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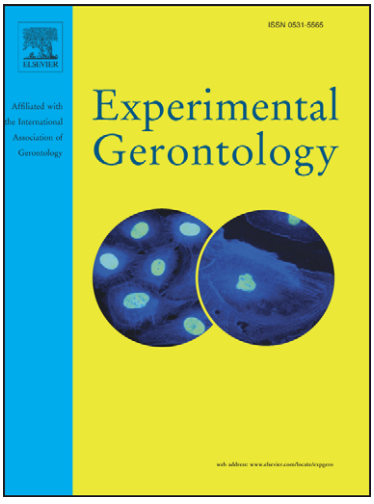

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Effects of interleukin-6 -174C/G and metallothionein 1A +647A/C single-nucleotide polymorphisms on zinc-regulated gene expression in ageing.

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Running Head: Zinc-regulated genes and IL-6 and MT SNPs

Key Words: IL-6, genetics, single nucleotide polymorphism, nutritional genomics, zinc, ageing, inflammation, metallothionein

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Abstract

Decreased zinc ion availability in ageing is associated with altered immune response. One of the main regulators of zinc availability is metallothionein. Metallothionein induction is under the control of interleukin-6, a pro-inflammatory cytokine whose production is associated with poor ageing. The production of interleukin-6 is controlled, in part, by variability in the -174 nucleotide position. Under conditions of chronic inflammation, such as in ageing, zinc release by metallothionein is limited and may reduce zinc availability. Understanding the precise nature of the interactions between interleukin-6 and metallothioneins will aid in identifying individuals who are at risk of zinc deficiency. In the current study, we used gene arrays to investigate the effects of *in vitro* zinc supplementation on gene expression in elderly donors with described interleukin-6 and metallothionein 1a polymorphisms. Ingenuity Pathway Analysis™ identified several zinc-responsive genetic networks uniquely regulated only in elderly individuals with the pro-inflammatory interleukin-6 polymorphism. These include zinc-dependent decreased transcription of pro-inflammatory cytokines and alterations in metabolic regulatory pathways. The genomics effects of zinc increased in significance in the presence of the metallothionein 1a +647 C/A transition, suggesting that the interleukin-6 and metallothionein 1a genes act in a concerted manner to control zinc-regulated gene expression.

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Introduction

In human ageing, zinc bioavailability is diminished resulting in impaired immune response and anti-oxidant activity (Mocchegiani et al. 1998). Metallothioneins (MTs) play an important role in regulating zinc homeostasis due to their high affinity for zinc. An important function of MTs is in releasing zinc in response to oxidative/nitrosative stress in order to up-regulate the zinc-dependent antioxidant enzymes (Mocchegiani et al. 2006). This response is inadequate in states of chronic inflammation and in ageing individuals (Mocchegiani et al. 2003).

A recent report has demonstrated that polymorphisms in the MT coding region are involved in regulating zinc release by MT and are associated with longevity (Cipriano et al. 2006). Cipriano et al. have shown that carriers of the C-allele at position +647 of the MT1a gene show enhanced zinc release, low metallothionein levels, and reduced IL-6 plasma concentrations. In addition, metallothionein gene polymorphisms have been associated with cardiovascular disease, hyperglycaemia and mild zinc deficiency in diabetic patients (Giacconi et al. 2005). Metallothionein induction and expression are under the control of IL-6 (Jacob et al. 1999). These data suggest that in the presence of heightened IL-6 production, the MT1a +647 C/A transition may impact negatively on inflammation through up-regulation of IL-6 pro-inflammatory cytokine production, thereby creating vicious pro-inflammatory feed-back cycle. Since increased IL-6 production has been linked to disease and is a poor indicator of survival (Fagiolo et al. 1993, Ferrucci et al 1999), these studies have profound implications for understanding the link between genes, health and longevity.

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It is critically important to determine the precise nature of the relationship between metallothionein status and IL-6 production. Heightened levels of metallothionein, IL-6 and low zinc ion availability are observed in diseases characterised by chronic inflammation and in syndromes of accelerated ageing (Mocchegiani et al. 2002). In addition, high levels of IL-6 production is common in ageing and is detrimental for successful ageing (Fagiolo et al. 1993, Ferrucci et al 1999). Genetic variation at the -174 region within the IL-6 promoter affects cytokine production. The presence of the C allele in the -174 position of the IL-6 promoter decreases the level of IL-6 production**.** Loss of the C-allele may be associated with risk of disease in ageing and may predict poor ageing outcome (Bonafe et al. 2001, Olivieri et al. 2001). In fact -174 C/G transitions have been associated with susceptibility to age-related diseases and may predict longevity (Bonafe et al. 2001, Franceschi et al. 2005, Olivieri et al. 2001). C-allele negative elderly individuals displayed impaired innate immune response, zinc deficiency, enhanced metallothionein expression, and low zinc ion availability compared to C-allele carriers (Giacconi et al. 2004). Taken together, these data suggest that the IL-6 C/G polymorphism may interact with MT to impact on zinc homeostasis.

In ageing, it is possible that enhanced IL-6 production may impact on metallothionein levels, thereby altering zinc availability. In turn, metallothionein homeostasis and regulation of zinc turnover may affect IL-6 production, causing a cycle by which increasing IL-6 expression further alters zinc homeostasis and depletes available zinc. Another relevant aspect to be taken into account is that in the presence of alterations in mechanisms regulating zinc homeostasis, zinc itself might also have side effects on oxidative stress by promoting the production of reactive oxygen species (Frazzini et

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al. 2006). Therefore, genetic variability at the IL6 -174 C/G locus may be useful for both screening of elderly subjects who are at risk for zinc deficiency and impaired immune response and also in identifying subjects that are more or less able to regulate zinc homeostasis (Mocchegiani et al 2006).

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Materials and Methods

Human Subjects and Sample Collection

Sixteen elderly subjects (age range 69-79 years) were selected for study participation. Elderly subjects were recruited in accordance to immunogeronotological 'SENIEUR' protocol (Ligthart 2001). 15 ml whole blood was collected and peripheral blood mononuclear cells (PBMC) were recovered. Cell cultures were prepared in zinc-free RPMI medium containing 10% of heat-inactivated FCS, 4 mM L-glutamine, 100 units/ml penicilin and streptomycin (Invitrogen, San Giuliano Milanese, MI, Italia). Cells were incubated for 24 h (37°C, 5% CO_2) with addition of zinc 50 μM (zinc-enriched medium). At the end of the 24 h of culture, cells were recovered, washed three times with RPMI medium, and counted. An aliquot of 2 x 10^6 cells was used for the determination of intracellular available zinc and MT expression by flow cytometry. Another aliquot (3-4 x 10^6 cells) was frozen and preserved for microarray analysis. The research protocol was approved by the Ethical Committee of INRCA (Ancona, Italy).

