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179	Abstract	<p><b>Aims/hypothesis:</b> Recent European guidelines for non-alcoholic fatty liver disease (NAFLD) call for reference values for HOMA-IR. In this study, we aimed to determine: (1) the upper limit of normal HOMA-IR in two population-based cohorts; (2) the HOMA-IR corresponding to NAFLD; (3) the effect of sex and <i>PNPLA3</i> genotype at rs738409 on HOMA-IR; and (4) inter-laboratory variations in HOMA-IR.</p> <p><b>Methods:</b> We identified healthy individuals in two population-based cohorts (FINRISK 2007 [<math>n = 5024</math>] and the Programme for Prevention of Type 2 Diabetes in Finland [FIN-D2D; <math>n = 2849</math>]) to define the upper 95th percentile of HOMA-IR. Non-obese individuals with normal fasting glucose levels, no excessive alcohol use, no known diseases and no use of any drugs were considered healthy. The optimal HOMA-IR cut-off for NAFLD (liver fat <math>\geq 5.56\%</math>, based on the Dallas Heart Study) was determined in 368 non-diabetic individuals (35% with NAFLD), whose liver fat was measured using proton magnetic resonance spectroscopy (<math>^1\text{H-MRS}</math>). Samples from ten individuals were simultaneously analysed for HOMA-IR in seven European laboratories.</p> <p><b>Results:</b> The upper 95th percentiles of HOMA-IR were 1.9 and 2.0 in healthy individuals in the FINRISK (<math>n = 1167</math>) and FIN-D2D (<math>n = 459</math>) cohorts. Sex or <i>PNPLA3</i> genotype did not influence these values. The optimal HOMA-IR cut-off for NAFLD was 1.9 (sensitivity 87%, specificity 79%). A HOMA-IR of 2.0 corresponded to normal liver fat (<math>&lt; 5.56\%</math> on <math>^1\text{H-MRS}</math>) in linear regression analysis. The 2.0 HOMA-IR measured in Helsinki corresponded to 1.3, 1.6, 1.8, 1.8, 2.0 and 2.1 in six other laboratories. The inter-laboratory CV% of</p>	

HOMA-IR was 25% due to inter-assay variation in insulin (25%) rather than glucose (5%) measurements.

**Conclusions/interpretation:** The upper limit of HOMA-IR in population-based cohorts closely corresponds to that of normal liver fat. Standardisation of insulin assays would be the first step towards definition of normal values for HOMA-IR.

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180	Keywords separated by ' - '	Insulin - Liver fat - NAFLD - <i>PNPLA3</i> - Reference values
181	Foot note information	The online version of this article (doi:10.1007/s00125-017-4340-1) contains supplementary material, which is available to authorized users.

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## Electronic supplementary material

**ESM 1**  
(PDF 777 kb)

# Use of HOMA-IR to diagnose non-alcoholic fatty liver disease: a population-based and inter-laboratory study

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## Abstract

**Aims/hypothesis** Recent European guidelines for non-alcoholic fatty liver disease (NAFLD) call for reference values for HOMA-IR. In this study, we aimed to determine: (1) the upper limit of normal HOMA-IR in two population-based cohorts; (2) the HOMA-IR corresponding to NAFLD; (3)

the effect of sex and *PNPLA3* genotype at rs738409 on HOMA-IR; and (4) inter-laboratory variations in HOMA-IR. **Methods** We identified healthy individuals in two population-based cohorts (FINRISK 2007 [ $n = 5024$ ] and the Programme for Prevention of Type 2 Diabetes in Finland [FIN-D2D;  $n = 2849$ ]) to define the upper 95th

