

1 **Widely used commercial ELISA does not detect preHP-2, but recognizes**
2 **properdin as a potential second member of the zonulin family**

3 Running Title: Commercial ELISA for human zonulin detects also properdin

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25 **Keywords:** diabetes - lipid metabolism - pre-Haptoglobin 2 - ELISA - intestinal permeability
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27
28 **Nonstandard abbreviations:** HP, haptoglobin; T2D, type 2 diabetes; WHR, waist-to-hip
29 ratio.

30 **Abstract**

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

35 **METHODS.** Serum zonulin was measured by using a widely used commercial ELISA kit in
36 376 subjects from the metabolically well-characterized cohort of Sorbs from Germany. In
37 addition, haptoglobin genotype was determined in DNA samples from all study subjects.

38 **RESULTS.** As zonulin concentrations did not correlate to the haptoglobin genotypes, we
39 investigated the specificity of the zonulin ELISA assay using antibody capture experiments,
40 mass spectrometry and Western blot analysis. Using serum samples that gave the highest or
41 lowest ELISA signals, we detected several proteins that are likely to be captured by the
42 antibody in the present kit. However, none of these proteins corresponds to pre-haptoglobin2.
43 We used increasing concentrations of recombinant pre-haptoglobin 2 and complement C3 as
44 one of the representative captured proteins and the ELISA kit did not detect either. Western
45 blot analysis using both the polyclonal antibodies used in this kit and monoclonal antibodies
46 rose against zonulin showed a similar protein recognition pattern but with different intensity
47 of detection. The protein(s) measured using the ELISA kit was (were) significantly increased
48 in patients with diabetes and obesity and correlated strongly with markers of the lipid and
49 glucose metabolism. Combining mass spectrometry and Western blot analysis using the
50 polyclonal antibodies used in the ELISA kit, we identified properdin as another member of
51 the zonulin family.

52 **CONCLUSIONS.** Our study suggests that the zonulin ELISA does not recognize pre-
53 haptoglobin 2, rather structural (and possibly functional) analogue proteins belonging to the
54 mannose-associated serine protease family, with properdin being the most likely possible
55 candidate.

56 **Introduction**

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

73 Intestinal barrier transport is mainly regulated by structures of the paracellular pathway called
74 tight junctions which form barriers between epithelial cells and regulate the transport of ions
75 and small molecules across the intestinal lumen. Intestinal permeability is a functional feature
76 of the intestinal barrier. It can be measured by analyzing flux rates of inert molecules across
77 the intestinal wall as a whole or across wall components (1). The gold standard for assessment
78 of intestinal permeability *in vivo* is an assay combining indigestible large and small
79 oligosaccharides, such as lactulose and mannitol; the larger oligosaccharide, lactulose, is only
80 transported via the paracellular pathway, whereas the smaller oligosaccharide, mannitol, is
81 taken up freely over the intestinal barrier via the transcellular route. However, these

82 oligosaccharide assays are expensive, laborious, poorly reproducible, and time consuming.
83 Therefore, identifying appropriate biomarkers for intestinal permeability is highly desirable.
84 Zonulin has been identified as a tight junction regulating protein which is, functionally, the
85 human counterpart of the *Vibrio cholera* endotoxin zonula occludens toxin (12, 13). Precisely,
86 subsequent studies recognized zonulin as the precursor of haptoglobin 2 (pre-HP2) (14).
87 Haptoglobin is a well-known protein involved in scavenging hemoglobin, whereas the
88 function of its precursor is largely unknown. Haptoglobin is first synthesized into a single
89 chain 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 differentiates
91 the HP1 from the HP2 allele. Due to this exon duplication the HP2 α -chain is 1.7kb longer
92 than in the HP1 allele. Haptoglobin is active as tetramer consisting of 2 α - and 2 β -chains
93 linked by disulfide bonds, resulting in three possible genotypes: homozygous HP1/1 and
94 HP2/2 as well as heterozygous HP1/2 (15, 16). About 15 % of the Caucasian population is
95 homozygous for HP (16, 17). Zonulin as pre-HP2 reversibly opens tight junctions and is
96 upregulated in diseases such as celiac disease and type 1 diabetes (T1D) (14, 18). Serum
97 zonulin concentrations are also increased in T2D and obesity (19–21) and strong correlations
98 were observed with various metabolic markers, including fasting plasma glucose, IL-6, HDL,
99 and triglyceride (TG) levels (19–21).

100 Here, we aimed at characterizing the relationship between circulating serum zonulin and traits
101 related to obesity in a metabolically well-characterized cohort of Sorbs from Germany. To
102 measure zonulin, we used the commercially available ELISA kit (Immundiagnostik,
103 Bensheim, Germany). In addition, we determined the haptoglobin genotypes in the entire
104 cohort. Due to a lack of correspondence between the observed circulating zonulin
105 concentrations and the haptoglobin genotypes in our study cohort, we further investigated the
106 possible identity of the product captured by the commercial ELISA assay. We found that the
107 ELISA kit used in the present study does not detect purified pre-HP2 but rather targets one or

108 more proteins from a range of candidate molecules possibly structurally and functionally
109 related to zonulin. Our data also showed that protein concentrations measured by this ELISA
110 correlated with parameters of obesity and related metabolic traits.

111

112 **Materials and Methods**

113 *Study subjects*

114 All subjects are part of a sample from an extensively clinically characterized population from
115 Eastern Germany, the Sorbs (22–24). Extensive phenotyping included standardized
116 questionnaires to assess past medical history and family history, collection of anthropometric
117 data (weight, height, waist-to-hip ratio (WHR)), and an oral glucose tolerance test. Glucose
118 was assessed by the Hexokinase method (Automated Analyzer Modular, Roche Diagnostics,
119 Mannheim, Germany) and serum insulin was measured using the AutoDELFI^A Insulin assay
120 (PerkinElmer Life and Analytical Sciences, Turku, Finland). Total serum cholesterol and TG
121 concentrations were measured by standard enzymatic methods (CHOD-PAP and GPO-PAP;
122 Roche Diagnostics). Serum LDL-C and HDL-C concentrations were determined using
123 commercial homogeneous direct measurement methods (Roche Diagnostics). All assays were
124 performed in an automated clinical chemistry analyzer (Hitachi/ Roche Diagnostics) at the
125 Institute of Laboratory Medicine, University Hospital Leipzig.

