Widely used commercial ELISA for human Zonulin reacts with Complement C3 rather than preHaptoglobin2

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Nonstandard abbreviations: HP, haptoglobin; T2D, type 2 diabetes; WHR, waist-to-hip ratio.

1

Abstract

BACKGROUND. There is increasing evidence for the role of impaired intestinal permeability in obesity and associated metabolic diseases. Zonulin is an established serum marker for intestinal permeability and supposedly identical to pre-haptoglobin2. Here, we aimed to investigate the relationship between intestinal permeability represented by circulating zonulin and metabolic traits related to obesity.

METHODS. Serum zonulin was measured by using a widely used commercially ELISA kit in 376 subjects from the metabolically well-characterized cohort of Sorbs from Germany. In addition, haptoglobin genotype was determined in DNA samples from all study subjects.

RESULTS. The genotype frequencies for haptoglobin genotypes HP1/1, HP1/2 and HP2/2 were 15.8%, 47.6% and 36.6% respectively. Since zonulin concentrations did not correspond to the haptoglobin genotypes we investigated the specificity of the zonulin ELISA assay using immunoprecipitation experiments, mass spectrometry and Western blot analysis. Thus we demonstrated by mass spectrometry that the kit is mainly capturing complement factor C3 and derived fragments. Circulating complement factor C3 was significantly increased in patients with diabetes and obesity and correlated strongly with markers of the lipid and glucose metabolism.

CONCLUSIONS. Our study supports the role of the complement C3 in the pathophysiology of obesity and necessitates critical revision of published data on zonulin as a marker for altered intestinal permeability from studies that have used the ELISA kit investigated here.

Introduction

The "intestinal barrier" is an established term, defined as a functional entity separating the gut lumen from the inner host, and consisting of mechanical, humoral, immunological, muscular and neurological elements. Intestinal barrier dysfunction is a characteristic feature of pathological states such as inflammatory bowel disease, celiac disease, nonalcoholic steatohepatitis and ulcerative colitis [1, 2]. There is also emerging evidence for the role of impaired intestinal permeability in metabolic diseases including obesity and type 2 diabetes (T2D) [3–5]. It has been hypothesized that gut bacteria and bacterial endotoxins may disrupt the intestinal barrier resulting in the so called "leaky gut" [4, 6]. The leakage of toxins, bacterial components or even live bacteria and their transfer to target organs such as adipose tissue might contribute to the development of obesity and T2D [6, 7]. Indeed, numerous studies in mouse models demonstrated that changes in the gut microbiota can alter the gut permeability and lead to an endotoxemia-induced inflammation in adipose tissue, and ultimately to obesity [3, 8, 9]. Results from experimental mouse models are supported by studies in humans by showing an increase in circulating endotoxin levels and circulating bacterial DNA in obese/diabetic patients, likely due to an increased intestinal permeability in affected subjects [10, 11].

Intestinal barrier transport is mainly regulated by structures of the paracellular pathway called tight junctions which form barriers between epithelial cells and regulate the transport of ions and small molecules across the intestinal lumen. Intestinal permeability is a functional feature of the intestinal barrier. It can be measured by analyzing flux rates of inert molecules across the intestinal wall as a whole or across wall components [1]. The gold standard for assessment of intestinal permeability in vivo is an assay combining indigestible large and small oligosaccharides, such as lactulose and mannitol; the larger oligosaccharide,

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79   lactulose, is only transported via the paracellular pathway, whereas the smaller

80   oligosaccharide, mannitol, is taken up freely over the intestinal barrier via the

81   transcellular route. However, these oligosaccharide assays are extensive, laborious

82   and time consuming. Therefore, identifying appropriate biomarkers for intestinal

83   permeability is highly desirable. Fasano et al. identified zonulin as a new tight

84   junction regulating protein which is, functionally, the human counterpart of the Vibrio

85   cholera endotoxin zonula occludens toxin [12, 13]. Later, the same group described

86   zonulin as the precursor of haptoglobin 2 (pre-HP2) [14]. Haptoglobin is a well-known

87   protein involved in the degradation of hemoglobin, whereas the function of its

88   precursor is largely unknown. Haptoglobin is first synthesized into a single chain

89   precursor protein, which is cleaved into a light N-terminal α-chain and heavy C-

90   terminal β-chain. An exon duplication of exons 3 and 4 of the haptoglobin gene

91   differentiates the HP1 from the HP2 allele. Due to this exon duplication the HP2 α-

92   chain is 1.7kb longer than in the HP1 allele. Haptoglobin is active as tetramer

93   consisting of 2 α- and 2 β-chains linked by disulfide bonds, resulting in three possible

94   genotypes: homozygous HP1/1 and HP2/2 as well as heterozygous HP1/2 [15, 16].