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27 percentile of HOMA-IR. Non-obese individuals with normal  
 28 fasting glucose levels, no excessive alcohol use, no  
 29 known diseases and no use of any drugs were considered  
 30 healthy. The optimal HOMA-IR cut-off for NAFLD (liver  
 31 fat  $\geq 5.56\%$ , based on the Dallas Heart Study) was deter-  
 32 mined in 368 non-diabetic individuals (35% with  
 33 NAFLD), whose liver fat was measured using proton  
 34 magnetic resonance spectroscopy ( $^1\text{H-MRS}$ ). Samples  
 35 from ten individuals were simultaneously analysed for  
 36 HOMA-IR in seven European laboratories.  
 37 **Results** The upper 95th percentiles of HOMA-IR were 1.9  
 38 and 2.0 in healthy individuals in the FINRISK ( $n = 1167$ )  
 39 and FIN-D2D ( $n = 459$ ) cohorts. Sex or *PNPLA3* genotype  
 40 did not influence these values. The optimal HOMA-IR cut-  
 41 off for NAFLD was 1.9 (sensitivity 87%, specificity 79%).  
 42 A HOMA-IR of 2.0 corresponded to normal liver fat  
 43 ( $< 5.56\%$  on  $^1\text{H-MRS}$ ) in linear regression analysis. The  
 44 2.0 HOMA-IR measured in Helsinki corresponded to 1.3,  
 45 1.6, 1.8, 1.8, 2.0 and 2.1 in six other laboratories. The  
 46 inter-laboratory CV% of HOMA-IR was 25% due to  
 47 inter-assay variation in insulin (25%) rather than glucose  
 48 (5%) measurements.  
 49 **Conclusions/interpretation** The upper limit of HOMA-IR  
 50 in population-based cohorts closely corresponds to that  
 51 of normal liver fat. Standardisation of insulin assays  
 52 would be the first step towards definition of normal  
 53 values for HOMA-IR.

54 **Keywords** Insulin · Liver fat · NAFLD · *PNPLA3* ·  
 55 Reference values

56 **Abbreviations**

58 ALT Alanine aminotransferase  
 60 AST Aspartate aminotransferase  
 63 AUROC Area under the receiver operating  
 64 characteristic (curve)  
 66 DHS Dallas Heart Study  
 68 DILGOM Dietary Lifestyle and Genetic Determinants  
 69 of the Development of Obesity and Metabolic  
 70 Syndrome study  
 72 FIN-D2D Programme for Prevention of Type 2  
 73 Diabetes in Finland  
 75 GGT  $\gamma$ -Glutamyltransferase  
 76  $^1\text{H-MRS}$  Proton magnetic resonance spectroscopy  
 78 NAFLD Non-alcoholic fatty liver disease  
 80 NPV Negative predictive value  
 83 *PNPLA3* Patatin-like phospholipase  
 84 domain-containing protein 3  
 86 PPV Positive predictive value  
 88 ROC Receiver operating characteristic

**Introduction**

91 Insulin resistance in individuals with non-alcoholic fatty liver  
 92 disease (NAFLD) is characterised by reduced whole body,  
 93 hepatic and adipose tissue insulin sensitivity [1, 2]. The liver,  
 94 once insulin resistant, overproduces glucose that stimulates  
 95 insulin secretion, resulting in mild hyperglycaemia and  
 96 hyperinsulinaemia. Therefore, the product of fasting glucose  
 97 and fasting insulin divided by a constant (i.e. HOMA-IR) [3]  
 98 is a good surrogate for insulin sensitivity in non-diabetic indi-  
 99 viduals [3–5]. Once the glucose concentration reaches the di-  
 100 agnostic threshold for type 2 diabetes, the insulin concentra-  
 101 tion starts to decline relative to glucose [6–9] and HOMA-IR  
 102 no longer exclusively reflects insulin sensitivity.