Zinc ion availability in lymphocytes and monocytes

The intracellular release of zinc from lymphocytes and monocytes and total metallothionein protein expression in PBMC were assessed by flow cytometry as previously described (Cipriano et al. 2006, Malavolta et al. 2006). Zinc ion availability is reported as the mean fluorescence detected with 20μM Zinpyr-1 normalized to the minimum fluorescence obtained after the addition of 50 μM N,N',N'-tetrakis (2-pyridylmethyl)ethylenediamine (TPEN) (Sigma–Aldrich, MI, Italy). Release of zinc is reported as the fluorescence increment obtained with 500μM

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of dyethilamine Nonoate acetoxymethylated (AcOM-DEA/NO, Calbiochem, VWR International s.r.l., Milan, It) normalized to the minimum fluorescence.

Metallothionein protein expression

Relative cellular fluorescence, reflecting cellular MT levels, was determined using flow cytometry as described previously (Malavolta et al. 2006, Yurkow et al. 1998). Samples probed with an irrelevant (isotypic) antibody (clone MOPC21, IgG1k, Sigma–Aldrich, MI, Italia) in conjunction with the fluorescein-conjugated secondary antibody served as staining controls. The MT-specific fluorescence of the un-gated population was determined by subtracting the mean fluorescence intensity obtained with the isotypic antibody from the one obtained with the MT-specific antibody.

Genetic Screening

Two single-nucleotide polymorphism (SNPs), IL-6 -174 G/C (rs1800795) and MT1a +647 A/C (rs11640851) were screened. The MT1a SNP corresponded to an amino acid transition from asparagine in the MT1a-A (adenine) to threonine in the MT1a-C (cytosine). PCR-restriction fragment length analysis (PCR-RFLP) for this SNP was performed using the following sense 5'- ACTTGGCTCAGCCCCAGATT-3' and antisense 5'-CACTCAGCTGGCAGCAT-TTG-3' primers.

Genomic DNA from peripheral blood was extracted with phenol/chloroform. Amplification reactions consisted of 25 µl of PCR mixture was composed of genomic DNA samples (200 ng), 300 µmol/L dNTPs, 2 mM $MgCl_2$, 1X Taq polymerase buffer, 10 pmol of each primer (as outlined above), 5% of DMSO, 2.5 U of Taq DNA polymerase (Eppendorf, Germany). PCR conditions were 94°C for 4 min; 40 cycles

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were at 94°C for 1 min, 60°C for 30 sec, 72°C for 1 minute and a final extension at 72° for 4 min. PCR products were digested with Mnl-I at 37°C overnight (Roche, Germany), then separated on 3% agarose gel and stained with ethidium bromide. The presence of adenine (A allele) at locus 647 within the coding region of MT1A produced one fragment of 187 base-pairs (bp), whereas the presence of cytosine (C allele) generated two fragments of 140 bp and 47 bp after Mnl-I digestion.

The G/C transition at locus –174 in the promoter region of IL-6 gene was evaluated using the following sense 5'-TTGTCAAGACATGCCAAGTGCT- 3' and antisense : 5'-GCCTCAGAGACATCTCCAGTCC-3' primers. 25 µl PCR reactions consisted of genomic DNA samples (200 ng), 200 µmol/L dNTPs, 2.25 mM $MgCl_2$, 1X Taq polymerase buffer, 1X Taq polymerase buffer, 0.5 µM of each primer, 2.5 U of Taq DNA polymerase (Eppendorf, Germany). Amplification conditions were 30 cycles of 95° for 30 sec, 62° for 30 sec ,72° for 1.5 min. The PCR products were digested at 37°C overnight with the enzyme NlaIII (New England Biolabs UK. as indicated by the manufacture's procedure then separated on 3% agarose gel and stained with ethidium bromide. The presence of C allele produced two fragments of 175 and 120 bp whereas G allele produced one fragment of 242 bp.

RNA Isolation

Total RNA was isolated from PBMC using TRIzol (Invitrogen, UK). The integrity of the RNA was confirmed with analysis by the Agilent 2100 bioanalyser (Palo Alto, CA, USA using the RNA 600 LabChip™ kit.

Target RNA Preparation, Hybridisation, and Microarray Analysis

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The human oligonucleotide library was purchased from Sigma-Compugen (Oligo library, release 1.0, Jamesburg, NJ, USA) with a further additional set of 700 oligonucleotides custom synthesised according to the manufacturer's standard design guidelines. Oligonucleotides were printed onto Codelink slides (GE Healthcare, Amersham, UK) according to manufacturer's guidelines using a Biorobotics TAS microarray system. Probe labelling was performed using the Genisphere 350HS labelling system (Hatfield, PA, USA) according to the manufacturer's standard protocol. Reverse transcription was performed using Powerscript RT (BD Biosciences, Oxford, UK) with a final dNTP concentration of 0.5mM each and using the 5pMol/ul modified oligo dT primer provided (Genisphere, Hatfield, PA, USA). Hybridisation was performed using a Lucidea SlidePro (GE Healthcare, Amersham, UK). Dendrimer hybridisation was performed with dextran concentration reduced to 2.5% final (w/v), hybridisation time was reduced to 4hrs at 50°C. Scanning was performed using a Perkin Elmer Scanarray 4000XL scanner (Buckinghamshire, UK) for both Cy3 and Cy5. Image analysis and data extraction was performed using Bluefuse V3.2 (Bluegnome Ltd, Cambridge, UK). Manual exclusion was further applied to remove outliers due to contamination.

Bioinformatics Analysis

Data sets were analysed using GeneSpring v7.2 (Silicon Genetics, CA, USA). Loess normalisations were applied to all data sets. A subset of genes for data interrogation was generated that excluded controls, spots of poor quality, and gene probes that were present in less than 50% of samples. From these selected genes, relative expression compared to untreated cells was determined and genes differentially expressed by a minimum of two-fold in the presence of zinc were determined. One-way, parametric,

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ANOVA tests were performed followed by Benjamini and Hochberg multiple test correction with a false discovery rate of 0.05. Microsoft Excel templates were prepared for each of the genotypes containing genes that were over- and under-expressed following zinc treatment. Ingenuity™ Pathway Analysis 3.0 was utilised to assemble functional networks altered by zinc status (Ingenuity™ Systems, CA, USA).

Real-time RT-PCR

Taqman (Applied Biosystems, Applera, UK) probes were used for RT-PCR (IL6: Hs00174131_m1; FABP-4: Hs00609791_m1; leptin: Hs00174877_m1; IL-1β: Hs00174097_m1; IL-1R2: Hs00174759_m1; GAPDH: Hs99999905_m1). RNA was prepared as before and *in vitro* transcription was performed using 1µg total RNA and random hexamer primers (Invitrogen, Paisley, UK). The Bio-rad I-Cycler (Bio-rad, Hercules, CA) with FAM-490 system detection was used for real time RT-PCR. PCR thermocycler conditions were 50°C for two minutes, 90°C for two minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Samples were run in triplicate with test probes and the control gene human GAPDH to control for differences in amount of starting material. Fold-changes (compared to untreated cells) were calculated by normalising the test crossing threshold (Ct) with the GAPDH amplified control Ct.

