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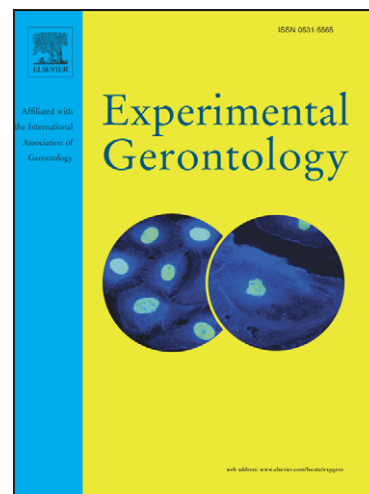
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4 1 **Effects of interleukin-6 -174C/G and metallothionein 1A +647A/C single-**
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6 **nucleotide polymorphisms on zinc-regulated gene expression in ageing.**
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10 4 **Mazzatti, D.J.¹, Malavolta, M.², White, A.J.³, Costarelli, L.², Giacconi, R.², Muti,**
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45 20
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27 **Abstract**

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29 Decreased zinc ion availability in ageing is associated with altered immune response.

30 One of the main regulators of zinc availability is metallothionein. Metallothionein

31 induction is under the control of interleukin-6, a pro-inflammatory cytokine whose

32 production is associated with poor ageing. The production of interleukin-6 is

33 controlled, in part, by variability in the -174 nucleotide position. Under conditions of

34 chronic inflammation, such as in ageing, zinc release by metallothionein is limited

35 and may reduce zinc availability. Understanding the precise nature of the interactions

36 between interleukin-6 and metallothioneins will aid in identifying individuals who are

37 at risk of zinc deficiency. In the current study, we used gene arrays to investigate the

38 effects of *in vitro* zinc supplementation on gene expression in elderly donors with

39 described interleukin-6 and metallothionein 1a polymorphisms. Ingenuity Pathway

40 Analysis™ identified several zinc-responsive genetic networks uniquely regulated

41 only in elderly individuals with the pro-inflammatory interleukin-6 polymorphism.

42 These include zinc-dependent decreased transcription of pro-inflammatory cytokines

43 and alterations in metabolic regulatory pathways. The genomics effects of zinc

44 increased in significance in the presence of the metallothionein 1a +647 C/A

45 transition, suggesting that the interleukin-6 and metallothionein 1a genes act in a

46 concerted manner to control zinc-regulated gene expression.

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4 **47 Introduction**
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7 48 In human ageing, zinc bioavailability is diminished resulting in impaired immune
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9 49 response and anti-oxidant activity (Mocchegiani et al. 1998). Metallothioneins (MTs)
10
11 50 play an important role in regulating zinc homeostasis due to their high affinity for
12
13 51 zinc. An important function of MTs is in releasing zinc in response to
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15 52 oxidative/nitrosative stress in order to up-regulate the zinc-dependent antioxidant
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17 53 enzymes (Mocchegiani et al. 2006). This response is inadequate in states of chronic
18
19 54 inflammation and in ageing individuals (Mocchegiani et al. 2003).
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24
25 56 A recent report has demonstrated that polymorphisms in the MT coding region are
26
27 57 involved in regulating zinc release by MT and are associated with longevity (Cipriano
28
29 58 et al. 2006). Cipriano et al. have shown that carriers of the C-allele at position +647
30
31 59 of the MT1a gene show enhanced zinc release, low metallothionein levels, and
32
33 60 reduced IL-6 plasma concentrations. In addition, metallothionein gene polymorphisms
34
35 61 have been associated with cardiovascular disease, hyperglycaemia and mild zinc
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37 62 deficiency in diabetic patients (Giacconi et al. 2005). Metallothionein induction and
38
39 63 expression are under the control of IL-6 (Jacob et al. 1999). These data suggest that
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41 64 in the presence of heightened IL-6 production, the MT1a +647 C/A transition may
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43 65 impact negatively on inflammation through up-regulation of IL-6 pro-inflammatory
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45 66 cytokine production, thereby creating vicious pro-inflammatory feed-back cycle.
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48 67 Since increased IL-6 production has been linked to disease and is a poor indicator of
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50 68 survival (Fagiolo et al. 1993, Ferrucci et al 1999), these studies have profound
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52 69 implications for understanding the link between genes, health and longevity.
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4 71 It is critically important to determine the precise nature of the relationship between
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6 72 metallothionein status and IL-6 production. Heightened levels of metallothionein, IL-
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8 73 6 and low zinc ion availability are observed in diseases characterised by chronic
9
10 74 inflammation and in syndromes of accelerated ageing (Mocchegiani et al. 2002). In
11
12 75 addition, high levels of IL-6 production is common in ageing and is detrimental for
13
14 76 successful ageing (Fagiolo et al. 1993, Ferrucci et al 1999). Genetic variation at the -
15
16 77 174 region within the IL-6 promoter affects cytokine production. The presence of the
17
18 78 C allele in the -174 position of the IL-6 promoter decreases the level of IL-6
19
20 79 production. Loss of the C-allele may be associated with risk of disease in ageing and
21
22 80 may predict poor ageing outcome (Bonafe et al. 2001, Olivieri et al. 2001). In fact -
23
24 81 174 C/G transitions have been associated with susceptibility to age-related diseases
25
26 82 and may predict longevity (Bonafe et al. 2001, Franceschi et al. 2005, Olivieri et al.
27
28 83 2001). C-allele negative elderly individuals displayed impaired innate immune
29
30 84 response, zinc deficiency, enhanced metallothionein expression, and low zinc ion
31
32 85 availability compared to C-allele carriers (Giacconi et al. 2004). Taken together,
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34 86 these data suggest that the IL-6 C/G polymorphism may interact with MT to impact
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36 87 on zinc homeostasis.
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45 89 In ageing, it is possible that enhanced IL-6 production may impact on metallothionein
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47 90 levels, thereby altering zinc availability. In turn, metallothionein homeostasis and
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49 91 regulation of zinc turnover may affect IL-6 production, causing a cycle by which
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51 92 increasing IL-6 expression further alters zinc homeostasis and depletes available zinc.
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53 93 Another relevant aspect to be taken into account is that in the presence of alterations
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55 94 in mechanisms regulating zinc homeostasis, zinc itself might also have side effects on
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57 95 oxidative stress by promoting the production of reactive oxygen species (Frazzini et
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4 96 al. 2006). Therefore, genetic variability at the IL6 -174 C/G locus may be useful for
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6 97 both screening of elderly subjects who are at risk for zinc deficiency and impaired
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8 98 immune response and also in identifying subjects that are more or less able to regulate
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11 99 zinc homeostasis (Mocchegiani et al 2006).
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4 **100 Materials and Methods**