103 A recent joint European practice guideline for NAFLD [10]  
 104 concluded: ‘HOMA-IR provides a surrogate estimate of insu-  
 105 lin resistance in persons without diabetes and can therefore be  
 106 recommended, provided proper reference values have been  
 107 established.’ A reference value can be defined as the mean +  
 108 2 SDs for normally distributed variables or the 95th percentile  
 109 for non-normally distributed variables of a population-based  
 110 sample [11, 12]. Definition of health then becomes dependent  
 111 on the underlying population. This is particularly relevant for  
 112 HOMA-IR, as obesity is highly prevalent and perhaps the  
 113 single most important cause of variation in insulin [13].  
 114 Thus, for HOMA-IR, it would seem wiser to use reference  
 115 values derived from healthy individuals, although definitions  
 116 of health can also vary [14–16]. Pre-analytical causes of var-  
 117 iation should also be considered [3, 4], and the inter-assay  
 118 variation of insulin [17] and glucose should be known.

119 Normal liver fat content, measured using proton magnetic  
 120 resonance spectroscopy ( $^1\text{H-MRS}$ ), was determined in the  
 121 population-based Dallas Heart Study (DHS) [18]. In healthy  
 122 individuals (BMI  $< 25 \text{ kg/m}^2$ , no diabetes, normal fasting glu-  
 123 cose levels, low alcohol consumption, no known liver disease  
 124 or risk factors for liver disease and normal alanine aminotrans-  
 125 ferase [ALT] levels;  $n = 345$ ), the 95th percentile for liver fat  
 126 content was 5.56%. It is unknown how HOMA-IR relates to  
 127 this amount of liver fat and whether this definition of normal  
 128 liver fat reflects what normal liver fat is elsewhere. The I148M  
 129 variant of the gene encoding patatin-like phospholipase  
 130 domain-containing protein 3 (*PNPLA3*) (rs738409 c.444  
 131 C>G, p.I148M) has a prevalence of 30–50% [19]. It increases  
 132 the risk of NAFLD, but not of insulin resistance [20]. The  
 133 impact of this gene variant on reference values for HOMA-  
 134 IR has not been studied.

135 In the present study, we aimed to determine: (1) the upper  
 136 95th percentile of HOMA-IR in two population-based co-  
 137 horts; (2) the HOMA-IR that best distinguishes between  
 138 NAFLD and normal liver fat content, as quantified by  $^1\text{H-}$   
 139 MRS in a cohort of non-diabetic individuals; (3) whether  
 140 sex or the *PNPLA3* genotype at rs738409 influences reference  
 141 values for HOMA-IR; and (4) the inter-laboratory variation in

142 HOMA-IR among European centres participating in the  
 143 Elucidating Pathways of Steatohepatitis (EPoS) consortium  
 144 ([www.epos-naflld.eu](http://www.epos-naflld.eu)).

145 **Methods**

146 **Study designs**

147 **Population-based cohorts for the determination of normal**  
 148 **HOMA-IR** To determine normal HOMA-IR, we studied non-  
 149 pregnant adults in two population-based cohorts: the National  
 150 FINRISK 2007/ Dietary Lifestyle and Genetic Determinants  
 151 of the Development of Obesity and Metabolic Syndrome  
 152 study (DILGOM) study ( $n = 5024$ ), conducted by the  
 153 National Institute for Health and Welfare in Finland between  
 154 January and July 2007 [21]; and the Programme for  
 155 Prevention of Type 2 Diabetes in Finland (FIN-D2D)  
 156 ( $n = 2849$ ), conducted between October and December 2007  
 157 [22] (see electronic supplementary material [ESM] **Methods**  
 158 and ESM Fig. 1). The definition of healthy was as in the  
 159 population-based DHS [18]: (1) alcohol use  $<30$  g/day in  
 160 men and  $<20$  g/day in women; (2) no diabetes, based on his-  
 161 tory and normal fasting plasma glucose levels ( $<6.1$  mmol/l);  
 162 (3) BMI  $<25$  kg/m<sup>2</sup>; (4) no regular use of drugs; and (5) no  
 163 clinical or biochemical evidence of liver or other disease, as  
 164 defined by history and biochemical examinations.