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Results

In order to examine the effect of zinc supplementation on gene expression profiling in elderly individuals, RNA samples were obtained from PBMC isolated from elderly donors selected on the basis of IL-6 -174 and MT1a +647 genotype and treated *in vitro* with normal media or media supplemented with zinc for a period of 24h. Donor information is shown in **Table 1**. Gene expression profiling of cells cultured in the presence of zinc was compared to cells cultured in the absence of exogenous zinc. Gene array analysis identified 61 genes that were differentially expressed in the presence of zinc. Of these, 17 were up-regulated and 44 down-regulated. To confirm that these genes identified were truly differentially expressed, stringent data analysis was performed. After exclusion of genes with missing spots, spots of poor quality, genes below detection threshold, and ANOVA testing, these 61 genes were considered to be truly differentially expressed.

In order to gain an understanding of which functional cellular processes may be most affected by exogenous zinc treatment, the 61 genes that were significantly altered in the presence of zinc were visualised by Ingenuity Pathway Analysis™. Ingenuity entry tool systematically encodes findings presented in peer-reviewed scientific publications into ontologies, or groups of genes /proteins related by common function. A molecular network of direct physical, transcriptional, and enzymatic interactions was computed from this knowledge base. The resulting network contains molecular relationships with a high degree of connectivity and every gene in the network is supported by published information. Genes identified through microarray analysis (focus genes), network score (probability of network being assembled by chance alone), and main cellular functions of the network are listed in **Figure 1a**. The two

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statistically significant molecular networks assembled using Ingenuity Pathway Analysis (IPA) profiling of genes differentially expressed in the presence of zinc, including interacting partners, are depicted in **Figure 1b and Figure 1c,** respectively. Genes demonstrated to be up-regulated or down-regulated in the presence of zinc are coloured in red and green, respectively. Class of function for each of the genes, including enzymes, growth factors, and receptors, are depicted by nodal shape, as identified in the figure legend. Interestingly, IPA identified interactions between IL-6 (gene expression decreased by zinc treatment) and metalloproteases (MMP9, MMP19) and pro-inflammatory mediators (IL1R2, CXCL3), all of which were down-regulated following zinc treatment. IPA further identified zinc-dependent down-regulation of several obesity-related genes: leptin, NR1H3 (LXR-α) and fatty acid binding protein 3 (FABP3). These data suggest that zinc may act as an important anti-inflammatory and anti-obesity agent. These results are consistent with the known age-dependent increases in pro-inflammatory status and fat mass.

In order to test the hypothesis that variability in IL-6 -174 and MT1a +647 loci influences the effects of zinc on the genomic level, donors were stratified by IL-6 and MT1a genotype. Individuals were determined to be either one of three genotypes: (1) MT1a +647 C-allele-/IL-6 -174-C-allele-positive carriers (C+C+), (2) MT1a +647 C-allele-positive/IL-6 -174-C-allele negative carriers (C+C-) or C-allele negative in both MT1a +647 and IL-6 -174 loci (C-C-). These three genotypes were distributed evenly throughout the donor population analysed, as shown in **Table 1**. The MT1a +647 C-allele-negative/IL-6 -174-C allele positive carriers (C-C+) were not considered for analysis due to high variability in gene expression in this group (data not shown for n=6 individuals) which we were not able to address by recruiting more individuals

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with this genotype due to the relatively low prevalence of these individuals within the population recruited. Gene expression data for each individual donor were grouped into three genotypes for analysis. For each of the three genotypes, average gene expression for each expressed gene was determined in the presence and absence of exogenous zinc. The number of genes which were differentially regulated by zinc as determined for each genotype is shown in **Figure 2a**. All of the 26 genes that were regulated by zinc (15 up-regulated, 11 down-regulated) in C+C+ donors were unique to this genotype. However, of the 45 and 38 genes that were regulated by zinc in the C+C- and C-C- donors, respectively, 24 genes were commonly regulated in these two genotypes. Five of these zinc-dependent IL-6 C-allele negative-regulated genes, IL-6, IL-1, IL-1R2, leptin, and FABP-4, listed in **Figure 2b** with associated p-values (compared to non-treated controls). These five genes were selected for confirmation of differential expression by real time RT-PCR. Real time RT-PCR confirmed that these genes were differentially regulated in individuals lacking the IL-6 -174C-allele following zinc treatment, as shown in **Figure 2c**. In addition to differential regulation of these individual genes, comparison analysis of the response of each of three genetic groups to zinc supplementation using Ingenuity™ tool identified several inflammatory canonical pathways which were differentially regulated by zinc depending on genetic background. These pathways are shown in **Figure 3**. Following zinc treatment, the IL-10, PPAR, IL-6, p38 MAPK, G-protein-coupled receptor and NFκB signalling pathways were affected in both C-C- and C+C- individuals. None of these pathways were differentially regulated by zinc in C+C+ individuals, supporting the hypothesis that zinc preferentially acts as an anti-inflammatory agent in individuals lacking the IL-6 C-allele. Of the pathways shown, only the pentose phosphate pathway was regulated by zinc in C+C+ individuals in

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addition to C-C- individuals, suggesting this pathway was regulated in a zinc-dependent IL-6/MT-independent manner. The interferon signalling pathway was regulated by zinc in only C-C- individuals, suggesting this pathway be co-ordinately regulated by MT and IL-6 interactions.

In order to assess whether variability in the IL-6 and MT1a loci influences zinc homeostasis or response to zinc supplementation, cellular zinc ion availability, NO-induced release, and metallothionein expression were determined for each genotype before and after zinc treatment, as previously described (Malavolta et al. 2006). Zinc availability (shown as mean normalized fluorescence) in lymphocytes (**Figure 4a**) and monocytes (**Figure 4c**) and NO-induced zinc release (shown as difference in the mean normalized fluorescence after the addition of AcOM-DEA/NO) from lymphocytes (**Figure 4b**) and monocytes (**Figure 4d**), was determined before and after *in vitro* culture with exogenous zinc, for MT1a/IL-6 C+C+, C+C-, and C-C- genotypes. The average total metallothionein protein expression across donors for each genotype as determined by mean fluorescence intensity is shown in **Figure 4e.** While increases were observed in zinc availability in lymphocytes and monocytes, and in metallothionein expression following zinc treatment, no statistically significant difference was observed between the IL-6 and MT1a genotypes. Similarly, there was no significant difference between IL-6 and MT1a C+ and C- carriers in zinc availability, NO-induced release of zinc, or metallothionein expression at the basal level, prior to zinc treatment. We observed no significant genotype-dependent difference between basal- and zinc-stimulated levels of zinc availability, NO-induced zinc release or metallothionein expression. Additionally, in the case of NO-induced zinc release in monocytes and lymphocytes derived from C+C+ carriers, we did not

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observe a significant increase in either parameter following zinc treatment. This was due to a surprising reduction of these parameters in some experimental samples and concomitant increased variability within this group. Lack of increased NO-stimulated zinc release in C+C+ carriers following zinc treatment corresponded with slightly increased basal levels of NO-stimulated zinc release in C+C+ monocytes compared to IL-6 -174 C- carriers, although this was not significant. These data suggest that the most significant responses to zinc treatment on the level of NO-stimulated zinc release in monocytes and lymphocytes occur in IL-6 C- carriers, which is consistent with the increased zinc-dependent gene expression in this group.