126 All blood samples were taken in the morning after an overnight fast and stored at -80°C until
127 analyses. From the 1040 Sorbs enrolled in the cohort, a subgroup of 376 subjects was
128 genotyped for haptoglobin and provided blood samples for zonulin measurements (Table 1).

129 Main metabolic characteristics of the study subjects are summarized in Table 2.

130 The study was approved by the ethics committee of the University of Leipzig and all subjects
131 gave written informed consent before taking part in the study.

132

133

134 ***ELISA measurements***

135 Circulating zonulin was measured by a competitive ELISA (Immundiagnostik AG, Bensheim,
136 Germany) in serum samples of 376 individuals according to the manufacturer's protocol.
137 Inter-assay coefficient of variation was 6.5%. When purified proteins zonulin and properdin
138 and the synthetic peptide AT1001 were tested by the ELISA, they were re-suspended in PBS
139 and diluted in Diluent buffer (IDK kit) to reach a final concentration of 5 µg/ml.

140

141 ***Genotyping***

142 Haptoglobin genotypes were determined by PCR using a method adapted from Koch et al.
143 (17). Briefly, the following two primer pairs were used: A (5'-
144 GAGGGGAGCTTGCCTTTCCATTG-3') and B (5'-GAGATTTTTGAGCCCTGGCTGGT-
145 3'), as well as C (5'-CCTGCCTCGTATTAAGTGCACC AT-3') and D (5'-
146 CCGAGTGCTCCACATAGCCATGT-3'). The primer pair A/B generates two bands: a
147 1,757-bp allele 1 specific band and a 3,481-bp allele 2 specific band. The primer pair C/D
148 produces one allele 2 specific band of 349 bp. The combination of the bands allows a
149 reproducible typing of the two common haptoglobin genotypes HP1 and HP2. In contrast, no
150 band is detectable for the rare HP deletion genotype, present in ~0.1% Caucasians (16).

151

152 ***Antibody capturing experiment***

153 We aimed to isolate the target protein of the ELISA antibody from serum samples utilizing
154 the immobilized anti-zonulin antibodies on the ELISA plates to perform antibody capturing
155 experiments. Based to the manufacturing information, these polyclonal antibodies were raised
156 against an octapeptide sequenced from the zonulin molecule (25). Equal amounts of undiluted
157 serum samples with highest and lowest concentrations of zonulin, as measured using the same
158 ELISA kit, were transferred to separate wells, incubated and washed according to the
159 manufacturer's protocol. Afterwards we eluted the captured protein(s) by incubation with 50

160 μ l of hot (95°C) SDS sample buffer with β -mercaptoethanol for 5 minutes. The captured
161 protein of high or low zonulin serum samples were pooled (N=8 for high (2 pooled groups)
162 and low (1 pooled group); measured protein concentrations using the zonulin ELISA of the
163 serum samples is given in Supplementary Table 1). Twenty μ l of these elution samples (high
164 or low zonulin) were separated by SDS-PAGE using precast Bolt 4-12% Bis-Tris Plus gels
165 (ThermoFisher, Waltham, MA, USA). Proteins were stained using the Pierce silver stain for
166 mass spectrometry (ThermoFisher) or detected by Western Blot.

167

168 *Mass spectrometry*

169 To identify proteins isolated from serum samples as described above, bands were excised
170 from silver-stained gels and in-gel digested with trypsin following a standard protocol (26).
171 After enzymatic digestion, the peptide mixtures were immediately analyzed by LC/MS/MS on
172 an U3000 RSLC nano-HPLC system (Thermo Fisher Scientific) coupled to an Orbitrap
173 Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). Samples were loaded onto a
174 pre-column (RP-C8, 300 μ m * 5 mm, 5 μ m, 100 Å, Thermo Fisher Scientific) and washed
175 with water containing 0.1% (v/v) TFA for 15 min, before the peptides were separated on the
176 separation column (RP-C18, 75 μ m * 250 mm, 2 μ m, 100 Å, Thermo Fisher Scientific) using
177 gradients from 1% to 40% (v/v) B (45 min), 40% to 85% (v/v) B (5 min) followed by 85% B
178 (5 min), with solvent A: 0.1% (v/v) formic acid (FA) in water and solvent B: 0.08% (v/v) FA
179 in acetonitrile. Data were acquired using data-dependent MS/MS mode where each high-
180 resolution full-scan in the orbitrap (m/z 198 to 1,500; R = 120,000) was followed by high-
181 resolution product ion scans in the orbitrap (higher energy collision-induced dissociation
182 (HCD), 27% normalized collision energy, R = 15,000, isolation window 2 Th) within 5 s,
183 starting with the most intense signal in the full-scan mass spectrum.

184 Data analysis was performed using the Proteome Discoverer 2.1 (Thermo Fisher Scientific).
185 MS/MS data of precursor ions (m/z range 350-5,000) were searched against the Swissprot

186 Database (version 11/2016, taxonomy human, 20,082 entries) and a contaminant database
187 using Sequest HT. Mass accuracy was set to 5 ppm and 20 mmu for precursor and fragment
188 ions, respectively. Carbamidomethylation of cysteines was set as fixed modification;
189 oxidation of methionines and N-terminal acetylation were set as variable modifications, two
190 missed cleavages of trypsin were allowed. Results were filtered for non-contaminant proteins
191 identified by at least three unique highly confident peptides (peptide FDR \leq 1%).