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6 **101**
7 **102 Human Subjects and Sample Collection**

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9 **103** Sixteen elderly subjects (age range 69-79 years) were selected for study participation.
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11 **104** Elderly subjects were recruited in accordance to immunogerontological 'SENIEUR'
12
13 **105** protocol (Ligthart 2001). 15 ml whole blood was collected and peripheral blood
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15 **106** mononuclear cells (PBMC) were recovered. Cell cultures were prepared in zinc-free
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17 **107** RPMI medium containing 10% of heat-inactivated FCS, 4 mM L-glutamine, 100
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19 **108** units/ml penicilin and streptomycin (Invitrogen, San Giuliano Milanese, MI, Italia).
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21 **109** Cells were incubated for 24 h (37°C, 5% CO₂) with addition of zinc 50 µM (zinc-
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23 **110** enriched medium). At the end of the 24 h of culture, cells were recovered, washed
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25 **111** three times with RPMI medium, and counted. An aliquot of 2 x 10⁶ cells was used for
26
27 **112** the determination of intracellular available zinc and MT expression by flow
28
29 **113** cytometry. Another aliquot (3-4 x 10⁶ cells) was frozen and preserved for microarray
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31 **114** analysis. The research protocol was approved by the Ethical Committee of INRCA
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33 **115** (Ancona, Italy).
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42 **117 Zinc ion availability in lymphocytes and monocytes**

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44 **118** The intracellular release of zinc from lymphocytes and monocytes and total
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46 **119** metallothionein protein expression in PBMC were assessed by flow cytometry as
47
48 **120** previously described (Cipriano et al. 2006, Malavolta et al. 2006). Zinc ion
49
50 **121** availability is reported as the mean fluorescence detected with 20µM Zinpyr-1
51
52 **122** normalized to the minimum fluorescence obtained after the addition of 50 µM
53
54 **123** N,N',N'-tetrakis (2-pyridylmethyl)ethylenediamine (TPEN) (Sigma-Aldrich, MI,
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56 **124** Italy). Release of zinc is reported as the fluorescence increment obtained with 500µM
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4 125 of diethylamine Nonoate acetoxymethylated (AcOM-DEA/NO, Calbiochem, VWR
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6 126 International s.r.l., Milan, It) normalized to the minimum fluorescence.

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10 128 **Metallothionein protein expression**

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13 129 Relative cellular fluorescence, reflecting cellular MT levels, was determined using
14
15 130 flow cytometry as described previously (Malavolta et al. 2006, Yurkow et al. 1998).
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17 131 Samples probed with an irrelevant (isotypic) antibody (clone MOPC21, IgG1k,
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19 132 Sigma–Aldrich, MI, Italia) in conjunction with the fluorescein-conjugated secondary
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21 133 antibody served as staining controls. The MT-specific fluorescence of the un-gated
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23 134 population was determined by subtracting the mean fluorescence intensity obtained
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25 135 with the isotypic antibody from the one obtained with the MT-specific antibody.
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32 137 **Genetic Screening**

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34 138 Two single-nucleotide polymorphism (SNPs), IL-6 -174 G/C (rs1800795) and MT1a
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36 139 +647 A/C (rs11640851) were screened. The MT1a SNP corresponded to an amino
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38 140 acid transition from asparagine in the MT1a-A (adenine) to threonine in the MT1a-C
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40 141 (cytosine). PCR-restriction fragment length analysis (PCR-RFLP) for this SNP was
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42 142 performed using the following sense 5'- ACTTGGCTCAGCCCCAGATT-3' and
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44 143 antisense 5'-CACTCAGCTGGCAGCAT-TTG-3' primers.
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52 145 Genomic DNA from peripheral blood was extracted with phenol/chloroform.
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54 146 Amplification reactions consisted of 25 µl of PCR mixture was composed of genomic
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56 147 DNA samples (200 ng), 300 µmol/L dNTPs, 2 mM MgCl₂, 1X Taq polymerase buffer,
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58 148 10 pmol of each primer (as outlined above), 5% of DMSO, 2.5 U of Taq DNA
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60 149 polymerase (Eppendorf, Germany). PCR conditions were 94°C for 4 min; 40 cycles
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4 150 were at 94°C for 1 min, 60°C for 30 sec, 72°C for 1 minute and a final extension at
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6 151 72° for 4 min. PCR products were digested with Mnl-I at 37°C overnight (Roche,
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8 152 Germany), then separated on 3% agarose gel and stained with ethidium bromide. The
9
10 153 presence of adenine (A allele) at locus 647 within the coding region of MT1A
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12 154 produced one fragment of 187 base-pairs (bp), whereas the presence of cytosine (C
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14 155 allele) generated two fragments of 140 bp and 47 bp after Mnl-I digestion.
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20 157 The G/C transition at locus -174 in the promoter region of IL-6 gene was evaluated
21
22 158 using the following sense 5'-TTGTCAAGACATGCCAAGTGCT- 3' and antisense :
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24 159 5'-GCCTCAGAGACATCTCCAGTCC-3' primers. 25 µl PCR reactions consisted
25
26 160 of genomic DNA samples (200 ng), 200 µmol/L dNTPs, 2.25 mM MgCl₂, 1X Taq
27
28 161 polymerase buffer, 1X Taq polymerase buffer, 0.5 µM of each primer, 2.5 U of Taq
29
30 162 DNA polymerase (Eppendorf, Germany). Amplification conditions were 30 cycles of
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32 163 95° for 30 sec, 62° for 30 sec ,72° for 1.5 min. The PCR products were digested at
33
34 164 37°C overnight with the enzyme NlaIII (New England Biolabs UK, as indicated by
35
36 165 the manufacture's procedure then separated on 3% agarose gel and stained with
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38 166 ethidium bromide. The presence of C allele produced two fragments of 175 and 120
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40 167 bp whereas G allele produced one fragment of 242 bp.
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48 169 **RNA Isolation**