165 **Liver fat cohort** Participants for the liver fat cohort were  
 166 recruited using newspaper advertisements, by contacting oc-  
 167 cupational health services and from individuals referred to the  
 168 Department of Gastroenterology, Helsinki University Hospital  
 169 (Helsinki, Finland), because of chronically elevated serum  
 170 transaminase concentrations using the following inclusion  
 171 criteria: (1) age 18–75 years; (2) no known acute or chronic  
 172 disease except obesity, hypertension or NAFLD based on  
 173 medical history, physical examination, standard laboratory  
 174 tests and ECG; (3) non-diabetic based on a fasting plasma  
 175 glucose level of  $\leq 6.9$  mmol/l; and (4) alcohol consumption  
 176 of  $\leq 20$  g per day in women and  $\leq 30$  g in men [23]. Study  
 177 physicians assessed alcohol intake using the same question-  
 178 naire as in the population-based studies. Exclusion criteria  
 179 included: (1) pregnancy; (2) serologic evidence of hepatitis  
 180 B/C or autoimmune hepatitis; (3) clinical signs or symptoms  
 181 of inborn errors of metabolism; (4) a history of predisposition  
 182 to toxins; (5) use of drugs associated with liver steatosis; and  
 183 (6) use of antihypertensive drugs or other drugs possibly  
 184 influencing glucose metabolism. The study protocol was ap-  
 185 proved by the ethics committee of the Helsinki University  
 186 Central Hospital and was carried out in accordance with the  
 187 Declaration of Helsinki. Each participant provided written in-  
 188 formed consent.

**Inter-laboratory variation in insulin assays** Ten non- 189  
 diabetic individuals covering a wide range of insulin sensitiv- 190  
 ities were recruited. The participants were healthy based on 191  
 medical history, physical examination and standard laboratory 192  
 tests, but eight of them were overweight or obese (BMI 193  
 $\geq 25$  kg/m<sup>2</sup>). Blood was drawn in Helsinki after a 12 h fast 194  
 for measurement of fasting insulin, glucose, HDL-cholesterol, 195  
 LDL-cholesterol, total cholesterol, triacylglycerol, aspartate 196  
 aminotransferase (AST), ALT,  $\gamma$ -glutamyltransferase (GGT), 197  
 ferritin and albumin. Measurements of laboratory variables 198  
 other than insulin or glucose were performed for comparison 199  
 to estimate their inter-assay CVs. The fresh samples were 200  
 analysed immediately in Helsinki. To study the effect of freez- 201  
 ing, another set of samples were immediately frozen to  $-80^{\circ}\text{C}$  202  
 and then melted and assayed on the same day in Helsinki. To 203  
 study the effect of time, a third set of samples were frozen to 204  
 $-80^{\circ}\text{C}$  and assayed after 2 weeks in Helsinki. At this same 205  
 time point, six additional sets of samples, which had been 206  
 shipped in dry ice, were assayed in Newcastle (UK), Paris 207  
 (France), Pisa (clinical and research laboratories; Italy), 208  
 Torino (Italy) and Mainz (Germany). The study protocol was 209  
 approved by the ethics committee of the Helsinki University 210  
 Central Hospital and was carried out in accordance with the 211  
 Declaration of Helsinki. Each participant provided written in- 212  
 formed consent. 213