192

193 *Western blot analysis*

194 Western blot experiments were performed to validate the results of mass spectrometric data
195 analysis and to compare the serum target proteins identified by the polyclonal antibodies used
196 by this ELISA kit compared to monoclonal anti-zonulin antibodies. Gels were blotted on a
197 PVDF membrane and Western blots were probed with anti-C3 β -chain (1:2000) (Biozol,
198 Eching, Germany), anti-haptoglobin (1:1000) (Abcam, Cambridge, UK), polyclonal anti-
199 zonulin (1:500) (kindly provided by Immundiagnostik), monoclonal anti-zonulin (1:5000)
200 (BioRad, Hercules, CA USA) antibodies. Purified C3c from plasma (Athens Research,
201 Athens, Georgia, USA) and recombinant zonulin were used as positive controls. Properdin
202 (R&D Systems Minneapolis, MN, U.S.A.) was also used to validate the potential protein
203 candidate identified by the ELISA. Incubation with primary and secondary antibodies (HRP-
204 conjugated) was done for 90 minutes at room temperature. Blots were visualized by enhanced
205 chemiluminescence using Pierce ECL Western Blotting Substrate (ThermoFisher).

206

207 *Statistical analysis*

208 Statistical analysis was performed using with SPSS 24 (IBM). All non-normally distributed
209 metric parameters were log transformed to generate a Gaussian normal distribution.
210 Spearman's rank correlation method was used to assess the relationship between metabolic
211 traits. To test for significant differences in distribution for ordinal values, the Kruskal-Wallis-

212 test was used. Exact differences between two groups were tested by both the Mann-Whitney-
213 U test and unpaired student's t-test. In addition, multiple linear regression analyses were done
214 to assess the linear relationship between continuous variables and genotypes. For all tests, a p-
215 value <0.05 was considered to be statistically significant.

216

217 **Results**

218 *Haptoglobin genotype*

219 Haptoglobin genotype HP1/1 was present in 15.8% of the subjects, HP1/2 in 47.6% and
220 HP2/2 in 36.6%. These frequencies are comparable to the distribution of HP genotypes in
221 cohorts of European ancestry reported by others (16, 17). We tested the association of the HP
222 genotypes with various anthropometric and metabolic traits in all study subjects. The analysis
223 revealed that blood hemoglobin levels significantly increase with the presence of at least one
224 HP2 allele (p=0.004 over all three groups, $p=4.2 \times 10^{-4}$ between HP1 homozygote and HP2
225 carriers). Furthermore, we observed that the total protein concentration in the urine
226 significantly differed between the three groups, with an increase in the HP2 carriers
227 (p=0.027). Interestingly, mean triiodothyronine (fT3) levels were lower in the HP1/1 group
228 than in the HP2/2 group (p=0.012) and in accordance, an increase in administered thyroid
229 gland hormones (p=0.023) was observed.

230

231 *Zonulin ELISA data do not match HP genotype distribution*

232 Strikingly, there were no significant differences in levels of the zonulin ELISA signal
233 between the three haptoglobin genotype groups (Figure 1; p=0.153 using ANOVA, p=0.07 for
234 the t-test comparing log transformed zonulin signals between HP1/1 vs. HP1/2 + HP2/2).
235 Assuming that the protein measured by the kit is zonulin (i.e. pre-HP2), subjects with the
236 HP1/1 genotype were expected to have no detectable zonulin levels. As the zonulin
237 concentrations measured in patient sera using the zonulin ELISA kit clearly did not reflect the

238 HP genotype distribution we therefore concluded that the protein measured by the kit is not
239 identical to zonulin as pre-HP2 or that, beside pre-HP2, the kit detects other unrelated
240 proteins. Consequently, we aimed at identifying the protein(s) detected by the alleged zonulin
241 ELISA kit.

242

243 ***The zonulin ELISA does not detect recombinant pre-HP2, but targets multiple proteins***

244 To attempt identifying proteins bound and quantified by the capturing antibody deployed in
245 the zonulin ELISA kit, we performed an immune-capturing experiment using patient sera and
246 the immobilized antibody of the ELISA kit as supplied. After incubation of the immobilized
247 ELISA kit antibodies with selected patient sera representing the highest and lowest measured
248 ELISA signals in the cohort, the captured proteins were separated by SDS-PAGE. Notably,
249 we could not measure the protein content of the eluted samples, but given that equal amounts
250 of serum were used, that the same washing and elution procedure was performed for all
251 samples and that 20 μ l of the pooled elution samples were used for SDS-PAGE and Western
252 blot, the detected amount of “captured” protein should resemble the amount of protein that
253 was present in the initial serum sample.

254 Silver staining revealed multiple bands, with the most intense band at ~70 kDa and further
255 prominent bands at ~55 kDa, ~180 kDa and >180kDa (Figure 2A). This band pattern was
256 incompatible with a band pattern that would be expected for pre-HP2 or haptoglobin-derived
257 proteins and further supported the results demonstrating the lack of correspondence of the
258 captured protein with HP genotypes. To further characterize major proteins captured by the
259 ELISA kit, protein bands were cut (Figure 2A) and subjected to MS analysis after tryptic
260 digestion (Supplementary Table 2). Mass spectrometry demonstrated that bands 1 (>180kDa),
261 2 (~150 kDa), 3 (~70 kDa) were all very likely representing the C3 protein or cleavage
262 products derived from the C3 protein, such as the C3 precursor (187 kDa), C3c (144 kDa) and
263 the C3 β -chain (71 kDa). Furthermore, the 55 kDa band was identified as properdin or factor

264 P (MW 53 kDa). To validate results from mass spectrometry, we performed Western blot
265 analyses. The major band at 70 kDa was clearly detected by an anti-C3 β -chain antibody
266 (Figure 2B).

267 Consequently, we tested several C3c proteins from different suppliers (Abcam, Cambridge,
268 UK; Athens Research, Athens, Georgia, USA; mybiosource, San Diego, California, USA), in
269 a range from 0.1 mg/ml to 0.1 ng/ml under native and denatured conditions, as well as diluted
270 in serum, with the respective ELISA kit. All results were negative (data not shown),
271 indicating that C3 might represent a contaminant only. Additionally, we tested C3,
272 recombinant zonulin, HP1, and HP2 at increasing concentrations (range 1-50 μ g/ml), along
273 with sera from celiac patients (both HP1-1 and HP2-2), healthy controls (both HP1-1 and
274 HP2-2), and our standard control (AF, HP2-2). The results showed in Figure 3 demonstrated
275 that while this kit does not recognize C3, zonulin, or mature haptoglobin (both HP1 and HP2),
276 it does recognize protein(s) both in HP1-1 and HP2-2 genotype subjects, irrespective of their
277 disease status.