95   About 15 % of the Caucasian population are homozygous for HP [16, 17]. Zonulin as

96   pre-HP2 reversibly opens tight junctions and is upregulated in diseases such as

97   celiac disease and type 1 diabetes (T1D) [14, 18]. Serum zonulin concentrations are

98   also increased in T2D and obesity [19–21] and strong correlations were observed

99   with various metabolic markers, including fasting plasma glucose, IL-6, HDL, and

100   triglyceride (TG) levels [19–21]. However, the rather small sample sizes in these

101   studies warrant further analyses to validate and strengthen the initial findings.

102   Here, we aimed to characterize the relationship between circulating serum zonulin

103   and traits related to obesity in a metabolically well-characterized cohort of Sorbs from

104   Germany. To measure zonulin, we used the widely acknowledged commercially

105 available ELISA kit (Immundiagnostik, Bensheim, Germany). In addition, we

106 determined the haptoglobin genotypes in the entire cohort. Due to a lack of

107 correspondence between the observed circulating zonulin concentrations and the

108 haptoglobin genotypes in our study cohort, we further investigated the identity of the

109 product captured by the commercial ELISA assay. We revealed that the ELISA kits

110 used in the present study actually targeted the complement factor C3 and C3-derived

111 fragments, which were strongly associated with altered lipid and glucose metabolism.

112

113 **Materials and Methods**

114 ***Study subjects***

115 All subjects are part of a sample from an extensively clinically characterized

116 population from Eastern Germany, the Sorbs [22–24][1–3]. Extensive phenotyping

117 included standardized questionnaires to assess past medical history and family

118 history, collection of anthropometric data (weight, height, waist-to-hip ratio (WHR)),

119 and an oral glucose tolerance test. Glucose was assessed by the Hexokinase

120 method (Automated Analyzer Modular, Roche Diagnostics, Mannheim, Germany)

121 and serum insulin was measured using the AutoDELFIA Insulin assay (PerkinElmer

122 Life and Analytical Sciences, Turku, Finland). Total serum cholesterol and TG

123 concentrations were measured by standard enzymatic methods (CHOD-PAP and

124 GPO-PAP; Roche Diagnostics). Serum LDL-C and HDL-C concentrations were

125 determined using commercial homogeneous direct measurement methods (Roche

126 Diagnostics). All assays were performed in an automated clinical chemistry analyzer

127 (Hitachi/ Roche Diagnostics) at the Institute of Laboratory Medicine, University

128 Hospital Leipzig.

129 All blood samples were taken in the morning after an overnight fast and stored at -

130 80°C until analyses. From the 1040 Sorbs enrolled in the cohort, a subgroup of 376

131 subjects was genotyped for haptoglobin and provided blood samples for zonulin

132 measurements (Table 1). Main metabolic characteristics of the study subjects are

133 summarized in Table 2.

134 **Table 1: Main characteristics of the study cohort included in zonulin**
135 **measurements.**

136

	all	*female*	*male*
n	376	233	143
Age (years)	53.3 ± 16.2	52.7 ± 15.8	54.2 ± 16.8
BMI (kg/m²)	28.1 ± 5.6	27.9 ±5.9	28.5 ± 5.1
NGT	191 (50.8%)	124 (53.2%)	67 (46.8%)
IGT	79 (21.0%)	49 (21.0%)	30 (21.0%)
T2D	106 (28.2 %)	60 (25.8%)	46 (32.2%)

137 Age and BMI are mean ± SD, NGT, IGT, T2D as absolute number (percentage);
138 NGT: normal glucose tolerance; IGT: impaired glucose tolerance; IFG: impaired
139 fasting glucose

140

141 **Table 2: Main characteristics of the study participants.**

142

	NGT	*IGT*	*T2D*
n	191	79	106
Age (years)	45.4 ± 15.6	59.7 ± 29.4**	62.9 ± 11.2**
BMI (kg/m²)	25.8 ± 5	29.7 ± 4.6**	30.97 ± 5.53**
WHR	0.84 ± 0.10	0.92 ± 0.09	0.94 ± 0.09**
FPG (mmol/l)	5.15 ± 0.50	5.68 ± 0.56**	7.72 ± 2.43** °°
120 min PG (mmol/l)	5.00 ± 1.40	8.95 ± 0.98**	5.78 ± 6.86 °°
Triglycerides (mmol/l)	1.29 ± 0.94	1.56 ± 0.92*	1.87 ± 1.24**
Fasting Insulin (pmol/l)	35.90 ± 25.98	53.56 ± 25.98**	62.38 ± 43.07**
120 min Insulin (pmol/l)	148.39 ± 133.58	409.28 ± 232.81**	182.22 ± 272.34 °°
HDL (mmol/l)	1.69 ± 0.40	1.56 ± 0.36*	1.43 ± 0.39** °
LDL (mmol/l)	3.20 ± 0.96	3.70 ± 1.06**	3.33 ± 0.95 °
Total cholesterol (mmol/l)	5.12 ± 1.03	5.57 ± 1.18*	5.27 ± 0.99
*HOMA IR (pmol/l*mmol/l)*	1.34 ± 1.06	2.30 ± 1.1**	3.58 ± 2.87** °°
HOMA IS (ratio)	1.07 ± 0.76	0.67 ± 0.78**	0.68 ± 1.63**
CRP (mg/l)	2.22 ± 4.20	3.19 ±4.54	3.87 ± 4.76**
Zonulin (ng/ml)	67.25 ± 25.45	71.88 ± 29.36	81.78 ± 25.31** °°