**Biochemical measurements** 214

**FINRISK/DILGOM and FIN-D2D** Biochemical assays 215  
 were performed in the Laboratory of Analytical 216  
 Biochemistry of the Institute of Health and Welfare 217  
 (Helsinki, Finland) using an Architect ci8200 analyser 218  
 (Abbott Laboratories, Abbott Park, IL, USA). Plasma glucose 219  
 was determined using the hexokinase method (Abbott 220  
 Laboratories) and serum insulin using a chemiluminescent 221  
 microparticle immunoassay (Abbott Laboratories). Serum to- 222  
 tal cholesterol, HDL-cholesterol and triacylglycerol concen- 223  
 trations were measured using enzymatic kits (Abbott 224  
 Laboratories), and the LDL-cholesterol concentration was cal- 225  
 culated using the Friedewald formula [24]. Total cholesterol 226  
 was measured using the CHOD-PAP assay (Abbott 227  
 Laboratories). Samples were stored at  $-80^{\circ}\text{C}$  before analysis. 228  
 In the FIN-D2D study, HbA<sub>1c</sub> was measured using an 229  
 immunoturbidimetric method (Abbott Laboratories), and se- 230  
 rum ALT, AST and GGT concentrations were measured using 231  
 International Federation of Clinical Chemistry photometric 232  
 methods (Abbott Laboratories). In the liver fat cohort, plasma 233  
 glucose was measured using the hexokinase method in an 234  
 autoanalyser (Roche Diagnostics Hitachi 917; Hitachi, 235  
 Tokyo, Japan) and serum insulin was measured in fresh serum 236  
 samples using a time-resolved fluoroimmunoassay with 237  
 AutoDELFLIA kits (Wallac, Turku, Finland). HbA<sub>1c</sub> was mea- 238  
 sured using HPLC using the fully automated analyser system 239



240 (Bio-Rad, Richmond, CA, USA). Serum triacylglycerol, total  
 241 cholesterol, LDL-cholesterol and HDL-cholesterol concentra-  
 242 tions were measured with enzymatic kits from Roche  
 243 Diagnostics using an autoanalyser (Roche Diagnostics  
 244 Hitachi 917; Hitachi). Serum ALT, AST and GGT activities  
 245 were determined as recommended by the European  
 246 Committee for Clinical Laboratory Standards using the  
 247 Roche Diagnostics Hitachi 917 (Hitachi). HOMA-IR was cal-  
 248 culated as described by Matthews et al [3]. The methods used  
 249 by the seven participating centres for HOMA-IR and the other  
 250 laboratory variables are shown in [ESM Methods](#).

251 **Genotyping of *PNPLA3* at rs738409**

252 **FINRISK/DILGOM** The *PNPLA3* genotype was deter-  
 253 mined from 1000G imputed genome-wide association study  
 254 data consisting of three subsets genotyped using the Illumina  
 255 HumanCoreExome, Illumina OmniExpress and Illumina  
 256 610K (Illumina, San Diego, CA, USA).

257 **FIN-D2D** Genomic DNA was extracted from whole blood  
 258 using automated Chemagen DNA extraction equipment  
 259 (PerkinElmer, Waltham, MA, USA) or a QIAamp DNA  
 260 Blood Maxi Kit (Qiagen, Hilden, Germany) following the  
 261 protocol of the kit with slight modifications. Genotyping  
 262 was performed using a TaqMan assay (Applied Biosystems,  
 263 Paisley, UK).

264 **Liver fat cohort** DNA was isolated from whole blood and the  
 265 *PNPLA3* genotype at rs738409 was determined as previously  
 266 described using a TaqMan assay (Applied Biosystems) [25].

267 **Measurement of liver fat content by <sup>1</sup>H-MRS**

268 Liver fat was measured by using <sup>1</sup>H-MRS as previously de-  
 269 scribed [23]. Liver fat content was expressed as a mass frac-  
 270 tion in percentage units [23]. NAFLD was defined as in the  
 271 DHS (liver fat  $\geq 5.56\%$  by <sup>1</sup>H-MRS) [18].

272 **Other measurements**

273 In all cohorts, body weight, height, BMI and waist and  
 274 hip circumferences were measured as previously de-  
 275 scribed [23, 26, 27].

