3.12) [18]. Differential gene expression analysis between gender-specific prior and post garlic consumption, was conducted on the gene expression measured by the microarrays using a paired limma t-test embedded in the limma R-package (version 3.47.16) [19]. All comparisons were adjusted for multiple testing using the false discovery rate (FDR), and genes with FDR ≤ 0.05 were considered as being significantly differentially expressed between the compared groups. Gene set enrichment analysis was performed using the fast gene set enrichment analysis (FGSEA) embedded in the fgsea R-package (version 3.13) [20], to determine if a given set of curated genes/ pathways showed statistically significant, concordant differences between the compared groups. FGSEA was run on pre-ranked individual expressed genes using the Log2 fold change as ranking metric. The collection of human KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/ kegg/), Reactome (https://reactome.org/), and gene ontology (GO) (http://www.geneontology.org/) defined gene sets were used as input for the analysis. Next, an enrichment score was calculated for each of these a priori defined gene sets, which is a metric of how significant the members of a gene set deviate from a random distributed ordered list compared to the input pre-ranked list. In our analysis, FGSEA was conducted using 1000 permutations, and minimum and maximum gene sets size was set to 15 and 500, respectively. Gene sets with FDR ≤ 0.05 were considered as being statistically significantly enriched. Data have been submitted to The Gene Expression Omnibus (GEO) with submission number GSE221486.

RESULTS

Unsupervised Pca-based Cluster Analysis

We first calculated the variation for all the expressed genes. Next, the genes with the top 100 most variation was used as input for a heatmap gene expression visualization and for PCA analysis and subsequent PCA-based cluster visualization, which separated the samples into two clusters (**Figures 1-2**). However, these two clusters did not reflect a post/prior garlic consumption separation of the samples, which were evident by the clusters being a mixture of post/prior consumption samples. Surprisingly, the clusters proved to be gender specific with the largest and smallest cluster, consisting of female or male participants, respectively (**Figure 2**).
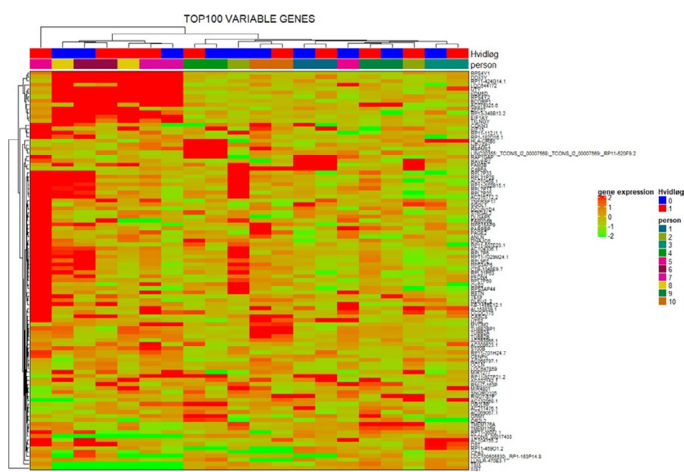
Figure 1: Heat map of the 100 top variable genes. The samples can be seen to cluster with largest relation being
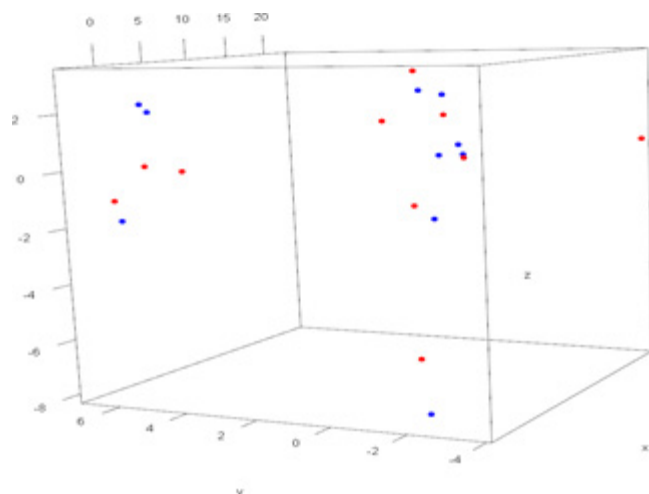

Figure 2: Performing PCA separates the samples into female (right) and male (left) clusters still with the largest relation between individual samples before (blue) and after (red) treatment

Analysis for Differentially Expressed Genes

Based on the observation of gender specific clustering, we conducted a paired limma t-test gene expression analysis on each gender separately. This resulted in 166 significantly differentially expressed genes in the female cluster after adjusting for multiple testing. Of these 166 genes, 115 were upregulated and 51 downregulated after garlic consumption compared to prior garlic intake. In contrast no genes were differentially expressed in the male samples, probably due to a low sample size (**Table S1**).