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Discussion

In the current study, we investigated whether variability in IL-6 -174 and MT1a +647 loci influenced zinc-regulated gene expression in ageing. The hypothesis was taken that an anti-inflammatory molecule such as zinc might impact positively on IL-6 production in the elderly. Since MT1a polymorphisms are associated with increased IL-6 production and could potentially impact on zinc homeostasis, we further segmented the elderly population recruited for investigation based on MT1a polymorphism. It was thought that MT1a genotype may exacerbate the IL-6 phenotype. By combining these two genetic polymorphisms we were able to identify an elderly sub-population who responded significantly to zinc treatment through modulation of inflammatory and metabolic gene expression profiles. This approach offers substantial benefit over attempting zinc intervention in a non-segmented population as many of the general ageing population are zinc sufficient. It is hoped that using nutrigenomic or nutrigenetic approaches to identify sub-populations who have the greatest susceptibility to nutritional deficiencies will aid in selecting individuals who will be afforded the greatest health benefits and will additionally provide the greatest probability of a successful nutritional intervention trial.

Of the genes that were differentially regulated on the basis of IL-6 genotype, the most interesting belong to either the IL-6 signalling pathway itself or are involved in regulation of energy balance/obesity, including IL-6, IL-1R2, IL-1, leptin, and FABP4. These data are consistent with the established links between serum IL-6 levels, IL-6 promoter polymorphisms and obesity (Klipstein-Grobusch et al. 2006; Qi et al. 2007; Wernstedt et al. 2004). Zinc supplementation has previously been shown to improve glycemic control in db/db and ob/ob mice (Begin-Heick et al. 1985, Simon

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and Taylor 2000). In fact, zinc-deficient mice have multiple diabetogenic traits including impaired glucose tolerance, insulin resistance and impaired insulin signalling (Cunnane et al. 1984, Gomot et al. 1992). A study carried out in obese children associated lower zinc serum concentrations with abdominal obesity (Beattie et al. 1998, Marreiro et al. 2004). Conversely, zinc supplementation has been demonstrated to increase insulin secretion and to improve glucose control in db/db and ob/ob mice (Begin-Heick et al. 1985, Simon and Taylor 2000).

In addition to associations between zinc status and obesity, an interaction between dietary zinc and adiposity was implicated in MT-null mice (Beattie et al. 1998). MT-deficient animals are obese, have elevated plasma leptin concomitant with adipose leptin mRNA expression compared to controls, and pronounced alterations in zinc homeostasis, suggesting that disrupted zinc availability may lead to alterations in energy regulation (Beattie et al. 1998). Furthermore, the obesity associated with these animals is characterised by elevated circulating leptin, a hormone that is produced by adipocytes (Rebuffe-Scrive et al. 1993, Van Heek M et al. 1997). Here we show that the addition of exogenous zinc led to increased MT expression concomitant with decreased leptin gene expression in IL-6 C-allele negative individuals. Wernstedt et al. have previously demonstrated that the IL-6 -174 C allele was associated with higher BMI and higher serum leptin levels (Wernstedt et al. 2004). Since several adipose-derived cytokines including leptin, tumor necrosis factor-α, and IL-6 are up-regulated in obesity and contribute to the development of diabetes and vascular disease, the findings that zinc could potentially decrease expression of these molecules is important for obesity-related conditions. Taken together, these data suggest that zinc intervention may be useful to restore normal levels of leptin and IL-6

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and could potentially impact positively on adipose mass in individuals with pro-inflammatory, obese phenotypes.

In the current study, we demonstrated that zinc treatment resulted in decreased FABP4 gene expression. Differential regulation of FABP4 has previously been demonstrated in zinc-deprived rats (Blanchard et al. 2001), suggesting this is a conserved effect that is observed *in vivo*. FABPs constitute a family of cytosolic lipid chaperones that share a conserved structure but exhibit a distinct, tissue-specific expression pattern (Haunerland and Spencer 2004). FABP4 (or A-FABP) is expressed predominantly in adipocytes and macrophages (Hotamisligil et al. 1996). Recent studies have demonstrated that FABP4 is important in systemic metabolic homeostasis. FABP4-deficient mice are partially protected against obesity-induced insulin resistance, type 2 diabetes, and hypercholesterolemic atherosclerosis in both dietary and genetic models (Makowski and Hotasmisligil 2004, Uysal KT et al. 1997). One elegant recent study performed by this same group examined the effect of leptin on FABP4/5 -/- mice (Cao et al. 2006). In the absence of leptin and despite severe obesity, these mice maintained euglycemic control and demonstrated improved peripheral insulin sensitivity. These data indicate that reductions in leptin and FABP4 by dietary zinc in IL-6 C-allele negative individuals may protect against insulin resistance and suggest an important mechanism by which zinc may protect against development of type 2 diabetes.

In addition to its role in the regulation of energy balance and obesity, evidence obtained recently indicates that FABPs may serve as master regulators in the control of cellular inflammatory responses through regulation of JNK and PPARγ (Hirosumin

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et al. 2002, Gao et al. 2002). These observations further establish the relationship between cellular stress responses, inflammatory cytokine production and obesity. Firstly, FABP4 may co-ordinate the lipid-mediated activation of stress kinases such as JNK or IkK under immune or metabolic stimuli thus linking lipid signalling to pro-inflammatory signalling and anti-insulin action (Hotamisligil et al. 1996). It has been hypothesised that the presence of FABP4 in periods of feast and famine may have been beneficial to maintain a strong immune response and adipose tissue in fitting with the concept of 'thrifty' phenotype to survive (Auwerx, Cock, and Knouff 2003). However, with increasing prevalence of excessive caloric intake, decreased energy expenditure and high stress lifestyle, FABPs may not be able to maintain inflammatory or metabolic homeostasis and the presence of these proteins may actually aid in the formation of obesity, dyslipidemia and inflammatory responses (Hotamisligil et al. 1996). Combined with the prevalent increased pro-inflammatory IL-6 production in elderly individuals, these alterations in inflammatory homeostasis have serious and detrimental implications for health in ageing populations.