278

279 **Anti-zonulin monoclonal antibodies and Immundiagnostik polyclonal antibodies**
280 **recognize same target proteins in Western blot.**

281 Our ELISA results clearly showed that this kit does not detect zonulin (alias, pre-HP2 as
282 originally claimed by the manufacturer) or complement C3, the protein captured by our
283 experiments described above. In order to further characterize the target protein(s) of this kit,
284 we performed Western blotting analysis of sera from a HP2-2 subject either under baseline
285 condition or after deglycosylation as we have previously described (14), using recombinant
286 zonulin as control. As anticipated, the zonulin monoclonal antibodies recognized recombinant
287 zonulin as well as a variety of bands in the serum sample, including bands with a MW of ~70,
288 52, 37, 27, and 16 kDa (Figure 4A). Based on similar patterns we detected when zonulin was
289 originally cloned (14), we predicted that the ~72 kDa corresponded to the glycosylated HP β -

290 chain, the 52 kDa zonulin, and the 16 kDa the HP2 α -chain. To confirm this interpretation, we
291 performed deglycosylation experiments showing the shift of the 72 kDa β -chain to a lower
292 MW, while, as anticipated, the zonulin band and the HP2 α -chain remained unchanged
293 (Figure 4A). Interestingly enough, the Immundiagnostik polyclonal antibodies raised against
294 the zonulin synthetic peptide inhibitor GGVLVQPG (AT1001) (25) recognized the same main
295 bands detected by the monoclonal antibodies but with different intensity, being the
296 recombinant zonulin and serum α -chain bands fainter compared to the monoclonal antibody
297 signal, while the serum β -chain and serum zonulin-like signals stronger (Figure 4A). When
298 combined to our ELISA results, these data suggest that, while the zonulin monoclonal
299 antibodies specifically detect in serum samples only zonulin at its predicted molecular weight,
300 most likely the polyclonal antibodies used in this kit are possibly detecting “zonulin-like”
301 protein(s) (as suggested by the much more intense signal of the ~52 kDa band compared to
302 the monoclonal antibodies) with similar molecular weight, structure, and possibly function.

303

304 **Immundiagnostik polyclonal antibodies recognize both zonulin as well as properdin as**
305 **an additional target in Western blot**

306 Among all the proteins we identified with our mass spec analysis, properdin fulfills the
307 structural-functional characteristics mentioned above and, therefore, may represent the most
308 likely candidate target detected by this ELISA kit. To explore this hypothesis, we performed
309 both Western blotting analysis using the Immunodiagnostik polyclonal antibodies and ELISA
310 test using commercially available properdin. The Western blot showed that beside
311 recombinant zonulin, the Immunodiagnostik polyclonal antibodies also detect properdin (Fig
312 4B) that migrated at the same molecular weight of zonulin and serum bands recognized by the
313 antibodies in both HP2-2 and HP 1-1 subjects (Fig 4B). Similar results were obtained with
314 polyclonal antibodies raised against recombinant zonulin (data not shown). The same samples
315 used for Western blotting analysis, including zonulin, properdin and AT1001 at a

316 concentration of 5 µg/ml were tested using the ELISA kit. The results showed that properdin
317 and AT1001 were both detected by the ELISA test, however, their amounts were substantially
318 underestimated by 914 (5.47 ng/ml) and 40 folds (126.04 ng/ml), respectively. However,
319 despite zonulin being detected in Western blot, it was not detected with the ELISA kit (Figure
320 3). In comparison, serum from subjects with HP 1-1 and HP 2-2 genotype were measured at a
321 concentration of 67.54 and 56.41 ng/ml, respectively (Table 3).

322

323 *Correlations of measured protein concentrations using the ELISA with metabolic traits*

324 In a sample of 376 subjects tested using the purchased zonulin ELISA, the product was
325 measured in a mean concentration of 72.2 ± 27.2 ng/ml (mean \pm standard deviation), ranging
326 from 17.8 to 207.1 ng/ml. The ELISA signal was significantly increased in subjects with T2D
327 (81.78 ± 25.31 ng/ml) compared to subjects with normal glucose tolerance (67.25 ± 25.45
328 ng/ml, Mann-Whitney-U test; $p=2.1 \times 10^{-8}$) or impaired glucose tolerance (71.88 ± 29.36
329 ng/ml, $p=0.0017$) (Figure 5A). Additionally, lean subjects had significantly lower values
330 (65.64 ± 25.23 ng/ml) than subjects with overweight (74.20 ± 30.68 ng/ml, $p=0.0082$) or
331 obesity (76.24 ± 24.17 ng/ml, $p=7.0 \times 10^{-5}$) (Figure 5B). We observed no gender differences or
332 any correlations with age (data not shown). The ELISA signal correlated with traits related to
333 glucose and lipid metabolism (Spearman's rank correlation test, adjusted for age, sex and
334 BMI; Table 4). It was positively correlated with BMI, HOMA-IR and -IS and fasting plasma
335 glucose (Table 4). Strong correlations were also observed for lipid metabolism parameters,
336 such as TG levels, total cholesterol, LDL and apolipoprotein B (Table 4). Correlations were
337 tested for a total of 95 accessible traits. After Bonferroni correction for multiple testing
338 (adjusted p-value for significance $p < 5.2 \times 10^{-4}$), correlations for BMI ($p=1.2 \times 10^{-5}$), fasting
339 glucose ($p=9.0 \times 10^{-6}$), TG ($p=4.2 \times 10^{-10}$), total cholesterol ($p=3.3 \times 10^{-5}$) and apolipoprotein B
340 ($p=2.3 \times 10^{-5}$) remained statistically significant.