143

144 *: $p < 0.05$ vs NGT; **: $p < 0.01$ vs NGT
145 °: $p < 0.05$ vs IGT; °°: $p < 0.01$ vs IGT
146 All data given as mean ± standard deviation, NGT: normal glucose tolerance, IGT:
147 impaired glucose tolerance, T2D: type 2 diabetes, CRP: C-reactive protein, HDL:
148 High density lipoprotein, LDL: Low density lipoprotein, FPG: Fasting plasma glucose,
149 WHR: Waist to hip ratio

150

151   The study was approved by the ethics committee of the University of Leipzig and all

152   subjects gave written informed consent before taking part in the study.

153

154   **ELISA measurements**

155   Circulating zonulin was measured by a competitive ELISA (Immundiagnostik AG,

156   Bensheim, Germany) in serum samples of 376 individuals.

157

158   **Genotyping**

159   Haptoglobin genotypes were determined by PCR using a method adapted from Koch

160   et al. [17]. Briefly, two primer pairs were used: A (5'-

161   GAGGGGAGCTTGCCTTTCCATTG-3') and B (5'-

162   GAGATTTTTGAGCCCTGGCTGGT-3') as well as C (5'-

163   CCTGCCTCGTATTAACTGCACC AT-3') and D (5'-

164   CCGAGTGCTCCACATAGCCATGT-3'). The primer pair A/B generates two bands: a

165   1,757-bp allele 1 specific band and a 3,481-bp allele 2 specific band. The primer pair

166   C/D produces one allele 2 specific band of 349 bp. The combination of the bands

167   allows a reproducible typing of the two common haptoglobin genotypes HP1 and

168   HP2. In contrast, no band is detectable for the rare HP deletion genotype, present in

169   ~0.1% Caucasians [16].

170

171   **Characterization of ELISA products**

172   *Immunoprecipitation*

173   Immunoprecipitation (IP) of target protein from serum samples utilizing the

174   immobilized anti-zonulin antibodies on the ELISA plates was performed. Undiluted

175   serum samples with highest and lowest concentrations of zonulin, as measured using

7

176 the same ELISA kit, were transferred to separate wells and incubated and washed

177 according to the manufacturer's protocol. Afterwards, 50 µl of hot (95°C) SDS sample

178 buffer with β-mercaptoethanol were added to the wells and incubated for 5 minutes to

179 release bound proteins from the immobilized capturing antibody. To increase the final

180 protein concentration, the initial 50 µl of SDS sample buffer were successively used

181 in further wells containing either high or low zonulin serum samples, respectively.

182 Proteins from 20 µl of each pooled sample (high or low zonulin) were separated by

183 SDS-PAGE using precast Bolt 4-12% Bis-Tris Plus gels (ThermoFisher, Waltham,

184 MA, USA). Proteins were stained using the Pierce Silver Stain for mass spectrometry

185 (ThermoFisher).

186

187 *Mass spectrometry*

188 To identify proteins isolated from serum samples using the zonulin ELISA kit as

189 described above, bands were excised from silver-stained gels and in-gel digested

190 with trypsin following a standard protocol [25]. After enzymatic digestion, the peptide

191 mixtures were immediately analyzed by LC/MS/MS on an U3000 RSLC nano-HPLC

192 system (Thermo Fisher Scientific) coupled to an Orbitrap Fusion Tribrid mass

193 spectrometer (Thermo Fisher Scientific). Samples were loaded onto a pre-column

194 (RP-C8, 300 µm * 5 mm, 5 µm, 100 Å, Thermo Fisher Scientific) and washed with

195 water containing 0.1% (v/v) TFA for 15 min, before the peptides were separated on

196 the separation column (RP-C18, 75 µm * 250 mm, 2 µm, 100 Å, Thermo Fisher

197 Scientific) using gradients from 1% to 40% (v/v) B (45 min), 40% to 85% (v/v) B (5

198 min) followed by 85% B (5 min), with solvent A: 0.1% (v/v) formic acid (FA) in water

199 and solvent B: 0.08% (v/v) FA in acetonitrile. Data were acquired using data-

200 dependent MS/MS mode where each high-resolution full-scan in the orbitrap (m/z

201 198 to 1,500; R = 120,000) was followed by high-resolution product ion scans in the

8

202 orbitrap (higher energy collision-induced dissociation (HCD), 27% normalized

203 collision energy, R = 15,000, isolation window 2 Th) within 5 s, starting with the most

204 intense signal in the full-scan mass spectrum.