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50 170 Total RNA was isolated from PBMC using TRIzol (Invitrogen, UK). The integrity of
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52 171 the RNA was confirmed with analysis by the Agilent 2100 bioanalyser (Palo Alto,
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54 172 CA, USA using the RNA 600 LabChip™ kit.
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59 174 **Target RNA Preparation, Hybridisation, and Microarray Analysis**

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4 175 The human oligonucleotide library was purchased from Sigma-Compugen (Oligo
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6 176 library, release 1.0, Jamesburg, NJ, USA) with a further additional set of 700
7
8 177 oligonucleotides custom synthesised according to the manufacturer's standard design
9
10 178 guidelines. Oligonucleotides were printed onto Codelink slides (GE Healthcare,
11
12 179 Amersham, UK) according to manufacturer's guidelines using a Biorobotics TAS
13
14 180 microarray system. Probe labelling was performed using the Genisphere 350HS
15
16 181 labelling system (Hatfield, PA, USA) according to the manufacturer's standard
17
18 182 protocol. Reverse transcription was performed using Powerscript RT (BD
19
20 183 Biosciences, Oxford, UK) with a final dNTP concentration of 0.5mM each and using
21
22 184 the 5pMol/ul modified oligo dT primer provided (Genisphere, Hatfield, PA, USA).
23
24 185 Hybridisation was performed using a Lucidea SlidePro (GE Healthcare, Amersham,
25
26 186 UK). Dendrimer hybridisation was performed with dextran concentration reduced to
27
28 187 2.5% final (w/v), hybridisation time was reduced to 4hrs at 50°C. Scanning was
29
30 188 performed using a Perkin Elmer Scanarray 4000XL scanner (Buckinghamshire, UK)
31
32 189 for both Cy3 and Cy5. Image analysis and data extraction was performed using
33
34 190 Bluefuse V3.2 (Bluegnome Ltd, Cambridge, UK). Manual exclusion was further
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36 191 applied to remove outliers due to contamination.
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45 **Bioinformatics Analysis**

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47 194 Data sets were analysed using GeneSpring v7.2 (Silicon Genetics, CA, USA). Loess
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49 195 normalisations were applied to all data sets. A subset of genes for data interrogation
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51 196 was generated that excluded controls, spots of poor quality, and gene probes that were
52
53 197 present in less than 50% of samples. From these selected genes, relative expression
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55 198 compared to untreated cells was determined and genes differentially expressed by a
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57 199 minimum of two-fold in the presence of zinc were determined. One-way, parametric,
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4 200 ANOVA tests were performed followed by Benjamini and Hochberg multiple test
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6 201 correction with a false discovery rate of 0.05. Microsoft Excel templates were
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8 202 prepared for each of the genotypes containing genes that were over- and under-
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10 203 expressed following zinc treatment. Ingenuity™ Pathway Analysis 3.0 was utilised to
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12 204 assemble functional networks altered by zinc status (Ingenuity™ Systems, CA, USA).
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18 206 **Real-time RT-PCR**

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20 207 Taqman (Applied Biosystems, Applied Biosystems, UK) probes were used for RT-PCR (IL6:
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22 208 Hs00174131_m1; FABP-4: Hs00609791_m1; leptin: Hs00174877_m1; IL-1β:
23
24 209 Hs00174097_m1; IL-1R2: Hs00174759_m1; GAPDH: Hs99999905_m1). RNA was
25
26 210 prepared as before and *in vitro* transcription was performed using 1µg total RNA and
27
28 211 random hexamer primers (Invitrogen, Paisley, UK). The Bio-rad I-Cycler (Bio-rad,
29
30 212 Hercules, CA) with FAM-490 system detection was used for real time RT-PCR. PCR
31
32 213 thermocycler conditions were 50°C for two minutes, 90°C for two minutes followed
33
34 214 by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Samples were run in
35
36 215 triplicate with test probes and the control gene human GAPDH to control for
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38 216 differences in amount of starting material. Fold-changes (compared to untreated
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40 217 cells) were calculated by normalising the test crossing threshold (Ct) with the
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42 218 GAPDH amplified control Ct.
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220 **Results**