341

342

343

344 **Discussion**

345 The role of impaired intestinal permeability in metabolic diseases including obesity and T2D
346 has recently been acknowledged in multiple studies (3, 4, 27). The tight junction regulator
347 zonulin, which was identified as pre-HP2 by Tripathi et al. (14), is an established circulating
348 marker of intestinal permeability in humans (28, 29). Here, we aimed to investigate the
349 relationship between intestinal permeability, represented by circulating zonulin, and metabolic
350 traits related to obesity and T2D. We set out to measure zonulin with a widely used
351 commercially available ELISA kit in a metabolically well-characterized cohort of Sorbs from
352 Germany. Considering the identity of zonulin as pre-HP2, we also genotyped the haptoglobin
353 gene in DNA samples from all subjects. The genotype frequency of the HP1/1 genotype
354 corresponded to previously reported frequencies of 15% in populations of European ancestry
355 (16, 17). Assuming that the measured zonulin is identical with pre-HP2 as reported previously
356 (13), we expected subjects with the HP1/1 genotype to have no detectable, or if taking into
357 account cross-reactions, at least significantly lower zonulin levels. However, concentrations
358 measured in the HP1/1 homozygous subjects were comparable with those found in HP2/2 and
359 HP1/2 carriers.

360 The epitope used to generate the capture antibody in the zonulin ELISA is based on the
361 sequence GGVLVQPG published by Wang et al. (25) (communication with customer
362 support; Immundiagnostik AG, Bensheim, Germany), which was initially thought to represent
363 the N-terminal sequence of fetal zonulin (25). However, this sequence is not present in pre-
364 HP2, which has since been proposed to be zonulin by the same authors (14). According to the
365 authors, the discrepancies between the previously reported zonulin sequence and the pre-HP2
366 sequence may be due to intraspecies variability associated with a high zonulin mutation rate
367 or due to sequencing errors at that time (14). In summary, the commercially available

368 competitive ELISA does very likely not detect preHP2 or zonulin, but rather unknown targets
369 bound by the antibody raised against the sequence reported by Wang et al (25).
370 Based on database searches, the epitope could correspond to Glu-Rich2, a protein which
371 shares 7 out of 8 amino acids. The zonulin/preHP2 binding partner CD163 shows some
372 conformity with the epitope (30). Our antibody capture experiment and subsequent mass
373 spectrometry analysis did not provide any evidence for either protein. The most abundant
374 protein identified by MS, C3, is evidently an unspecific product overshadowing the real
375 targets. Indeed, the respective ELISA kit did not detect any complement C proteins obtained
376 from different suppliers when tested under native and denatured conditions, as well as diluted
377 in serum. Also, the same kit did not detect recombinant zonulin, mature HP1 or mature HP2.
378 Considering the additional MS hits, a few proteins stand out, although, without further
379 validation, we interpret these data with caution, since only more abundant proteins may be
380 identified by MS analysis, while our protein(s) of interest may be in low abundance in serum
381 samples and, therefore, not identifiable with this approach. The van Willebrand factor (vWF,
382 band A, Figure 2) is involved in the intrinsic coagulation pathway and the acute phase
383 response and known to be increased in inflammatory bowel disease and bacterial diarrhea
384 (31). Inter-alpha-inhibitor heavy chain 4 (band B, Figure 2) a large glycoprotein cleaved into
385 smaller fragments by Kallikrein which is also involved in the intrinsic coagulation pathway.
386 One of these fragments, called urinary trypsin-inhibitor, attenuates LPS-induced endothelial
387 barrier dysfunction by upregulation of vascular endothelial-cadherin expression (32).
388 Complement component 9 (C9, band C, Figure 2) is an important component of the
389 membrane attacking complex within the complement cascade and is required for complement-
390 mediated lipopolysaccharide release and outer membrane damage in bacteria (33). Protein
391 S100-A8, or Calprotectin, (band D, Figure 2) is a calcium- and zinc-binding protein which
392 plays a prominent role in the regulation of inflammatory processes and immune response (34).
393 However, based on the data presented in Figure 4, our most likely candidate(s) should be in

394 the ~50 kDa range, where the polyclonal antibody signal was stronger. Therefore, the most
395 interesting candidate protein we have identified is properdin or factor P (band D, Figure 2), a
396 member of the complement alternative pathway that has a molecular weight (53 kDa), within
397 the range of those proteins recognized by the Immundiagnostik anti-AT1001 polyclonal
398 antibodies (Figures 4A and B) and serum levels (~25 ng/ml) (35) similar to the range of
399 detection of the kit. Our combined Western blot analysis (Figure 4B) and ELISA test
400 confirmed that the polyclonal antibodies raised against AT1001 detect properdin amongst
401 other proteins. However, when purified proteins/peptides, including the AT1001 peptide used
402 to raise the polyclonal antibodies, which is also used as internal control in the ELISA kit,
403 were tested by ELISA, they were highly under-estimated by the test. One possible explanation
404 for these results is that zonulin and also properdin are not the main targets detected by the
405 ELISA, however the fact that even AT1001 was under-estimated seems to suggest that this
406 hypothesis cannot entirely explain our results. Alternatively, it is possible that tertiary and
407 quaternary (multimers) structure arrangements present in sera samples but not in recombinant
408 proteins are necessary in order to properly detect both zonulin and properdin by this ELISA.
409 If this is the case and/or that the main target of this ELISA is/are additional proteins in the
410 ~50kDa range present in human serum remains to be established.

411 Once released from neutrophils, T cells and macrophages in response to acute microbial
412 exposure, properdin causes production of chemotactic anaphylatoxin C3a and C5a with
413 subsequent formation of immune complexes that cause increased endothelial permeability
414 (35). Intriguingly, zonulin as pre-HP2 also causes generation of C3a and C5a, with
415 subsequent increased vascular permeability in several districts, including the lung, with
416 subsequent onset of acute lung injury (36). Another striking similarity between zonulin and
417 properdin is the fact that both are associated to viral respiratory tract infections (37, 38).