205 Data analysis was performed using the Proteome Discoverer 2.1 (Thermo Fisher

206 Scientific). MS/MS data of precursor ions (m/z range 350-5,000) were searched

207 against the Swissprot Database (version 11/2016, taxonomy human, 20,082 entries)

208 and a contaminant database using Sequest HT. Mass accuracy was set to 5 ppm

209 and 20 mmu for precursor and fragment ions, respectively. Carbamidomethylation of

210 cysteines was set as fixed modification; oxidation of methionines and N-terminal

211 acetylation were set as variable modifications, two missed cleavages of trypsin were

212 allowed. Results were filtered for non-contaminant proteins identified by at least three

213 unique highly confident peptides (peptide FDR ≤ 1%).

214

215 *Western blot analysis*

216 Western blot experiments were performed to validate the results of mass

217 spectrometric data analysis. Gels were blotted on a PVDF membrane and Western

218 blots were probed with anti-C3 β-chain (1:2000) (Biozol, Eching, Germany) and anti-

219 haptoglobin (1:1000) (Abcam, Cambridge, UK) antibodies. Purified C3c from plasma

220 (Athens Research, Athens, Georgia, USA) was used as a positive control. Incubation

221 with primary and secondary antibodies (HRP-conjugated) was done for 90 minutes at

222 room temperature. Blots were visualized by enhanced chemiluminescence using

223 Pierce ECL Western Blotting Substrate (ThermoFisher).

224

225 ***Statistical analysis***

226 Statistical analysis was performed using with SPSS 24 (IBM). All non-normally

227 distributed metric parameters were log transformed to generate a Gaussian normal

9

228   distribution. Spearman's rank correlation method was used to assess the relationship

229   between metabolic traits. To test for significant differences in distribution for ordinal

230   values, the Kruskal-Wallis-test was used. Exact differences between two groups were

231   tested by both the Mann-Whitney-U test and unpaired student's t-test. In addition,

232   multiple linear regression analyses were done to assess the linear relationship

233   between continuous variables and genotypes. For all tests, a p-value <0.05 was

234   considered to be statistically significant. These statistical tests were used as

235   referenced in the results.

236

237   **Results**

238   ***Haptoglobin genotype***

239   Haptoglobin genotype HP1/1 was present in 15.8% of the subjects, HP1/2 in 47.6%

240   and HP2/2 in 36.6%. These frequencies are comparable to the distribution of HP

241   genotypes in Caucasians reported by others [16, 17]. We tested the association of

242   the HP genotypes with various anthropometric and metabolic traits in all study

243   subjects. The analysis essentially revealed that hemoglobin levels significantly

244   increase with the presence of at least one HP2 allele (p=0.004 over all three groups,

245   p=4.2×10^{-4}) between HP1 homozygote and HP2 carriers). Furthermore, we observed

246   that the total protein concentration in the urine significantly differed between the three

247   groups, with an increase in the HP2 carriers having increased concentrations

248   (p=0.027). Interestingly, mean triiodothyronine (fT3) levels were lower in the HP1/1

249   group than in the HP2/2 group (p=0.012) and in accordance, an increase in

250   administered thyroid gland hormones (p=0.023) was observed.

251

10

252 ***Zonulin ELISA data do not reflect HP genotype distribution***

253 Strikingly, there were no significant differences in levels of the zonulin ELISA signal

254 between the three haptoglobin genotype groups (Figure 1; $p=0.153$ using ANOVA,

255 $p=0.07$ for the t-test comparing log transformed zonulin signals between HP1/1 vs.

256 HP1/2 + HP2/2). Assuming that the protein measured by the kit is zonulin (i.e. pre-

257 HP2), subjects with the HP1/1 genotype, which has a frequency of ~15% in

258 Caucasians [16, 17], were expected to have no detectable zonulin levels. As the

259 zonulin concentrations measured in patient sera using the zonulin ELISA kit clearly

260 did not reflect the HP genotype distribution we therefore concluded that the protein

261 measured by the kit is not identical to zonulin as pre-HP2. Consequently, we aimed

262 to unravel the identity of the protein(s) detected by the alleged zonulin ELISA kit.