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

233

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

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4 245 statistically significant molecular networks assembled using Ingenuity Pathway
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6 246 Analysis (IPA) profiling of genes differentially expressed in the presence of zinc,
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8 247 including interacting partners, are depicted in **Figure 1b** and **Figure 1c**, respectively.
9
10 248 Genes demonstrated to be up-regulated or down-regulated in the presence of zinc are
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12 249 coloured in red and green, respectively. Class of function for each of the genes,
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14 250 including enzymes, growth factors, and receptors, are depicted by nodal shape, as
15
16 251 identified in the figure legend. Interestingly, IPA identified interactions between IL-6
17
18 252 (gene expression decreased by zinc treatment) and metalloproteases (MMP9,
19
20 253 MMP19) and pro-inflammatory mediators (IL1R2, CXCL3), all of which were down-
21
22 254 regulated following zinc treatment. IPA further identified zinc-dependent down-
23
24 255 regulation of several obesity-related genes: leptin, NR1H3 (LXR- α) and fatty acid
25
26 256 binding protein 3 (FABP3). These data suggest that zinc may act as an important
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28 257 anti-inflammatory and anti-obesity agent. These results are consistent with the known
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30 258 age-dependent increases in pro-inflammatory status and fat mass.
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38 260 In order to test the hypothesis that variability in IL-6 -174 and MT1a +647 loci
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40 261 influences the effects of zinc on the genomic level, donors were stratified by IL-6 and
41
42 262 MT1a genotype. Individuals were determined to be either one of three genotypes: (1)
43
44 263 MT1a +647 C-allele-/IL-6 -174-C-allele-positive carriers (C+C+), (2) MT1a +647 C-
45
46 264 allele-positive/IL-6 -174-C-allele negative carriers (C+C-) or C-allele negative in both
47
48 265 MT1a +647 and IL-6 -174 loci (C-C-). These three genotypes were distributed evenly
49
50 266 throughout the donor population analysed, as shown in **Table 1**. The MT1a +647 C-
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52 267 allele-negative/IL-6 -174-C allele positive carriers (C-C+) were not considered for
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54 268 analysis due to high variability in gene expression in this group (data not shown for
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56 269 n=6 individuals) which we were not able to address by recruiting more individuals
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4 270 with this genotype due to the relatively low prevalence of these individuals within the
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6 271 population recruited. Gene expression data for each individual donor were grouped
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8 272 into three genotypes for analysis. For each of the three genotypes, average gene
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10 273 expression for each expressed gene was determined in the presence and absence of
11
12 274 exogenous zinc. The number of genes which were differentially regulated by zinc as
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15 275 determined for each genotype is shown in **Figure 2a**. All of the 26 genes that were
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17 276 regulated by zinc (15 up-regulated, 11 down-regulated) in C+C+ donors were unique
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19 277 to this genotype. However, of the 45 and 38 genes that were regulated by zinc in the
20
21
22 278 C+C- and C-C- donors, respectively, 24 genes were commonly regulated in these two
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24 279 genotypes. Five of these zinc-dependent IL-6 C-allele negative-regulated genes, IL-6,
25
26 280 IL-1, IL-1R2, leptin, and FABP-4, listed in **Figure 2b** with associated p-values
27
28 281 (compared to non-treated controls). These five genes were selected for confirmation
29
30 282 of differential expression by real time RT-PCR. Real time RT-PCR confirmed that
31
32 283 these genes were differentially regulated in individuals lacking the IL-6 -174C-allele
33
34 284 following zinc treatment, as shown in **Figure 2c**. In addition to differential regulation
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36 285 of these individual genes, comparison analysis of the response of each of three genetic
37
38 286 groups to zinc supplementation using IngenuityTM tool identified several
39
40 287 inflammatory canonical pathways which were differentially regulated by zinc
41
42 288 depending on genetic background. These pathways are shown in **Figure 3**.
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44 289 Following zinc treatment, the IL-10, PPAR, IL-6, p38 MAPK, G-protein-coupled
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46 290 receptor and NFκB signalling pathways were affected in both C-C- and C+C-
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48 291 individuals. None of these pathways were differentially regulated by zinc in C+C+
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50 292 individuals, supporting the hypothesis that zinc preferentially acts as an anti-
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52 293 inflammatory agent in individuals lacking the IL-6 C-allele. Of the pathways shown,
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56 294 only the pentose phosphate pathway was regulated by zinc in C+C+ individuals in
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4 295 addition to C-C- individuals, suggesting this pathway was regulated in a zinc-
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6 296 dependent IL-6/MT-independent manner. The interferon signalling pathway was
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8 297 regulated by zinc in only C-C- individuals, suggesting this pathway be co-ordinately
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10 298 regulated by MT and IL-6 interactions.

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15 300 In order to assess whether variability in the IL-6 and MT1a loci influences zinc
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17 301 homeostasis or response to zinc supplementation, cellular zinc ion availability, NO-
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19 302 induced release, and metallothionein expression were determined for each genotype
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21 303 before and after zinc treatment, as previously described (Malavolta et al. 2006). Zinc
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23 304 availability (shown as mean normalized fluorescence) in lymphocytes (**Figure 4a**)
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25 305 and monocytes (**Figure 4c**) and NO-induced zinc release (shown as difference in the
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27 306 mean normalized fluorescence after the addition of AcOM-DEA/NO) from
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29 307 lymphocytes (**Figure 4b**) and monocytes (**Figure 4d**), was determined before and
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31 308 after *in vitro* culture with exogenous zinc, for MT1a/IL-6 C+C+, C+C-, and C-C-
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33 309 genotypes. The average total metallothionein protein expression across donors for
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35 310 each genotype as determined by mean fluorescence intensity is shown in **Figure 4e**.
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37 311 While increases were observed in zinc availability in lymphocytes and monocytes,
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39 312 and in metallothionein expression following zinc treatment, no statistically significant
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41 313 difference was observed between the IL-6 and MT1a genotypes. Similarly, there was
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43 314 no significant difference between IL-6 and MT1a C+ and C- carriers in zinc
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45 315 availability, NO-induced release of zinc, or metallothionein expression at the basal
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47 316 level, prior to zinc treatment. We observed no significant genotype-dependent
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49 317 difference between basal- and zinc-stimulated levels of zinc availability, NO-induced
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51 318 zinc release or metallothionein expression. Additionally, in the case of NO-induced
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53 319 zinc release in monocytes and lymphocytes derived from C+C+ carriers, we did not
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4 320 observe a significant increase in either parameter following zinc treatment. This was
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6 321 due to a surprising reduction of these parameters in some experimental samples and
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8 322 concomitant increased variability within this group. Lack of increased NO-stimulated
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10 323 zinc release in C+C+ carriers following zinc treatment corresponded with slightly
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12 324 increased basal levels of NO-stimulated zinc release in C+C+ monocytes compared to
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15 325 IL-6 -174 C- carriers, although this was not significant. These data suggest that the
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17 326 most significant responses to zinc treatment on the level of NO-stimulated zinc
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19 327 release in monocytes and lymphocytes occur in IL-6 C- carriers, which is consistent
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21 328 with the increased zinc-dependent gene expression in this group.
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4 **329 Discussion**