Additionally, we did neither detect any significant differentially expressed genes when conducting non-gender specific limma paired t-test, reflecting the observation of greater variation between genders than between prior/post garlic intake.

Gene Set Analysis

To identify signaling pathways and gene sets that are differentially activated or deactivated after garlic consumption, we conducted fast gene set enrichment analysis (FGSEA) between post and prior garlic intake, among the males and females separately. In the male cluster 16 and 385 gene sets showed significant up or down-regulation, respectively, after correction for

multiple testing (FDR ≤ 0.05), while in the female cluster 54 and 318 gene sets displayed significant up or down-regulation respectively after correction for multiple testing (**Table S1**).

Of the 16 significant upregulated pathways in men 7 are related to the immune system and 5 related to olfactory function, and among the top 50 downregulated pathways in men; 32 were associated to RNA transcription and processing, 9 to protein metabolic processes, 2 to oxidative phosphorylation and 2 to tumor genesis (**Table S1**) (Male_pos and Male_neg). Among the 50 significant upregulated pathways in women: 14 were associated to translational processes, 6 pathways were related to RNA catabolic processes, 10 were related to oxidative phosphorylation and 2 to protein metabolism. Furthermore, of the top 50 downregulated pathways in women 19 were related to the immune system, 12 to chromosome organization, 6 to transcriptional activity, 4 to protein catabolic processes, 2 to telomer maintenance (**Table S1**) (Female_pos and Female_neg).

DISCUSSION

Smell is critical to understand and provide information about the environment. Olfaction facilitates identification of food, partners, predators, and serves both to recognize sensual pleasure and warning against danger. Olfactory sensory neurons (OSNs) are responsible for detection of chemical stimuli in the environment but are also continuously exposed to microorganisms. After an odorant binds to an olfactory receptor, axons expressing sensation at the olfactory receptor converge on the olfactory bulb. Signals are relayed to areas of the cerebral cortex responsible for conscious thought and to the limbic system, which produces emotion [21]. The limbic system is composed of numerous centers, including the amygdala, septal nucleus, prepyramidal cortex, entorhinal cortex, hippocampus and regulates motivation, emotion, and memory [20]. With further projections reaching the thalamus and frontal cortex, this constates a highly interactive and dynamic system. In mammals, approximately 1000 genes encode olfactory receptors (ORs), but only 347 encode functional receptors [21]. OR genes are distributed on all human chromosomes except chromosomes 20 and Y, with nearly 80% clustered between 6 and 138 genes [15]. One of the largest OR gene clusters is located at the distal end of the major histocompatibility complex (MHC) class I on chromosome 6 [22]. It can be speculated that these OR genes are not only physically linked to MHC, but also have some functional association The MHC-associated olfactory gene cluster has 36 OR genes at a density of one OR gene per 23 kb [22]. 25 or genes are located in the major region 135 kb away from the HLA-F locus and 11 in the minor region 2000 kb away from the HLA-F locus. Recently, OSN was shown to mediate rapid pro-inflammatory innate immune responses in teleosts when presented with viral pathogens [23]. This is consistent with the finding of a highly upregulated humoral immune response and a concomitant similar increase in olfactory signalling in male samples. The observation that men and woman have differences of both the innate and the adaptive immune system has previously been reported [22]. It has previously been show that females show an increased immune reactivity his greater immunocompetence may cause an improved resilience to both infectious and some non-infectious diseases. It has been shown that, women have both a stronger humoral

and cellular [24,25]. Immune response to antigen than males [26]. High immune reactivity may predispose women to develop autoimmune diseases [27]. Although they do not have higher B lymphocyte counts than men, they still have higher levels of circulating antibodies [28] and higher levels of autantibodies, which can lead to autoimmune disease [29]. Rheumatoid arthritis, psoriasis, Sjögren's syndrome, multiple sclerosis, Graves' disease and Systemic Lupus Erythematosus (SLE) are more prevalent in women [30]. It has also been documented that women are more prone to developing autoimmune diseases and that garlic may have a positive impact on these conditions [31-35].