263

264 ***Identification of the (major) protein bound by the zonulin ELISA capturing***

265 ***antibody***

266 To identify proteins bound and therefore quantified by the capturing antibody

267 deployed in the zonulin ELISA kit, which was used in this and many other studies

268 (Supplementary Table 1), we performed an immunoprecipitation experiment using

269 patient sera and the immobilized antibody of the ELISA kit as supplied. After

270 incubation of the immobilized ELISA kit antibodies with selected patient sera

271 representing the highest and lowest measured ELISA signals in the cohort, the

272 captured proteins were separated by SDS-PAGE. Silver staining revealed multiple

273 bands, with the most intense band at ~70 kDa and further prominent bands at ~55

274 kDa, ~180 kDa and >180kDA (Figure 2A). This band pattern was incompatible with a

275 band pattern that would be expected for pre-HP2 or haptoglobin-derived proteins and

276 further supported the results demonstrating the lack of correspondence of the

277 captured protein with HP genotypes. To unequivocally identify all major proteins

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278   captured by the ELISA kit, protein bands were cut (Figure 2A) and subjected to MS

279   analysis after tryptic digestion (Supplementary Table 2). Mass spectrometry

280   demonstrated that bands 1 (>180kDa), 2 (~150 kDa), 3 (~70 kDa) were all very likely

281   representing the C3 protein or cleavage products derived from the C3 protein, such

282   as the C3 precursor (187 kDa), C3c (144 kDa) and the C3 β-chain (71 kDa) (Figure

283   2C). Furthermore, the 55 kDa band was identified as properdin or factor P (MW 51

284   kDa). To validate results from mass spectrometry, we performed Western blot

285   analyses and indeed, the major band at 70 kDa was clearly detected by the anti-

286   C3 β-chain antibody (Figure 2B). Importantly, the band intensities clearly reflected

287   the signal intensities measured using the zonulin ELISA, with high-level "zonulin"

288   sera yielding very intense C3 β-chain bands after immunoprecipitation, and sera with

289   a low ELISA signal resulting in low Western blot signals.

290

Circulating complement factor C3 correlates with metabolic traits

291

292   In a sample of 376 subjects tested using the purchased zonulin ELISA, the product

293   (circulating C3) was measured in a mean concentration of 72.2 ± 27.2 ng/ml (mean ±

294   standard deviation), ranging from 17.8 to 207.1 ng/ml. The C3 protein was

295   significantly increased in subjects with T2D (81.78 ± 25.31 ng/ml) compared to

296   subjects with normal glucose tolerance (67.25 ± 25.45 ng/ml, Mann-Whitney-U test;

297   p=2.1x10^{-8}) or impaired glucose tolerance (71.88 ± 29.36 ng/ml, p=0.0017) (Figure

298   3A). Additionally, lean subjects had significantly lower values (65.64 ± 25.23 ng/ml)

299   than subjects with overweight (74.20 ± 30.68 ng/ml, p=0.0082) or obesity (76.24 ±

300   24.17 ng/ml, p=7.0x10^{-5}) (Figure 3B). We observed no gender differences or any

301   correlations with age (data not shown). Circulating complement factor C3 correlated

302   with traits related to glucose and lipid metabolism (Spearman's rank correlation test,

303   adjusted for age, sex and BMI; Table 3). It was positively correlated with BMI, HOMA-

304   IR and -IS and fasting plasma glucose (Table 3). Strong correlations were also

305   observed for lipid metabolism parameters, such as TG levels, total cholesterol, LDL

306   and apolipoprotein B (Table 3). Correlations were tested for a total of 95 accessible

307   traits. After Bonferroni correction for multiple testing (adjusted p-value for significance

308     $p < 5.2 \times 10^{-4}$), correlations for BMI ($p=1.2 \times 10^{-5}$), fasting glucose ($p=9.0 \times 10^{-6}$), TG

309   ($p=4.2 \times 10^{-10}$), total cholesterol ($p=3.3 \times 10^{-5}$) and apolipoprotein B ($p=2.3 \times 10^{-5}$)

310   remained statistically significant.