418 Notably, the peptide sequence used for the generation of the antibody is also not present in
419 any of the discussed proteins. The five proteins mentioned above, including properdin, have

420 ~50% similarity to this epitope. Zonulin as preHP-2 is a member of a larger family of tight
421 junction regulating proteins. Indeed, phylogenetic analyses suggest that haptoglobins evolved
422 from mannose-associated serine protease (MASP), a complement-associated protein (like
423 properdin), with their alpha-chain containing a complement control protein (CCP) (this
424 domain activates complement similarly to properdin), while the β -chain is related to
425 chymotrypsin-like serine proteases (SP domain) (39, 40). However, the SP domain of HP
426 lacks the essential catalytic amino acid residues required for protease function; structure-
427 function analyses have implicated this domain in receptor recognition and binding (41).
428 Although not a serine protease, zonulin shares approximately 19% amino acid sequence
429 homology with chymotrypsin, and their genes both map on chromosome 16. Alignment of the
430 β -chain sequence of zonulin to that of several serine proteases is remarkably consistent except
431 for an insertion of 16 residues in the region corresponding to the methionyl loop of the serine
432 proteases. Comparison of the zonulin α - β junction region with the heavy-light-chain junction
433 of tissue-type plasminogen activator strengthens the evolutionary homology of zonulin and
434 serine proteases. The active-site residues typical of the serine proteases, His57 and Ser195,
435 are replaced in zonulin by lysine and alanine, respectively. Because of these mutations, during
436 evolution zonulin most likely lost its protease activity despite that zonulin and serine
437 proteases evolved from a common ancestor (18). Therefore, zonulin, and the serine proteases
438 represent a striking example of homologous proteins with different biological functions but
439 with the common characteristic of complement activation. Beside zonulin and properdin,
440 other members of the MASP family include a series of plasminogen-related growth factors
441 (epidermal growth factor (EGF), hepatocyte growth factor (HGF), etc.) involved in cell
442 growth, proliferation, differentiation and migration, and disruption of intercellular junctions.
443 In light of these considerations, other MASP members identified in our capturing experiments
444 in general, and properdin in particular, are intriguing possible targets (36).

445 Analyzing the protein concentrations measured using this ELISA in subjects who have been
446 extensively characterized for metabolic phenotypes, our data suggest that it is upregulated
447 both in diabetic and obese patients. This is in line with previously reported findings using this
448 ELISA kit (19–21). Our data shows, that the ELISA target is potentially involved in the lipid
449 metabolism by showing in various linear stepwise regression models that triglyceride levels
450 and fasting glucose are the strongest independent available variables explaining the observed
451 variance in measured protein concentrations (Supplementary Table 3).

452 It is important to note, that our study, as it has not been initially designed to address the
453 question of ELISA specificity, has clear limitations. To obtain amounts of isolated protein in
454 the antibody capture experiments, we needed to change the experimental protocol provided by
455 the manufacturer. We used undiluted serum samples instead of a 50-fold dilution, which very
456 likely increased the risk of non-specific binding. Yet, we used sera from patient that exhibited
457 the highest and the lowest concentrations measured by the ELISA kit using the
458 manufacturer's protocol. Thus, any non-specific binding should be detected as equally strong
459 bands in the silver stained gels after the antibody capture experiment. Yet, we observed band
460 intensities of affinity purified protein that clearly correlated with the concentrations measured
461 using the ELISA, in the silver stained gels (total protein) and in the Western blot using the
462 anti-C3- β -chain antibody, indicating a specific isolation of proteins recognized by the kit
463 antibody. Nevertheless, ELISA results that failed to recognize C3 disputed the notion that
464 complement C3 is the target of this kit. Also, we have performed this experiment using two
465 different Lot no. of the ELISA, using sera from eight different patients of each high and low
466 concentration and obtained the same results.

467 In conclusion, based on our data we suggest that the Immundiagnostik ELISA kit supposedly
468 testing serum zonulin (pre-HP2) levels could identify a variety of proteins structurally and
469 possibly functionally related to zonulin, suggesting the existence of a family of zonulin
470 proteins as previously hypothesized (42), rather than a single member of permeability-

471 regulating proteins. Additional studies are necessary to establish the primary target proteins
472 (zonulin, properdin and/or other structurally similar proteins) detected by this commercially
473 available ELISA.

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485

486 **Author contributions**

487 LS, PK and JTH conceived the study, designed and conducted experiments, analyzed data and
488 wrote the paper. AT recruited patients. CHI performed mass spectrometry experiments. AC,
489 HH, DS and MS interpreted and analyzed data. RF performed the zonulin ELISA test, MF
490 performed the Western blotting analysis, and AF critically revised the manuscript, contributed
491 to the study design of some of the performed experiments, and provided critical interpretation
492 of the data. All authors discussed results, edited and commented on the manuscript. All the
493 authors have accepted responsibility for the entire content of this submitted manuscript and
494 approved submission.

495

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

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623 **Tables**

624 **Table 1: Composition of the study cohort included in zonulin measurements.**

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

625 Age and BMI are mean ± SD, NGT, IGT, T2D as absolute number (percentage); NGT:
 626 normal glucose tolerance; IGT: impaired glucose tolerance; IFG: impaired fasting glucose
 627

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

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

629

630 *: p<0.05 vs NGT; **: p<0.01 vs NGT

631 °: p<0.05 vs IGT; °°: p<0.01 vs IGT

632 All data given as mean ± standard deviation, NGT: normal glucose tolerance, IGT: impaired
 633 glucose tolerance, T2D: type 2 diabetes, CRP: C-reactive protein, HDL: High density
 634 lipoprotein, LDL: Low density lipoprotein, FPG: Fasting plasma glucose, WHR: Waist to hip
 635 ratio

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Table 3: Correlation of ELISA signal with metabolic phenotypes.