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7 332 In the current study, we investigated whether variability in IL-6 -174 and MT1a +647

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9 333 loci influenced zinc-regulated gene expression in ageing. The hypothesis was taken

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11 334 that an anti-inflammatory molecule such as zinc might impact positively on IL-6

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13 335 production in the elderly. Since MT1a polymorphisms are associated with increased

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15 336 IL-6 production and could potentially impact on zinc homeostasis, we further

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17 337 segmented the elderly population recruited for investigation based on MT1a

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19 338 polymorphism. It was thought that MT1a genotype may exacerbate the IL-6

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21 339 phenotype. By combining these two genetic polymorphisms we were able to identify

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23 340 an elderly sub-population who responded significantly to zinc treatment through

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25 341 modulation of inflammatory and metabolic gene expression profiles. This approach

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27 342 offers substantial benefit over attempting zinc intervention in a non-segmented

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29 343 population as many of the general ageing population are zinc sufficient. It is hoped

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31 344 that using nutrigenomic or nutrigenetic approaches to identify sub-populations who

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33 345 have the greatest susceptibility to nutritional deficiencies will aid in selecting

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35 346 individuals who will be afforded the greatest health benefits and will additionally

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37 347 provide the greatest probability of a successful nutritional intervention trial.

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41 349 Of the genes that were differentially regulated on the basis of IL-6 genotype, the most

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43 350 interesting belong to either the IL-6 signalling pathway itself or are involved in

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45 351 regulation of energy balance/obesity, including IL-6, IL-1R2, IL-1, leptin, and

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47 352 FABP4. These data are consistent with the established links between serum IL-6

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49 353 levels, IL-6 promoter polymorphisms and obesity (Klipstein-Grobusch et al. 2006; Qi

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51 354 et al. 2007; Wernstedt et al. 2004). Zinc supplementation has previously been shown

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53 355 to improve glycemic control in db/db and ob/ob mice (Begin-Heick et al. 1985, Simon

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4 356 and Taylor 2000). In fact, zinc-deficient mice have multiple diabetogenic traits
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6 357 including impaired glucose tolerance, insulin resistance and impaired insulin
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8 358 signalling (Cunnane et al. 1984, Gomot et al. 1992). A study carried out in obese
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10 359 children associated lower zinc serum concentrations with abdominal obesity (Beattie
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12 360 et al. 1998, Marreiro et al. 2004). Conversely, zinc supplementation has been
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15 361 demonstrated to increase insulin secretion and to improve glucose control in db/db
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17 362 and ob/ob mice (Begin-Heick et al. 1985, Simon and Taylor 2000).

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22 364 In addition to associations between zinc status and obesity, an interaction between
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24 365 dietary zinc and adiposity was implicated in MT-null mice (Beattie et al. 1998). MT-
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26 366 deficient animals are obese, have elevated plasma leptin concomitant with adipose
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28 367 leptin mRNA expression compared to controls, and pronounced alterations in zinc
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30 368 homeostasis, suggesting that disrupted zinc availability may lead to alterations in
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32 369 energy regulation (Beattie et al. 1998). Furthermore, the obesity associated with these
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34 370 animals is characterised by elevated circulating leptin, a hormone that is produced by
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36 371 adipocytes (Rebuffe-Scrive et al. 1993, Van Heek M et al. 1997). Here we show that
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38 372 the addition of exogenous zinc led to increased MT expression concomitant with
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40 373 decreased leptin gene expression in IL-6 C-allele negative individuals. Wernstedt et
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42 374 al. have previously demonstrated that the IL-6 -174 C allele was associated with
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44 375 higher BMI and higher serum leptin levels (Wernstedt et al. 2004). Since several
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46 376 adipose-derived cytokines including leptin, tumor necrosis factor- α , and IL-6 are up-
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48 377 regulated in obesity and contribute to the development of diabetes and vascular
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50 378 disease, the findings that zinc could potentially decrease expression of these
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52 379 molecules is important for obesity-related conditions. Taken together, these data
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54 380 suggest that zinc intervention may be useful to restore normal levels of leptin and IL-6
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4 381 and could potentially impact positively on adipose mass in individuals with pro-
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6 382 inflammatory, obese phenotypes.

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10 384 In the current study, we demonstrated that zinc treatment resulted in decreased
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12 385 FABP4 gene expression. Differential regulation of FABP4 has previously been
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15 386 demonstrated in zinc-deprived rats (Blanchard et al. 2001), suggesting this is a
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17 387 conserved effect that is observed *in vivo*. FABPs constitute a family of cytosolic lipid
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19 388 chaperones that share a conserved structure but exhibit a distinct, tissue-specific
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21 389 expression pattern (Haunerland and Spencer 2004). FABP4 (or A-FABP) is
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23 390 expressed predominantly in adipocytes and macrophages (Hotamisligil et al. 1996).
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25 391 Recent studies have demonstrated that FABP4 is important in systemic metabolic
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27 392 homeostasis. FABP4-deficient mice are partially protected against obesity-induced
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29 393 insulin resistance, type 2 diabetes, and hypercholesterolemic atherosclerosis in both
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31 394 dietary and genetic models (Makowski and Hotamisligil 2004, Uysal KT et al. 1997).
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33 395 One elegant recent study performed by this same group examined the effect of leptin
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35 396 on FABP4/5 *-/-* mice (Cao et al. 2006). In the absence of leptin and despite severe
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37 397 obesity, these mice maintained euglycemic control and demonstrated improved
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39 398 peripheral insulin sensitivity. These data indicate that reductions in leptin and FABP4
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41 399 by dietary zinc in IL-6 C-allele negative individuals may protect against insulin
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43 400 resistance and suggest an important mechanism by which zinc may protect against
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45 401 development of type 2 diabetes.