311

312 **Table 3: Correlation of complement factor C3 with metabolic phenotypes.**

313

	Non-adjusted		Adjusted for age, sex, BMI	
	r	p-value	r	p-value
Anthropometric trait				
Age	0.31	0.55	-	-
Sex	-0.005	0.93	-	-
BMI	**0.221**	**1.2×10^{-5}**	-	-
WHR	**0.172**	**7.3×10^{-4}**	**0.118**	**2.1×10^{-2}**
Glucose metabolism				
Glucose (0 min)	**0.294**	**6.6×10^{-9}**	**0.227**	**9.0×10^{-6}**
Glucose (120 min)	-0.039	0.45	-0.046	0.38
Insulin (0 min)	**0.173**	**7.7×10^{-4}**	0.079	0.13
Insulin (120 min)	-0.039	0.46	-0.044	0.39
HOMA IR	**0.244**	**2.0×10^{-6}**	**0.149**	**4.0×10^{-3}**
HOMA IS	**-0.243**	**2.0×10^{-6}**	**-0.145**	**4.9×10^{-3}**
Lipid metabolism				
Triglycerides	**0.370**	**6.5×10^{-14}**	**0.312**	**4.2×10^{-10}**
Total cholesterol	**0.219**	**1.5×10^{-5}**	**0.211**	**3.3×10^{-5}**
LDL	**0.182**	**3.4×10^{-4}**	0,160	1.7×10^{-3}
HDL	**-0.136**	**7.7×10^{-3}**	-0.063	0.22
Apolipoprotein B	**0.247**	**9.9×10^{-7}**	**0.215**	**2.3×10^{-5}**
Adipokines				
Adiponectin	**-0.215**	**2.2×10^{-6}**	**-0.176**	**5.4×10^{-4}**
Progranulin	**0.151**	**3.0×10^{-3}**	**0.129**	**1.1×10^{-2}**
Vaspin	0.027	0.6	0.05	0.33
Chemerin	**0.103**	**4.3×10^{-2}**	0.065	0.21
FGF21	**0.165**	**2.1×10^{-3}**	**0.152**	**4.7×10^{-3}**
Other				
C-reactive protein	**0.232**	**4.0×10^{-6}**	**0.166**	**1.1×10^{-3}**
Total protein	**0.124**	**1.5×10^{-2}**	0,134	8.6×10^{-3}
Hemoglobin	**0.201**	**7.4×10^{-5}**	**0.143**	**5.1×10^{-3}**
Uric acid	**0.176**	**5.2×10^{-4}**	**0.110**	**3.1×10^{-2}**

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13

bold: significant correlations after adjustment, <u>underlined</u>: significant after Bonferroni adjustment for multiple testing (p<5.2x10$^{-4)}$; r: Spearman rank correlation coefficient, p: significance level; BMI: Body Mass Index; HDL: High density lipoprotein, LDL: Low density lipoprotein, WHR: Waist to hip ratio, FGF21: Fibroblast growth factor 21, HOMA-IR/IS: Homeostasis Model Assessment Insulin Resistance/ Insulin Sensitivity.

Discussion

The role of impaired intestinal permeability in metabolic diseases including obesity and T2D has recently been acknowledged in multiple studies [3, 4, 26]. The tight junction regulator zonulin, which was identified as pre-HP2 by Tripathi et al. [14], is an established circulating marker of intestinal permeability in humans [27, 28]. Here, we aimed to investigate the relationship between intestinal permeability, represented by circulating zonulin, and metabolic traits related to obesity and T2D. We set out to measure zonulin with a widely used commercially available ELISA kit in a metabolically well-characterized cohort of Sorbs from Germany. Considering the identity of zonulin as pre-HP2, we also genotyped the haptoglobin gene in DNA samples from all subjects. The genotype frequency of the HP1/1 genotype corresponded to previously reported frequencies of 15% in a general Caucasian population [16, 17]. Assuming that the measured zonulin is identical with pre-HP2 as reported previously [13], we expected subjects with the HP1/1 genotype to have no detectable, or if taking into account cross-reactions, at least significantly lower zonulin levels. However, concentrations measured in the HP1/1 homozygous subjects were comparable with those found in HP2/2 and HP1/2 carriers.

The epitope used to generate the capture antibody in the zonulin ELISA is based on the sequence GGVLVQPG published by Wang et al. [29] (communication with customer support; Immundiagnostik AG, Bensheim, Germany), which was initially thought to represent the N-terminal sequence of fetal zonulin [29]. However, this sequence is not present in pre-HP2, which has since been proposed to be zonulin by

14

343 the same authors [14]. According to the authors, the discrepancies between the

344 previously reported zonulin sequence and the pre-HP2 sequence may be due to

345 intraspecies variability associated with a high zonulin mutation rate or due to

346 sequencing errors at that time [14]. In summary, the commercially available

347 competitive ELISA does very likely not detect preHP2 or zonulin, but rather unknown

348 targets bound by the antibody raised against the sequence reported by Wang et al

349 [29]. Based on database searches, the epitope would most likely correspond to Glu-

350 Rich2, a protein which shares 7 out of 8 amino acids. The zonulin/preHP2 binding

351 partner CD163 shows some conformity with the epitope [30]. Yet, our mass

352 spectrometry data did not provide any evidence for either protein, but clearly

353 indicated that the major proteins targeted by the ELISA are the complement factor C3

354 and C3 derived fragments, in particular the C3 beta chain (Supplementary Table 2

355 and Figure 2). Properdin, which was also identified in immunoprecipitation

356 experiments, was likely co-immunoprecipitated, as this serum protein binds the

357 complex of C3b and activated complement factor B to positively regulate complement

358 activation via the alternative pathway [31].