	<i>Non-adjusted</i>		<i>Adjusted for age, sex, BMI</i>	
	<i>r</i>	<i>p-value</i>	<i>r</i>	<i>p-value</i>
<i>Anthropometric trait</i>				
<i>Age</i>	0.31	0.55	-	-
<i>Sex</i>	-0.005	0.93	-	-
<i>BMI</i>	0.221	1.2x10⁻⁵	-	-
<i>WHR</i>	0.172	7.3x10⁻⁴	0.118	2.1 x10⁻²
<i>Glucose metabolism</i>				
<i>Glucose (0 min)</i>	0.294	6.6x10⁻⁹	0.227	9.0 x10⁻⁶
<i>Glucose (120 min)</i>	-0.039	0.45	-0.046	0.38
<i>Insulin (0 min)</i>	0.173	7.7x10⁻⁴	0.079	0.13
<i>Insulin (120 min)</i>	-0.039	0.46	-0.044	0.39
<i>HOMA IR</i>	0.244	2.0x10⁻⁶	0.149	4.0 x10⁻³

<i>HOMA IS</i>	-0.243	2.0x10⁻⁶	-0.145	4.9 x10⁻³
<i>Lipid metabolism</i>				
<i>Triglycerides</i>	<u>0.370</u>	<u>6.5x10⁻¹⁴</u>	<u>0.312</u>	<u>4.2 x10⁻¹⁰</u>
<i>Total cholesterol</i>	<u>0.219</u>	<u>1.5x10⁻⁵</u>	<u>0.211</u>	<u>3.3x10⁻⁵</u>
<i>LDL</i>	0.182	3.4x10⁻⁴	0.160	1.7 x10⁻³
<i>HDL</i>	-0.136	7.7x10⁻³	-0.063	0.22
<i>Apolipoprotein B</i>	<u>0.247</u>	<u>9.9x10⁻⁷</u>	<u>0.215</u>	<u>2.3x10⁻⁵</u>
<i>Adipokines</i>				
<i>Adiponectin</i>	-0.215	2.2x10⁻⁶	-0.176	5.4x10⁻⁴
<i>Progranulin</i>	0.151	3.0x10⁻³	0.129	1.1 x10⁻²
<i>Vaspin</i>	0.027	0.6	0.05	0.33
<i>Chemerin</i>	0.103	4.3x10⁻²	0.065	0.21
<i>FGF21</i>	0.165	2.1x10⁻³	0.152	4.7x10⁻³
<i>Other</i>				
<i>C-reactive protein</i>	0.232	4.0x10⁻⁶	0.166	1.1x10⁻³
<i>Total protein</i>	0.124	1.5x10⁻²	0.134	8.6x10⁻³
<i>Hemoglobin</i>	0.201	7.4x10⁻⁵	0.143	5.1x10⁻³
<i>Uric acid</i>	0.176	5.2x10⁻⁴	0.110	3.1 x10⁻²

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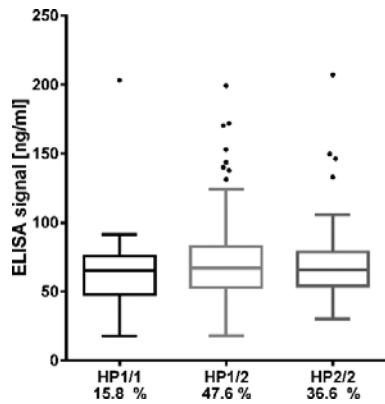
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bold: significant correlations after adjustment, underlined: significant after Bonferroni adjustment for multiple testing ($p < 5.2 \times 10^{-4}$); r: Spearman rank correlation coefficient, p: significance level; BMI: Body Mass Index; HDL: High density 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.

645 **Figure legends**

646



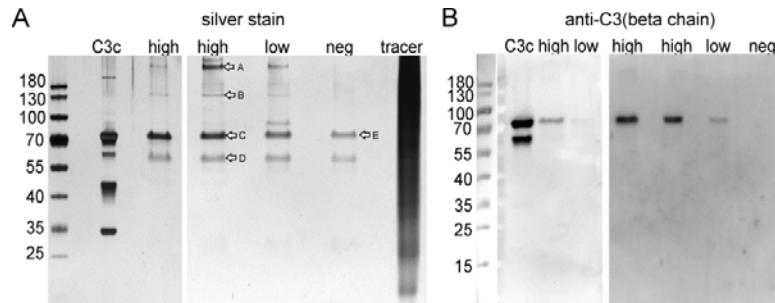
647

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

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

650

651



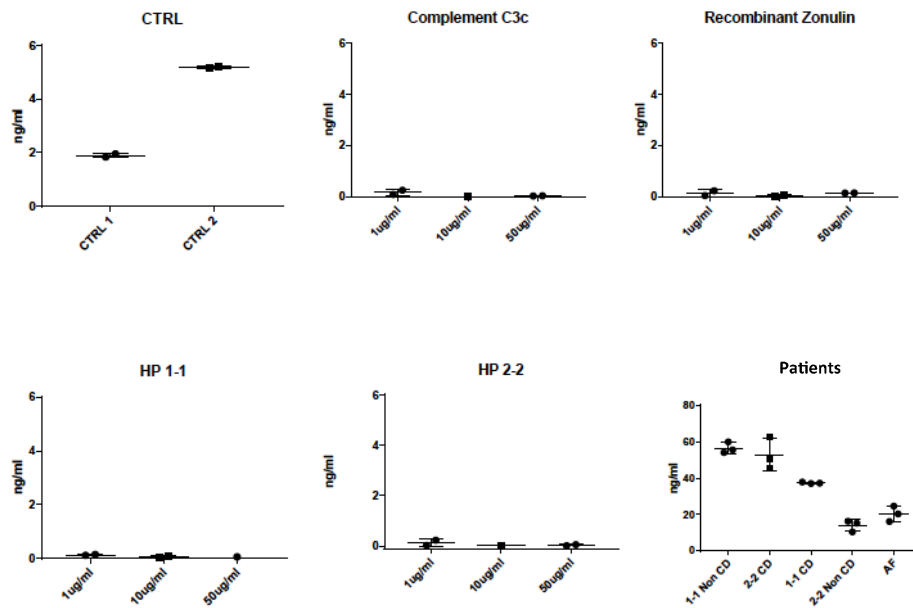
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653 **Fig 2. A) Silver stain of immunoprecipitated ELISA products and B) Western blot**
654 **analyses using an anti-C3 β -chain antibody.**

655 Commercially available C3c protein isolated from human plasma was run as positive control.
656 high: pooled IP samples of sera that gave highest ELISA signals; low: pooled IP samples of
657 sera that gave lowest ELISA signals; neg: negative control using dilution buffer from the
658 ELISA kit; tracer: competitive tracer reagent from the ELISA kit. Letters in (A) indicated
659 bands analyzed by MS after tryptic in gel digestion (Supplementary Table 2).