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49 403 In addition to its role in the regulation of energy balance and obesity, evidence
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51 404 obtained recently indicates that FABPs may serve as master regulators in the control
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53 405 of cellular inflammatory responses through regulation of JNK and PPAR γ (Hirosumin
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4 406 et al. 2002, Gao et al. 2002). These observations further establish the relationship
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6 407 between cellular stress responses, inflammatory cytokine production and obesity.
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8 408 Firstly, FABP4 may co-ordinate the lipid-mediated activation of stress kinases such as
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10 409 JNK or I κ K under immune or metabolic stimuli thus linking lipid signalling to pro-
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12 410 inflammatory signalling and anti-insulin action (Hotamisligil et al. 1996). It has been
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14 411 hypothesised that the presence of FABP4 in periods of feast and famine may have
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16 412 been beneficial to maintain a strong immune response and adipose tissue in fitting
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18 413 with the concept of ‘thrifty’ phenotype to survive (Auwerx, Cock, and Knouff 2003).
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20 414 However, with increasing prevalence of excessive caloric intake, decreased energy
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22 415 expenditure and high stress lifestyle, FABPs may not be able to maintain
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24 416 inflammatory or metabolic homeostasis and the presence of these proteins may
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26 417 actually aid in the formation of obesity, dyslipidemia and inflammatory responses
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28 418 (Hotamisligil et al. 1996). Combined with the prevalent increased pro-inflammatory
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30 419 IL-6 production in elderly individuals, these alterations in inflammatory homeostasis
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32 420 have serious and detrimental implications for health in ageing populations.
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40 422 In conclusion, the observations that zinc may alleviate the increased pro-
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42 423 inflammatory/pro-atherogenic status of elderly individuals lacking the IL-6 C- allele
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44 424 suggest that this population would receive increased health benefits from zinc
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46 425 supplementation compared to the general population. Because high levels of
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48 426 circulating pro-inflammatory cytokines are prevalent in both the general ageing
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50 427 population and due to the increased prevalence of obesity in the non-aged population,
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52 428 it remains feasible that zinc supplementation may also improve health status in these
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54 429 populations. Further phenotype-specific interventions (e.g. in obese individuals with
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56 430 high levels of adipokine secretion) are needed to test these hypotheses.
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4 **431 Acknowledgements**

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15 **437 Author contributions**

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17 438 DJM analysed microarray data, performed RT-PCR, and wrote the manuscript; AJW
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19 439 performed the microarrays; MM performed metallothionein, zinc availability and zinc
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21 440 release analyses. LC, RG, E Muti and CC recruited subjects, performed prospective
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23 441 genotyping, collected blood, isolated PBMC, and cultured and treated cells. JRP and
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25 442 E Mocchegiani supervised and designed the study.

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31 444 The authors declare no competing interests.
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566 **Tables.**

Donor ID	Gender	Age	Genotype +647 A/C	MT1a	+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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4 **568 Figure Legends**