359 Complement factor C3 is the merging point of the three routes of the complement

360 system pathways and is a well-known protein involved in various aspects of the

361 primary immune response [32]. By measuring circulating C3 beta chain

362 concentrations in subjects, whose metabolic phenotypes have been extensively

363 characterized, our data suggest that it is upregulated both in diabetic and obese

364 patients. This is in line with previously reported findings showing associations

365 between C3c levels and insulin resistance as measured by HOMA-IR [33]. Moreover,

366 C3 is an established player in lipid metabolism [34, 35]. It is not only produced by

367 hepatocytes, but also by macrophages and adipocytes and it binds to various

368 lipoprotein particles including Apo-A1 complexes, chylomicrons and HDL and is

15

369   potentially involved in the transport of this molecules [34]. Furthermore, C3a-desArg,

370   a constitutively produced C3 fragment (Figure 2C) more commonly known as

371   Acylation-stimulating protein (ASP), is a lipogenic hormone involved in lipid storage

372   and energy homeostasis [36]. ASP is increased in lean and obese diabetic patients

373   [37]. Knockout of the C3a-desArg receptor CC3aR$^{-/-}$ prevents mice from diet-induced

374   insulin resistance [38]. In addition, treatment of these knockout mice as well as

375   knockout rats with C3aR (and C51R) peptide antagonists results in elevated plasma

376   triglycerides [34, 38]. Our data further support a role for C3 in lipid metabolism by

377   showing in various linear stepwise regression models that triglyceride levels and

378   fasting glucose are the strongest independent available variables explaining the

379   observed variance in C3 (Supplementary Table 3). It is noteworthy that numerous

380   studies employing the ELISA kit used in this study reported associations of circulating

381   zonulin with obesity and diabetes similar to our results [19–21], thus strengthening

382   the metabolic correlations observed in our study. Nonetheless, we strongly believe

383   that many of these studies using zonulin as a marker for intestinal permeability would

384   need to revise, or at least reconsider their conclusions (please see Supplementary

385   Table 1 which shows, to the best of our knowledge, a complete listing of all

386   PubMed listed publications using the respective ELISA kit). Rather than showing any

387   relation to intestinal permeability, studies employing the here tested zonulin ELISA kit

388   most likely suggest associations between complement C3 and metabolic traits,

389   although it cannot be ruled out that C3, as a major complement system factor, might

390   be associated with increased intestinal permeability. The factor C3 strongly correlates

391   with insulin resistance [1] and elevated C3 may also be a response to inflammation

392   and bacteria from the "leaky gut" [7, 39, 40]. Further studies are warranted to

393   establish a mechanistic link between C3, intestinal permeability and insulin

394   resistance. Finally, it is also of note, that e.g. in Germany, the zonulin kits analyzed in

16

395 our lab are approved for in-vitro diagnostics of altered intestinal permeability

396 (http://www.immundiagnostik.com/ fileadmin/pdf/Zonulin_K5601.pdf). It is

397 questionable, that even after publication of the protein identity of zonulin as pre-HP2,

398 the commercial ELISA is still sold as specific for zonulin without mention of the

399 inconsistencies in sequence of pre-HP2 and the peptide used for immunization.

400 In conclusion, our study supports the role of the complement C3 β-chain in the

401 pathophysiology of obesity and suggests reconsidering commercially available ELISA

402 kits when investigating zonulin as a marker for altered intestinal permeability.

403

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407

408

409 **Author contributions**

410 LS, PK and JTH conceived the study, designed and conducted experiments,

411 analyzed data and wrote the paper. AT recruited patients. CHI performed mass

412 spectrometry experiments. AC, HH, DS and MS interpreted and analyzed data. All

413 authors discussed results, edited and commented on the manuscript. All the authors

414 have accepted responsibility for the entire content of this submitted manuscript and

415 approved submission.

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417 **Disclosure statement:** The authors have no conflicts of interest to declare.