In conclusion, the observations that zinc may alleviate the increased pro-inflammatory/pro-atherogenic status of elderly individuals lacking the IL-6 C- allele suggest that this population would receive increased health benefits from zinc supplementation compared to the general population. Because high levels of circulating pro-inflammatory cytokines are prevalent in both the general ageing population and due to the increased prevalence of obesity in the non-aged population, it remains feasible that zinc supplementation may also improve health status in these populations. Further phenotype-specific interventions (e.g. in obese individuals with high levels of adipokine secretion) are needed to test these hypotheses.

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Author contributions

DJM analysed microarray data, performed RT-PCR, and wrote the manuscript; AJW performed the microarrays; MM performed metallothionein, zinc availability and zinc release analyses. LC, RG, E Muti and CC recruited subjects, performed prospective genotyping, collected blood, isolated PBMC, and cultured and treated cells. JRP and E Mocchegiani supervised and designed the study.

The authors declare no competing interests.

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566 **Tables.**

Donor ID	Gender	Age	Genotype MT1a +647 A/C	+647	Genotype IL-6 -174 C/G	-174
297	M	72	AA	C-	GG	C-
271	F	73	AA	C-	GG	C-
260	F	72	AA	C-	GG	C-
259	M	74	AA	C-	GG	C-
286	F	75	AA	C-	GG	C-
1396	M	74	AC	C+	CC	C+
1399	M	74	AC	C+	GC	C+
143	M	77	AC	C+	GC	C+
188	M	79	AC	C+	GC	C+
1344	F	78	AC	C+	CC	C+
307	M	78	AC	C+	GG	C-
112	M	72	AC	C+	GG	C-
48	M	72	AC	C+	GG	C-
63	F	75	AC	C+	GG	C-
65	F	75	AC	C+	GG	C-
173	M	69	AC	C+	GG	C-

Table 1: Donor sex, age, IL-6 and MT1a genotype, and frequency of the C-allele (C+ or C-) for each locus are shown.

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Figure Legends

Figure 1: Ingenuity™ networks of genes differentially expressed in PBMC derived from elderly donors cultured in the presence of zinc. The 61 genes that were differentially regulated by zinc were analysed by the Ingenuity™ Pathway Analysis tool. Two genetic networks were found to be significantly regulated by zinc ($p<0.001$). The network genes identified through microarray analysis (focus genes, shown in bold), network score, and main cellular functions of the network are listed (a). The networks shown were significantly associated with cellular growth and proliferation, cancer, and cellular movement (b) and immune response, cellular growth and proliferation and cellular morphology (c). Coloured genes were identified by microarray analysis as differentially up-regulated or down-regulated in the presence of zinc (red and green colouring, respectively). Other nodal genes are directly or indirectly associated with the differentially expressed genes and were either not spotted on the array or not found to be differentially expressed following zinc treatment. The meaning of the nodal shapes is indicated in the figure legend.

Figure 2: Genes differentially expressed in MT1aC+/IL-6 C+, MT1aC+/IL-6 C-, MT1aC-/IL-6 C- donors in the presence of zinc. The number of genes differentially expressed in PBMC segmented by variability in the IL-6 and MT1a loci, following addition of exogenous zinc is shown (a). Zinc-dependent differential expression of five selected genes (IL-6, IL-1, IL-1R2, leptin and FABP4) of the 24 genes commonly regulated in MT1a C+/IL-6 C-donors and MT1a C-/IL-6 C- by microarray analysis is shown, with associated p-value compared to non-treated controls (b). Differential expression of IL-6, IL-1, IL1R2, leptin and FABP4 following zinc treatment was determined by real-time RT-PCR (c). RNA was

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prepared and *in vitro* transcription was performed using 1µg total RNA and random hexamer primers (Invitrogen, Paisley, UK). The Bio-rad I-Cycler (Bio-rad, Hercules, CA) with FAM-490 system detection was used for real time RT-PCR. Taqman (Applied Biosystems, Applera, UK) probes were used as detailed in *Subjects and Methods*. Samples were grouped by genotype MT1a/IL-6- C+C+ (black bars), - C+C- (grey bars), and - C-C- (white bars) and analysed before and after 24h zinc supplementation. Samples were run in triplicate with test probes and the control gene human GAPDH to control for differences in amount of starting material. Fold-changes were calculated by normalising the test gene crossing threshold (Ct) with the GAPDH amplified control. Fold-change values were normalised to gene expression in non-treated controls. *denotes $p<0.05$; ** denotes $p<0.005$; ***denotes ;<0.0005 by ANOVA (compared to non-treated controls).

Figure 3: Canonical pathways differentially expressed in PBMC derived from elderly donors following zinc treatment. Genes that were differentially expressed in PBMC isolated from elderly donors stratified by MT1a/IL-6 haplotype following zinc treatment were clustered into canonical pathways using Ingenuity™ tool. The relative proportion of genes (y-axis) differentially expressed in each canonical pathway (Interferon signalling, IL-10 signalling, PPAR signalling, Pentose phosphate pathway, IL-6 signalling, p38 MAPK signalling, G-protein coupled receptor signalling, and NFkB signalling) following zinc treatment (x-axis) is demonstrated for each of the three genotypes: MT1a/IL-6 C+C+ (black bars), MT1a/IL-6 C+C- (dark grey bars), and MT1a/IL-6 C-C- (white bars). No bar represented depicts no haplotype-specific affect of zinc on the pathway.

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Figure 4: Zinc availability, NO-induced release of zinc, and metallothionein expression in elderly PBMC following zinc treatment. Zinc ion availability and NO-induced release of zinc were evaluated by flow cytometry in PBMC isolated from elderly donors (stratified by IL-6/MT-1allele carriers) before (0h) and after (24h) zinc treatment. Zinc ion availability in lymphocyte (a) and monocyte (c) sub-population selected by FW and SS scatter was calculated as the mean normalized fluorescence intensity (Fn). Fn was calculated by the ratio of the mean fluorescence intensity and the minimum mean fluorescence intensity detected after the addition of the zinc chelator TPEN. The NO-induced release of zinc [Fn(NO) – Fn)] in lymphocyte (b) and monocyte (d) selected by FW and SS scatter was calculated by subtracting Fn to the mean normalized fluorescence obtained after incubating PBMC for 30' with AcOM-DEA/NO. Data represent mean ± SD (n = 5 for C+C+ and C-C- carriers and n = 6 for C+C- carriers). Metallothionein protein expression was evaluated by Flow Cytometry in PBMC isolated from elderly donors (stratified by IL-6/MT1allele carriers) before (0h) and after (24h) zinc treatment (e). The MT-specific mean fluorescence intensity (MFI) was determined by subtracting the fluorescence obtained with an isotypic antibody (clone MOPC21, IgG1k, Sigma–Aldrich, MI, Italia) from the one obtained with the MT-specific antibody (monoclonal mouse anti-horse metallothionein clone E9, from DAKOCYTOMATION, Denmark). Data represent mean ± SD (n = 5 for C+C+ and C-C- carriers and n = 6 for C+C- carriers).* denotes $p < 0.05$; ** denotes $p < 0.01$ by ANOVA.