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6 **569**
7 **570 Figure 1: Ingenuity™ networks of genes differentially expressed in PBMC**
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9 **571 derived from elderly donors cultured in the presence of zinc.** The 61 genes that
10 were differentially regulated by zinc were analysed by the Ingenuity™ Pathway
11 Analysis tool. Two genetic networks were found to be significantly regulated by zinc
12 (p<0.001). The network genes identified through microarray analysis (focus genes,
13 shown in bold), network score, and main cellular functions of the network are listed
14 (a). The networks shown were significantly associated with cellular growth and
15 proliferation, cancer, and cellular movement (b) and immune response, cellular
16 growth and proliferation and cellular morphology (c). Coloured genes were identified
17 by microarray analysis as differentially up-regulated or down-regulated in the
18 presence of zinc (red and green colouring, respectively). Other nodal genes are
19 directly or indirectly associated with the differentially expressed genes and were
20 either not spotted on the array or not found to be differentially expressed following
21 zinc treatment. The meaning of the nodal shapes is indicated in the figure legend.
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41 **585 Figure 2: Genes differentially expressed in MT1aC+/IL-6 C+, MT1aC+/IL-6 C-,**
42 **586 MT1aC-/IL-6 C- donors in the presence of zinc.** The number of genes
43 differentially expressed in PBMC segmented by variability in the IL-6 and MT1a loci,
44 following addition of exogenous zinc is shown (a). Zinc-dependent differential
45 expression of five selected genes (IL-6, IL-1, IL-1R2, leptin and FABP4) of the 24
46 genes commonly regulated in MT1a C+/IL-6 C-donors and MT1a C-/IL-6 C- by
47 microarray analysis is shown, with associated p-value compared to non-treated
48 controls (b). Differential expression of IL-6, IL-1, IL1R2, leptin and FABP4
49 following zinc treatment was determined by real-time RT-PCR (c). RNA was
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4 594 prepared and *in vitro* transcription was performed using 1µg total RNA and random
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6 595 hexamer primers (Invitrogen, Paisley, UK). The Bio-rad I-Cycler (Bio-rad, Hercules,
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8 596 CA) with FAM-490 system detection was used for real time RT-PCR. Taqman
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10 597 (Applied Biosystems, Applera, UK) probes were used as detailed in *Subjects and*
11
12 598 *Methods*. Samples were grouped by genotype MT1a/IL-6- C+C+ (black bars), -
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14 599 C+C- (grey bars), and - C-C- (white bars) and analysed before and after 24h zinc
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16 600 supplementation. Samples were run in triplicate with test probes and the control gene
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18 601 human GAPDH to control for differences in amount of starting material. Fold-
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20 602 changes were calculated by normalising the test gene crossing threshold (Ct) with the
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22 603 GAPDH amplified control. Fold-change values were normalised to gene expression
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24 604 in non-treated controls. *denotes $p < 0.05$; ** denotes $p < 0.005$; ***denotes $p < 0.0005$
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26 605 by ANOVA (compared to non-treated controls).
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34 607 **Figure 3: Canonical pathways differentially expressed in PBMC derived from**
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36 608 **elderly donors following zinc treatment.** Genes that were differentially expressed
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38 609 in PBMC isolated from elderly donors stratified by MT1a/IL-6 haplotype following
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40 610 zinc treatment were clustered into canonical pathways using IngenuityTM tool. The
41
42 611 relative proportion of genes (y-axis) differentially expressed in each canonical
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44 612 pathway (Interferon signalling, IL-10 signalling, PPAR signalling, Pentose phosphate
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46 613 pathway, IL-6 signalling, p38 MAPK signalling, G-protein coupled receptor
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48 614 signalling, and NFkB signalling) following zinc treatment (x-axis) is demonstrated for
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50 615 each of the three genotypes: MT1a/IL-6 C+C+ (black bars), MT1a/IL-6 C+C- (dark
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52 616 grey bars), and MT1a/IL-6 C-C- (white bars). No bar represented depicts no
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54 617 haplotype-specific affect of zinc on the pathway.
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4 619 **Figure 4: Zinc availability, NO-induced release of zinc, and metallothionein**
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6 620 **expression in elderly PBMC following zinc treatment.** Zinc ion availability and
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8 621 NO-induced release of zinc were evaluated by flow cytometry in PBMC isolated from
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10 622 elderly donors (stratified by IL-6/MT-1 allele carriers) before (0h) and after (24h) zinc
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12 623 treatment. Zinc ion availability in lymphocyte (a) and monocyte (c) sub-population
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14 624 selected by FW and SS scatter was calculated as the mean normalized fluorescence
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16 625 intensity (Fn). Fn was calculated by the ratio of the mean fluorescence intensity and
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18 626 the minimum mean fluorescence intensity detected after the addition of the zinc
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20 627 chelator TPEN. The NO-induced release of zinc [$Fn(NO) - Fn$] in lymphocyte (b)
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22 628 and monocyte (d) selected by FW and SS scatter was calculated by subtracting Fn to
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24 629 the mean normalized fluorescence obtained after incubating PBMC for 30' with
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26 630 AcOM-DEA/NO. Data represent mean \pm SD (n = 5 for C+C+ and C-C- carriers and n
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28 631 = 6 for C+C- carriers). Metallothionein protein expression was evaluated by Flow
29
30 632 Cytometry in PBMC isolated from elderly donors (stratified by IL-6/MT1 allele
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32 633 carriers) before (0h) and after (24h) zinc treatment (e). The MT-specific mean
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34 634 fluorescence intensity (MFI) was determined by subtracting the fluorescence obtained
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36 635 with an isotypic antibody (clone MOPC21, IgG1k, Sigma-Aldrich, MI, Italia) from
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38 636 the one obtained with the MT-specific antibody (monoclonal mouse anti-horse
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40 637 metallothionein clone E9, from DAKOCYTOMATION, Denmark). Data represent
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42 638 mean \pm SD (n = 5 for C+C+ and C-C- carriers and n = 6 for C+C- carriers). * denotes
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44 639 $p < 0.05$; ** denotes $p < 0.01$ by ANOVA.
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USCRIPT

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, BID, 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. 1c

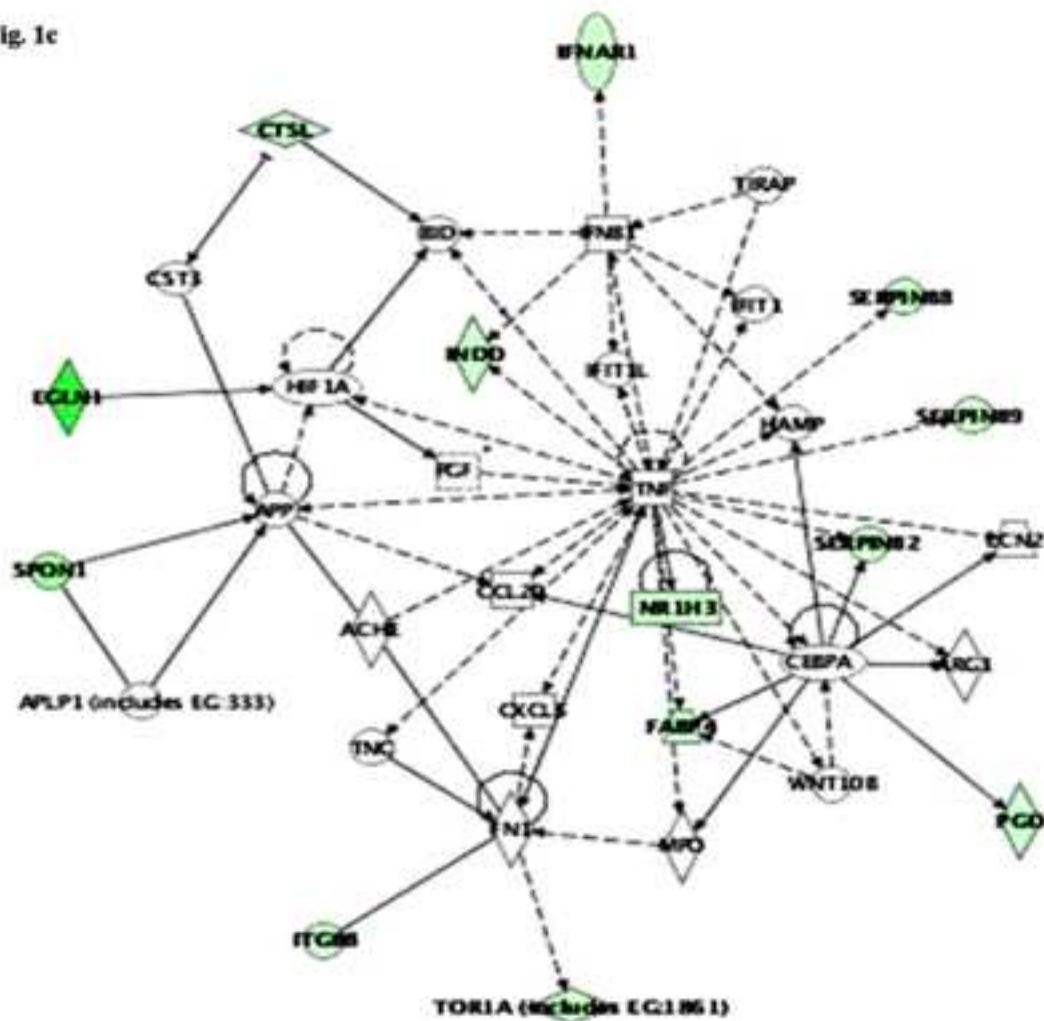


Fig. 2

a.