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528 **Supporting Information**

529 **S1 Table: Publications using the analyzed ELISA kit**

530 **S2 Table: Mass spectrometry results of tryptic digests**

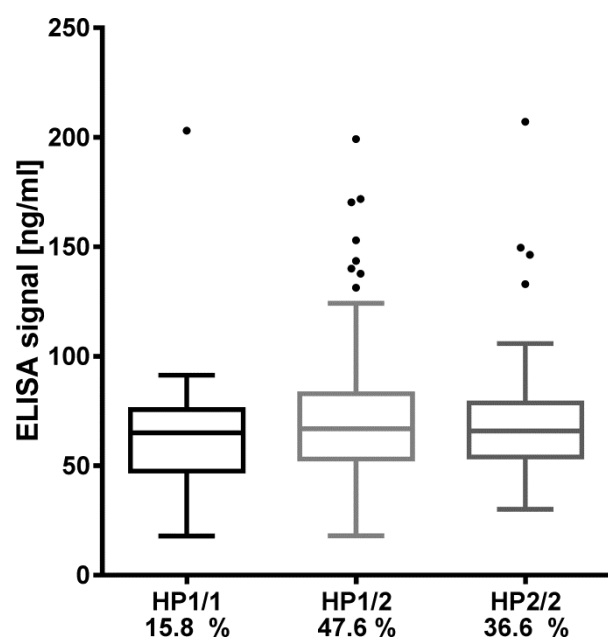531 **S3 Table: Multiple stepwise linear regression analysis**

532

Fig 1. Distribution of zonulin ELISA values according to haptoglobin genotypes.

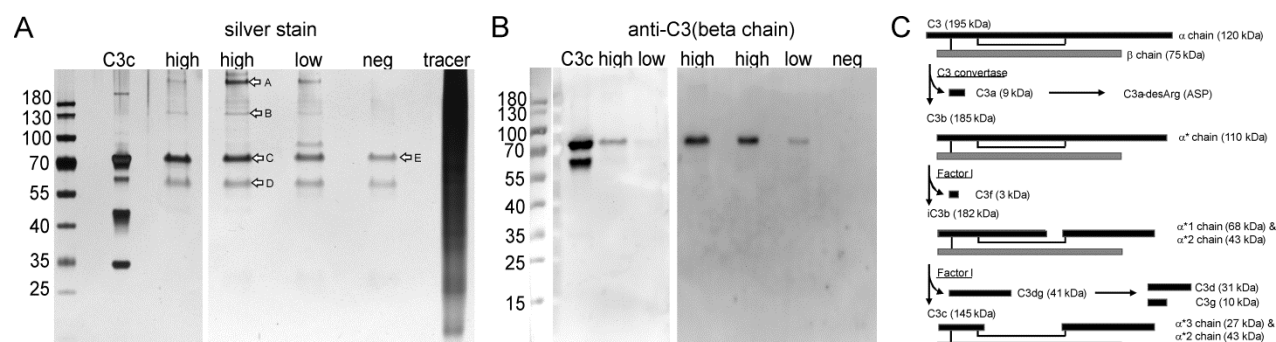Data is presented as boxplots with Turkey-Whiskers and outliers.

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Fig 2. A) Silver stain of immunoprecipitated ELISA products and B) Western blot analyses using an anti-C3 β-chain antibody. (C) Proteolytic processing and resulting fragments of complement C3 protein with corresponding molecular weights. Commercially available C3c protein isolated from human plasma was run as positive control. high: pooled IP samples of sera that gave highest ELISA signals; low: pooled IP samples of sera that gave lowest ELISA signals; neg: negative control using dilution buffer from the ELISA kit; tracer: competitive tracer reagent from the ELISA kit. Letters in (A) indicated bands analyzed by MS after tryptic in gel digestion (Supplementary Table 2).
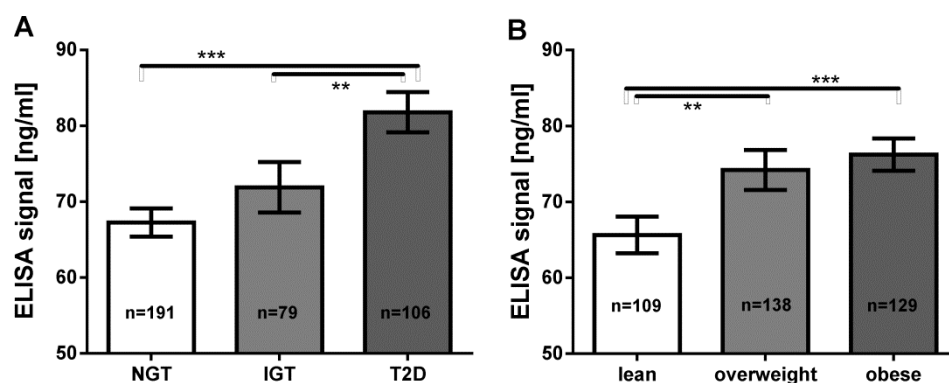
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Fig 3. A) Mean zonulin ELISA values according to glucose tolerance groups B) mean ELISA values according to BMI groups. **: p<0.01; ***: p<0.001

NGT: normal glucose tolerance, IGT: impaired glucose tolerance, T2D: type 2 diabetes