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Fig. 1a

Genes	Score	Focus Genes	Top Functions
AGT, **BUB1**, CAST, CMA1, **CXCL3**, EDG2, EDG4, EDG7, EGR2, HOXB7, **IL-6**, IL-8, **IL-24**, IL8RAP, **IL1R2**, **INDO**, **ITGB3**, **KITLG**, **LEP**, **MMP9**, **MMP19**, **NXPH4**, OSBPL1A, **P2RY6**, PGF, PNPT1, PROS1, PTGER1, SC5D1, SDHC, **SLC14A2**, SLC16A1, TFF2, **IFPI2**	27	15	Cellular growth and proliferation; Cancer; Cellular movement
ACH3, APLP1, APP, ARG1, B1D, CCL20, CEBP1, CST3, **CTSL**, CXCL5, **EGLN1**, **FABP4**, FN1, HAMP, HIF1A, IFIT1, IFIT1L, **IFNAR1**, IFNB1, **INDO**, **ITGB8**, LCN2, MPO, **NR1H3**, **PGD**, PGF, **SERPINB2**, **SERPINB8**, **SERPINB9**, **SPON1**, TIRAP, TNC, TNF, **TOR1A**, WNT10B	23	13	Immune response; Cellular growth and proliferation; Cellular morphology
Focus genes are shown in bold. A score of >3 was considered statistically significant (p<0.001).			

Fig. 1b

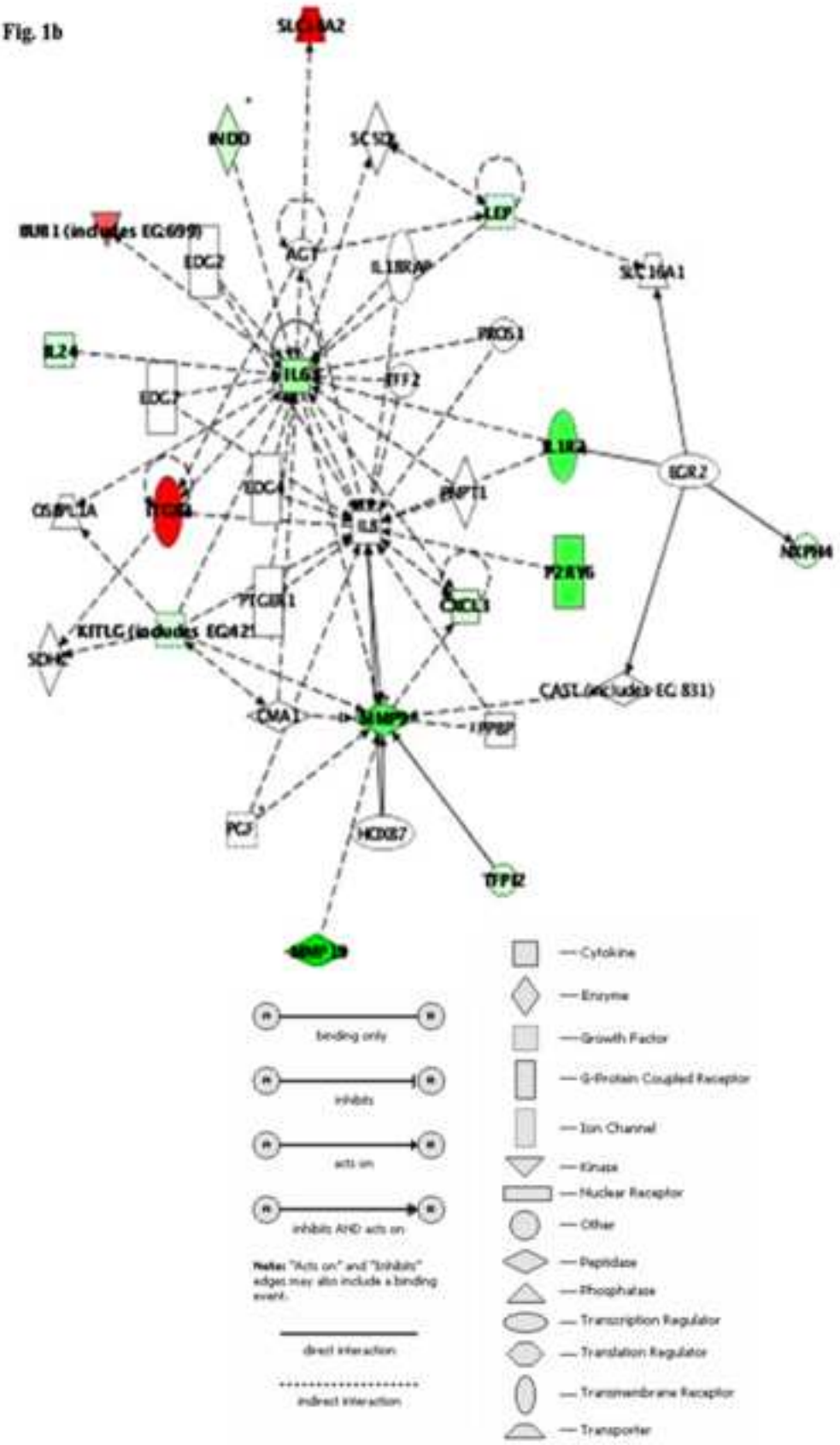
SLC16A2
INDO
SCS2
LEP
BUB1 (includes EG:699)
EDC2
AGT
IL18RAP
SLC16A1
PRCS1
IL24
IL6
TTF2
EDC7
IL1R2
ECR2
EDC4
PAPT1
OSBPL1A
IL5
NEPH4
P2RY6
PTGER1
CXCL3
KITLG (includes EG:42)
SDHC
CASL (includes EG:831)
CMA1
MMP14
IPPBP
PCJ
HCDH27
TFR2
Cytokine
Enzyme
Growth Factor
G-Protein Coupled Receptor
Ion Channel
Kinase
Nuclear Receptor
Other
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
binding only
inhibits
acts on
inhibits AND acts on
Note: "Acts on" and "Inhibits" edges may also include a binding event.
direct interaction
indirect interaction