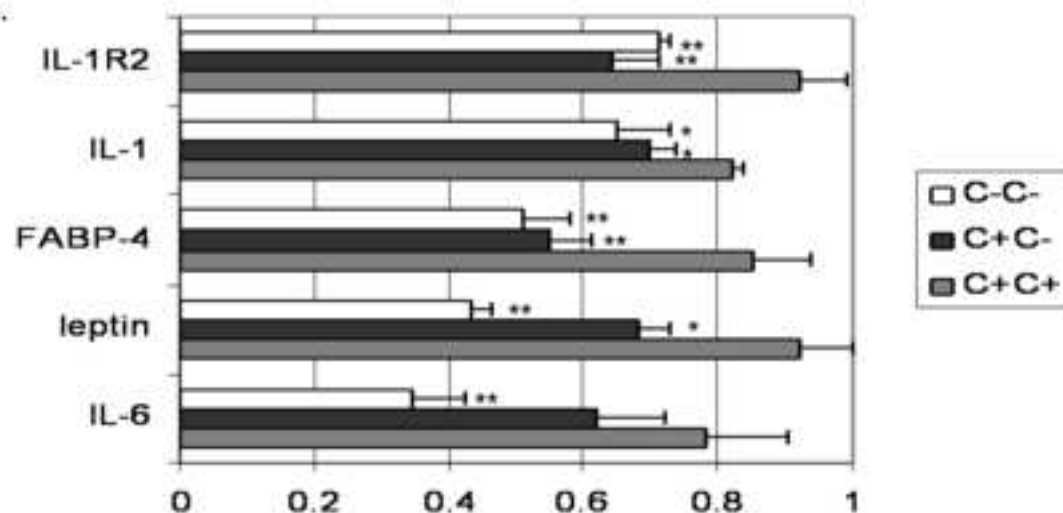
Genes altered in PBMC derived from elderly donors cultured in the presence of zinc			
Genotype	MT1a C+/IL-6 C+ (C+C+)	MT1a C+/ IL-6 C- (C+C-)	MT1a C- /IL-6 C- (C-C-)
Up-regulated	15	15	10
Down-regulated	11	30	28
Total	26	45	38

b.

Gene Name	p-value	
	MT1a C+/IL-6 C-	MT1a C-/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.



MANUSCRIPT

Fig. 3

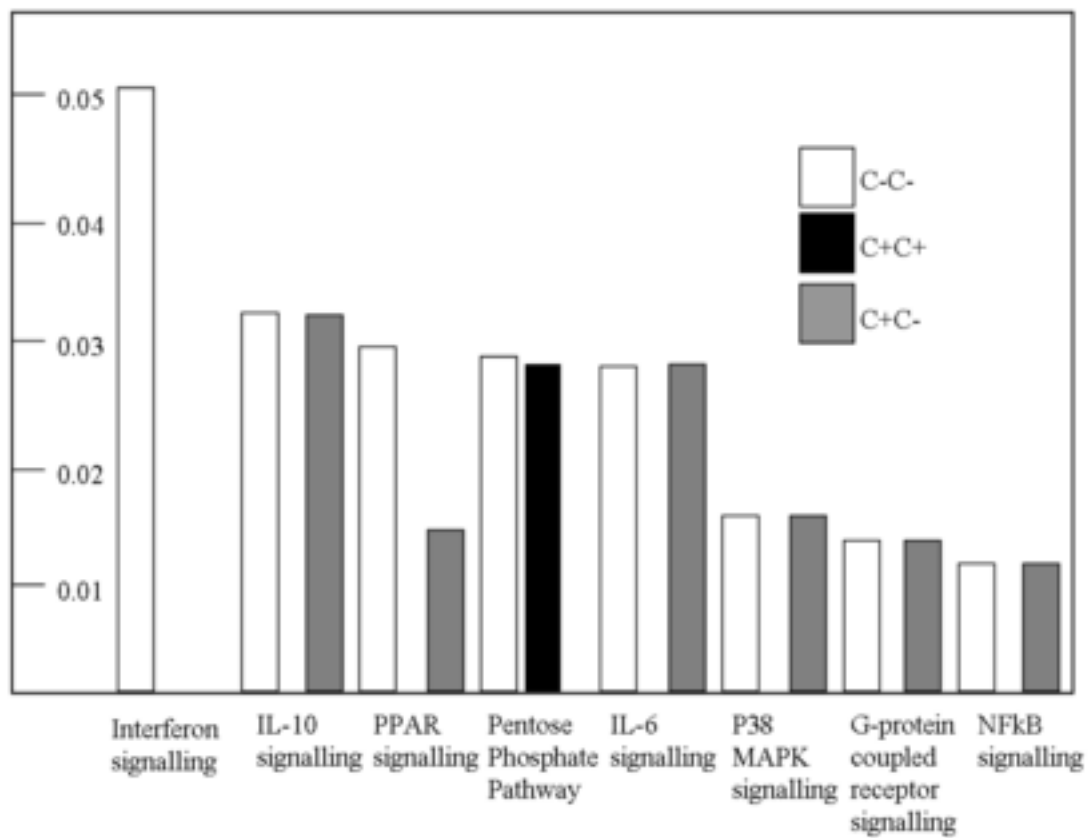


Fig. 4