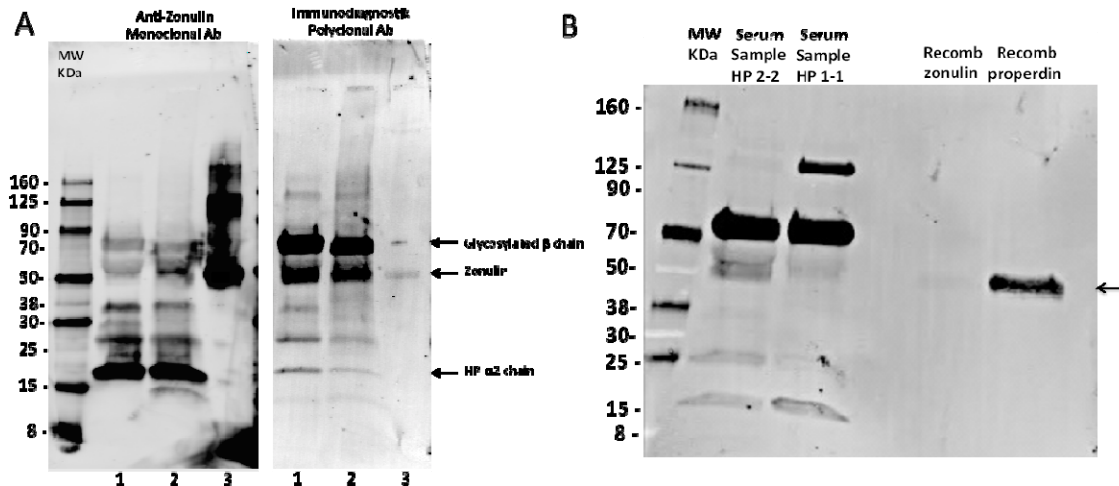
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663 **Fig 3. Mean zonulin ELISA values obtained by testing specific candidate target proteins**
 664 **and patients' serum samples.** This ELISA kit did not detect increasing concentrations (range
 665 1-50 µg/ml) of complement, recombinant zonulin (pre-HP2), mature HP1 or mature HP2.
 666 Conversely, a strong signal was detected in sera of both celiac disease patients (CD) and
 667 healthy controls (non-CD, internal control AF with HP2-2), irrespective of their HP genotype.
 668 All samples are technical triplicates, kit controls are shown for comparison.
 669



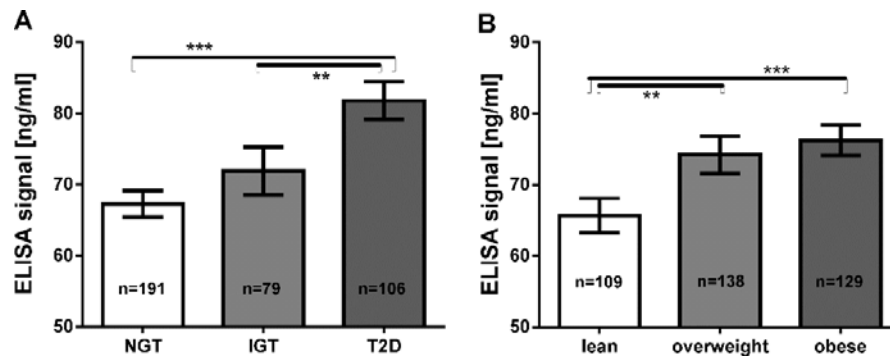
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672 **Fig 4. Western blot analyses of a prototype human serum sample (AF HP2-2) using both**
 673 **anti-zonulin monoclonal antibodies and Immundiagnostik polyclonal antibodies.** (A)
 674 Prototype serum sample of a HP2-2 homozygous subject, either untreated (lane 1) or after
 675 deglycosylation (lane 2) was resolved and then immunoblotted using either anti-zonulin
 676 monoclonal antibodies (left panel) or Immundiagnostik polyclonal antibodies (right panel).
 677 Recombinant zonulin was added as control (lane 3). As anticipated, zonulin monoclonal
 678 antibodies recognize the recombinant protein as well as a series of serum bands, with the
 679 strongest signal being related to the 16kDa zonulin α2-chain. The Immundiagnostik
 680 polyclonal antibodies also recognize recombinant zonulin but with a much weaker signal

681 compared to the monoclonal antibodies. These antibodies also recognize the same serum
682 bands detected by the zonulin monoclonal antibodies, with the ~70 and 52 kDa bands being
683 highlighted with the strongest signal. Sample's deglycosylation showed the shift of the 70
684 kDa band to a lower MW, suggesting that this may represent the zonulin β -chain as we have
685 previously shown (14). (B) Prototype serum samples of a HP1-1 and a HP2-2 homozygous
686 subject were resolved and immunoblotted using the Immundiagnostik polyclonal antibodies.
687 Recombinant zonulin and properdin were added as control. The antibodies also detect
688 properdin that migrated at the same molecular weight of zonulin and serum bands recognized
689 by the antibodies in both HP2-2 and HP 1-1 subjects.
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693 **Fig 5. A) Mean zonulin ELISA values according to glucose tolerance groups B) mean**
694 **ELISA values according to BMI groups.** **: p<0.01; ***: p<0.001
695 NGT: normal glucose tolerance, IGT: impaired glucose tolerance, T2D: type 2 diabetes
696

697 Supporting Information

698 S1 Table: Characteristics of serum samples used for antibody capture experiments

699 S2 Table: Mass spectrometry results of tryptic digests

700 S3 Table: Multiple stepwise linear regression analysis