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Fig. 1c

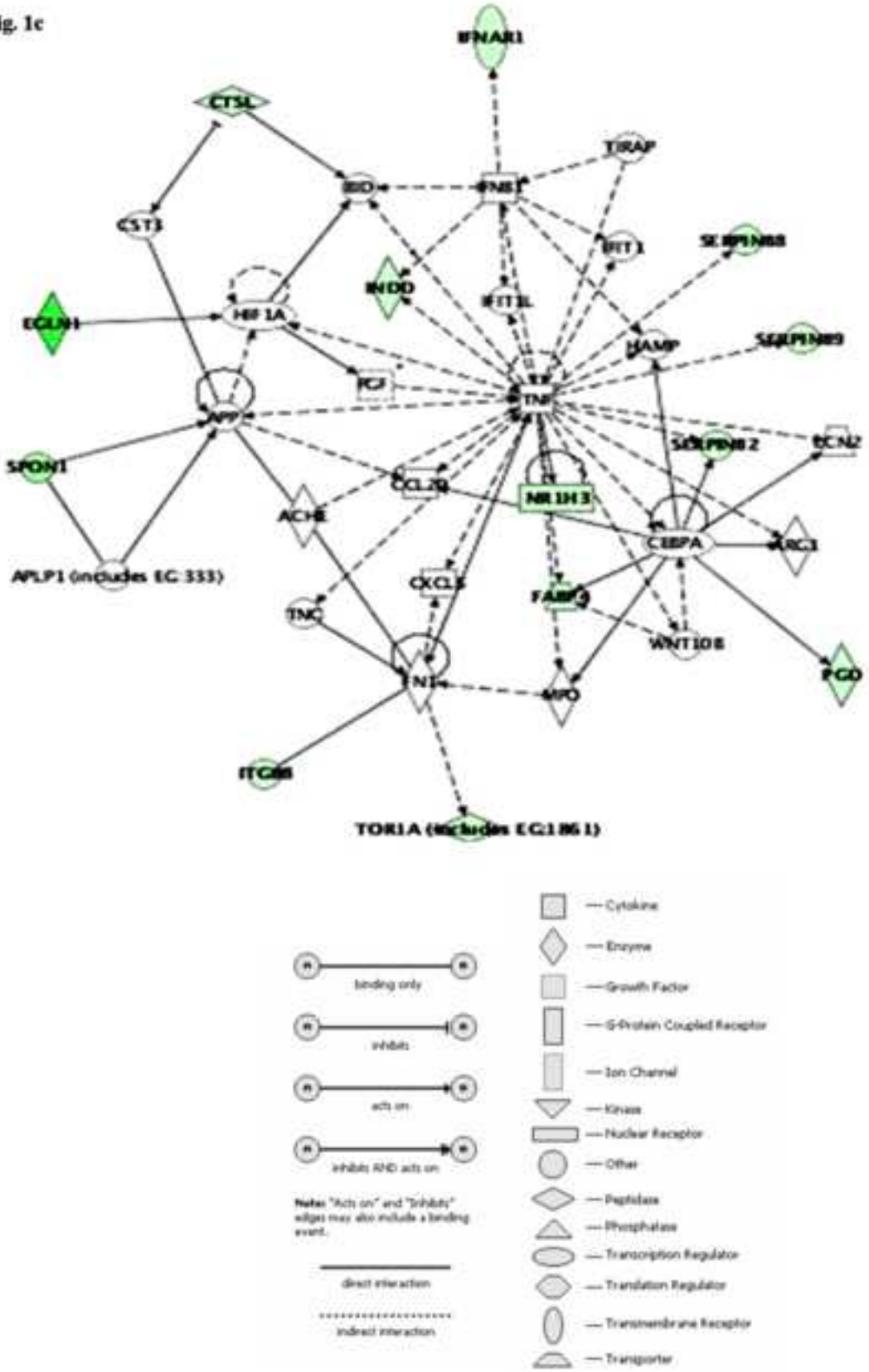

Fig. 2

a.

Genes altered in PBMC derived from elderly donors cultured in the presence of zinc			
Genotype	MT1a C+/IL-6 C+ (C+C+)	MT1a C+/ IL-6 C- (C+C-)	MT1a C- /IL-6 C- (C-C-)
Up-regulated	15	15	10
Down-regulated	11	30	28
Total	**26**	**45**	**38**

b.

Gene Name	p-value	
	MT1a C+/IL-6 C-	MT1a C-l/IL-6 C-
IL-6	0.07	0.03*
IL-1	0.03*	0.01*
IL-1R	0.002**	0.00006***
Leptin	0.08	0.04*
FABP-4 (adipose)	0.09	0.05*
*** Denotes p<0.05, **p<0.005, ***p<0.0005**		

c.