Here we show that the difference between male and female immune system is nivellated by the ingestion of garlic, with significant downregulation of both the humoral and cellular immune systems in females. Our results demonstrated the downregulation of female T helper 2 (TH2) cells by downregulating the adaptive immune response, particularly the cytokines IL4, IL6, IL10, and B-cell antibody production, which precedes protection against these autoimmune diseases. Moreover, proinflammatory TLR4, which is generally more highly expressed in male neutrophils, is further downregulated by garlic ingestion in female samples [36]. On the other hand, upregulation in males is reflected in increased complement-dependent phagocytic capacity, which may provide better protection against infection. Women not only have a higher prevalence of Alzheimer's Disease (AD) than age-matched men, but also show age-related faster decline [37]. Allthough Parkinson's Disease (PD) is more frequent in men the Unified Parkinson´s Disease Rating Scale (UPDRS) instability score have shown an earlier onset of tremor in women. Garlic has in a number of different formulations, either as chloroform extracts or aged garlic extract (AGE) shown to be able to inhibit reactive oxygen species (ROS) formation [38]. Reactive oxygen species (ROS) serve as cell signaling molecules for normal biologic processes. However, the generation of ROS can also provoke damage to multiple cellular organelles and processes, which can ultimately disrupt normal physiology. ROS is *via* the formation of aggregates linked to both AD, depression, schizophrenia and PD. Especially, women present with a higher prevalence of AD and a faster age-related decline, and a higher prevalence of depression and PD than men. In our study we see a significant upregulation in several pathways related to oxidative stress in women especially before garlic intake while these pathways are downregulated in men albeit with a higher significance in women.

In line with these observations, it has been documented that AD may appear together with psychiatric symptoms related to a primary disease mechanism rather than just being a co-morbidity and disease burden [39]. Increased hospitalization of patients with RA, SLE, and ankylosing spondylitis suffering from neuropsychiatric disorder has previously been demonstrated [40]. Additionally, anxiety and depression has been shown associated to Sjogren's syndrome [41,42]. A wide spectrum of autoantibodies is associated with a great diversity of clinical manifestations in SLE [42]. Especially, intraventricular ICV injection of SLE prevalent anti-ribosomal-P protein autoantibodies in mice induce depression-like behavior [43,44]. These mice demonstrated a reduced sensitivity to smell. Additionally,

patients with MS also show a significant association between decreased odor identification ability and symptoms of anxiety and depression [45,46]. These findings support the notion of a link between olfaction, neurology and the immune system and imply that a common mechanism explains the link between garlic ingestion and the previous reported pleiotropic positive effects.

Our results are based on analysis of blood samples and the expression profiles are hence mainly reflecting the transcriptionally activity of leucocytes and hence, are not informative in relation to metabolic and malignant pathways in other tissues. In this perspective the overall downregulation of transcriptional and increase in RNA catabolic activity may solely reflect the overall decrease in immunological activity up-on garlic ingestion in woman and the reverse picture in men reflect the complementary situation. Gender specific immune-dimorphism due to the presence of different sex hormones was previously recognized [46]. Estrogen has been shown to modulate immune responses by interfering with the negative selection of high-affinity autoreactive B cells, modulating B cell function, and leading to Th2 responses [47-54].

CONCLUSION

Estrogen influences physiological functions through ERs expressed in brain, intestinal epithelial cells, lymphoid tissue cells, and immune cells. Interestingly, phytoestrogens have likewise been reported to affect sex hormones by altering sex hormone-binding globulin (SHBG) levels in ER-independent mechanisms of action. Circulating estrogens and androgens are primarily bound to albumin and SHBG, with only a small part being free. Hence, since estrogens and androgens are biologically active only in their free form, SHBG affects steroid activity. *In vitro* studies have shown that isoflavonoids, a group of phytoestrogens, stimulate the synthesis of SHBG by liver cancer cells. In addition, phytoestrogens inhibit aromatase and other enzymes involved in the synthesis of steroid hormones. Garlic contains high concentrations of the isoflavones daidzein, genistein and glycitein. It can be speculated if the gender difference demonstrated here can be explained by the isoflavonoid content alone or additional factors are at play.

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CONFLICT OF INTEREST

None.

DECLARATIONS

Ethical Consent

Informed consent was obtained for all individuals. The local ethical committee of Southern Denmark has approved the protocol under number S-20160190. All methods were carried out in accordance with relevant guidelines and regulations.

Copyright

Credit Author Statement

Funding Source

GEO Submission

Data has been deposited under GEO number GSE221486.

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