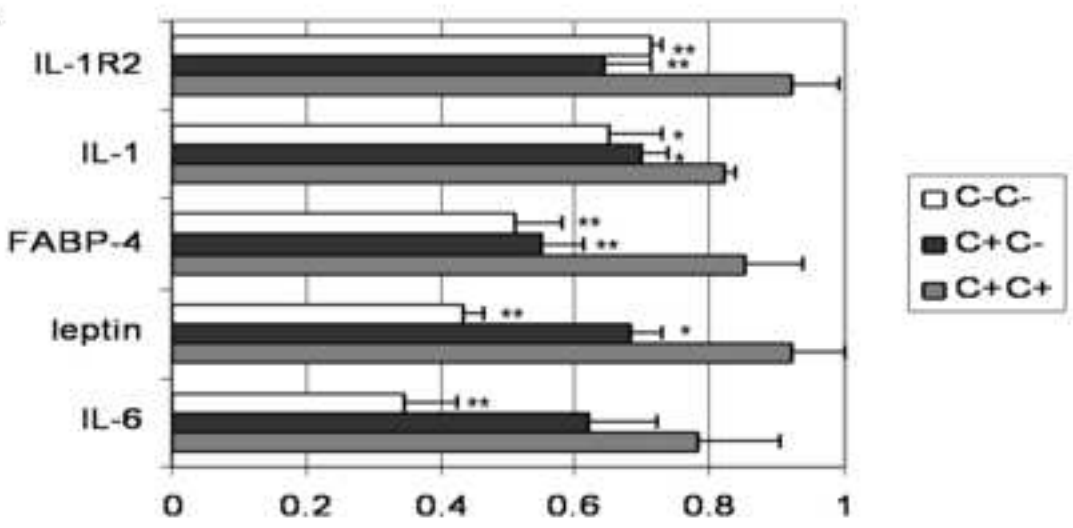

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Fig. 3

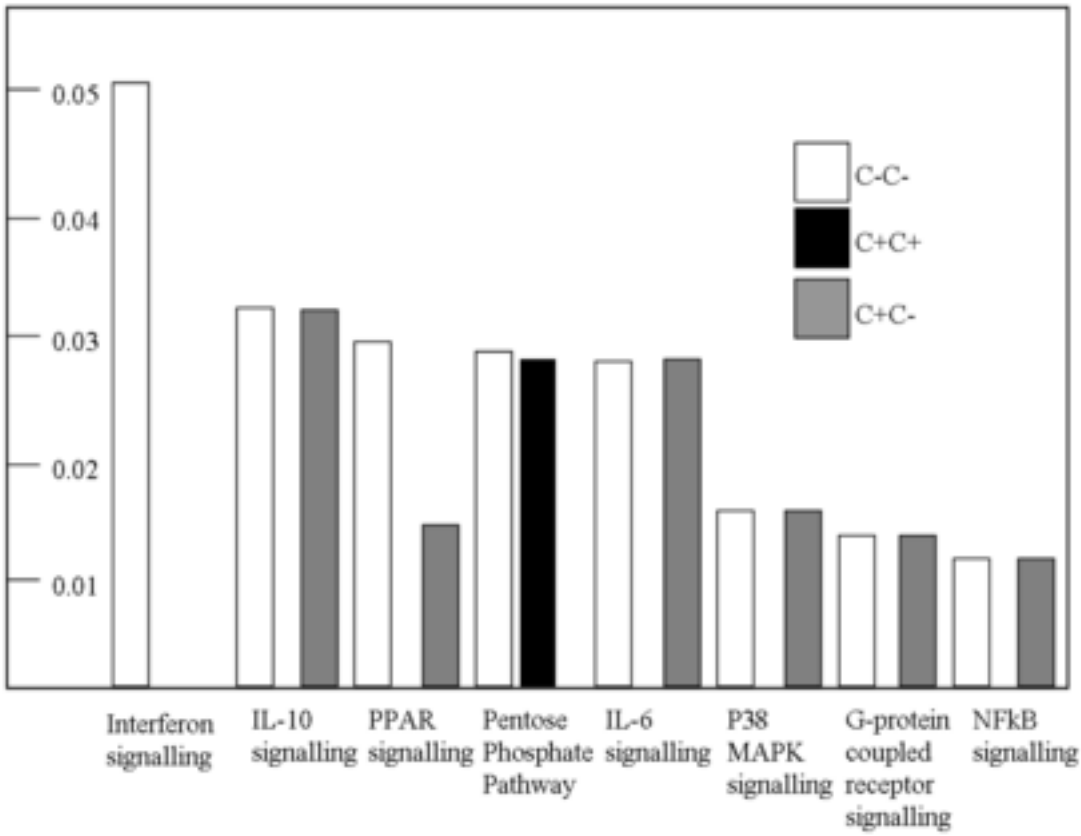
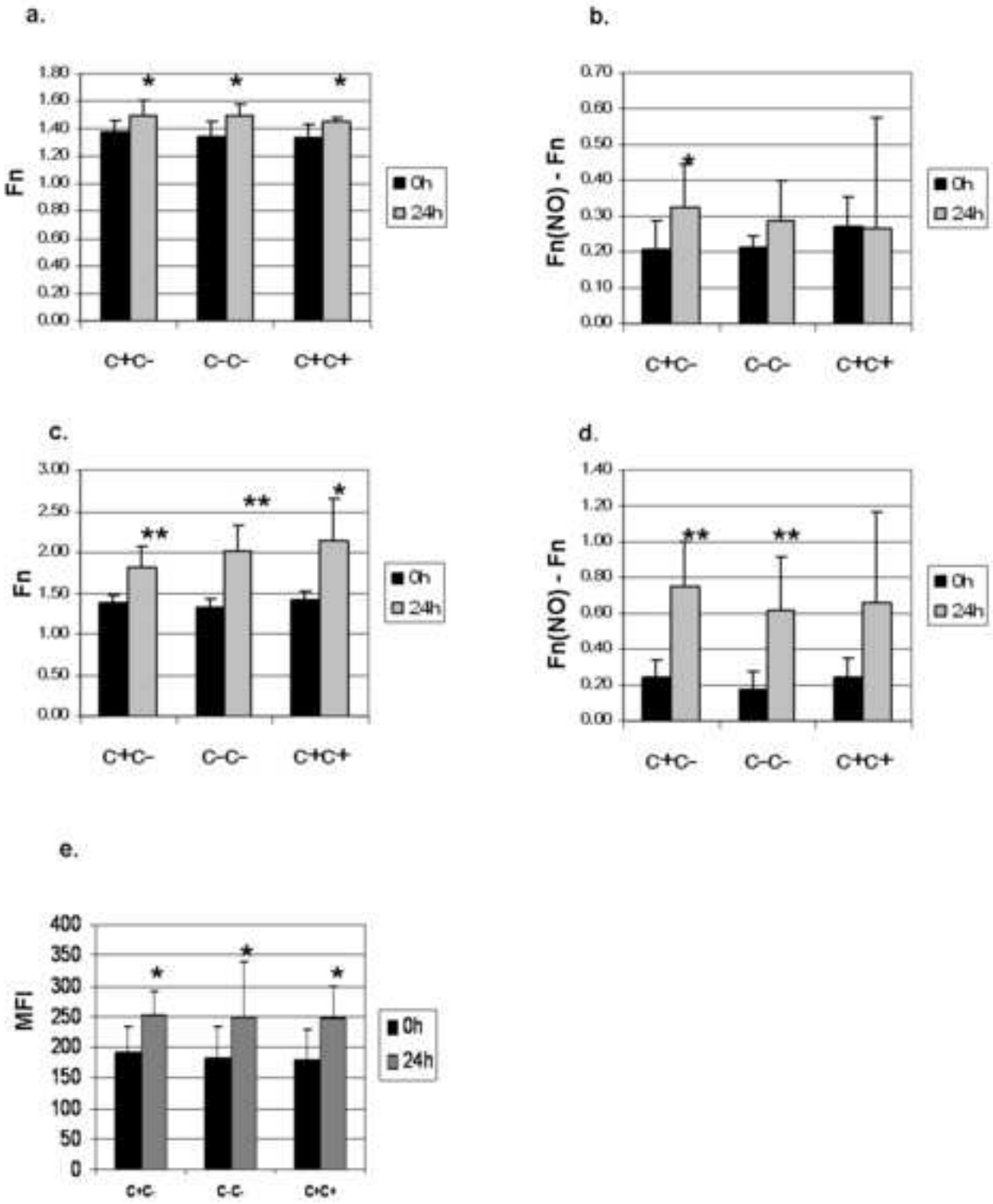


Fig. 4


a.
Fn
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
C+C-
C-C-
C+C+
0h
24h
b.
Fn(NO) - Fn
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
C+C-
C-C-
C+C+
0h
24h
c.
Fn
3.00
2.50
2.00
1.50
1.00
0.50
0.00
C+C-
C-C-
C+C+
0h
24h
d.
Fn(NO) - Fn
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
C+C-
C-C-
C+C+
0h
24h
e.
MFI
400
350
300
250
200
150
100
50
0
C+C-
C-C-
C+C+
0h
24h