276 **Statistical analysis**

277 Distribution of continuous variables was analysed for normal-  
 278 ity using the Kolmogorov–Smirnov test. Data are shown as  
 279 means  $\pm$  SD for normally distributed data and as medians (25–  
 280 75%) for non-normally distributed data. To compare charac-  
 281 teristics among groups, the unpaired *t* test and the Mann–  
 282 Whitney *U* test were used for continuous variables, and

Fisher’s exact test and the  $\chi^2$  test were used for categorical  
 variables, where appropriate. Logarithmic transformation was  
 performed for non-normally distributed data if needed.  
 Correlation analyses were performed using Pearson’s correla-  
 tion coefficient.

Healthy individuals in the FINRISK/DILGOM ( $n = 1167$ )  
 and FIN-D2D ( $n = 459$ ) cohorts were identified. HOMA-IR  
 was not normally distributed, and therefore the 95th percentile  
 (90% CI) rather than the mean + 2 SD was used to determine  
 the upper reference value for HOMA-IR [12]. After log<sub>2</sub> trans-  
 formation, HOMA-IR values were adjusted in a generalised  
 linear model, using age and BMI as covariates.

We used two methods to identify a cut-off value of HOMA-  
 IR for NAFLD. First, we calculated the HOMA-IR value that  
 corresponded to the normal liver fat content based on the DHS  
 (liver fat  $< 5.56\%$  [18]) using linear regression analysis. We  
 tested whether the slopes and intercepts in linear regression  
 analysis differed between men and women, and carriers and  
 non-carriers of the *PNPLA3* I148M variant. The 95th percen-  
 tile was used to define normal liver fat content in healthy  
 individuals in the liver fat cohort, as in the DHS [18].  
 Second, we determined the receiver operating characteristic  
 (ROC) curve to calculate the area under the ROC curve  
 (AUROC [95% CI]). The Youden index [28] was used to  
 identify the optimal cut-off of HOMA-IR. For this, individuals  
 were randomly divided into discovery (two-thirds of the  
 individuals) and validation (one-third) groups. The discovery  
 group was used to determine the ROC curve for HOMA-IR.  
 The validation group and all individuals were used for valida-  
 tion. For additional validation, we generated 1000 random sets  
 of samples and used the bootstrap method to validate the  
 model in the sample sets. The AUROC of each set was esti-  
 mated, and the average of these estimates provided the overall  
 prediction accuracy of the model. Power analysis was con-  
 ducted to estimate the appropriate sample size for correlation  
 analysis and ROC analysis. To detect a correlation coefficient  
 of 0.2 between HOMA-IR and liver fat content with a power  
 of 0.8, a sample size of at least 193 was required. By setting  
 the ratio of sample sizes between negative and positive groups  
 at 2, at least 23 cases and 46 control participants were needed  
 to reach a statistical power of 0.8 to detect the minimum  
 AUROC of 0.7.

The inter-laboratory CVs of fasting insulin, glucose,  
 HOMA-IR, lipids, liver enzymes, ferritin and albumin among  
 laboratories were calculated. Linear regression analyses were  
 performed to compare insulin, glucose and HOMA-IR mea-  
 surements in Helsinki to those in other centres. The HOMA-  
 IR in each centre corresponding to the upper limit of normal  
 HOMA-IR in Helsinki was defined from linear regression  
 equations.

We considered a *p* value of  $< 0.05$  to be statistically signif-  
 icant. Calculations were made using R Project for Statistical  
 Computing version 3.1.1 ([www.r-project.org](http://www.r-project.org), Vienna,

336 Austria) and GraphPad Prism version 6.00 for Mac OS X  
337 (GraphPad Software, San Diego, CA, USA).

338 **Results**

339 **Reference values for HOMA-IR in two population-based**  
340 **cohorts**

341 Characteristics of the healthy individuals in the two  
342 population-based cohorts ( $n = 1167$  in FINRISK/DILGOM,  
343  $n = 459$  in FIN-D2D) are shown in Table 1. Characteristics of  
344 these individuals subgrouped based on their *PNPLA3* geno-  
345 type at rs738409 are shown in ESM Table 1. The upper limit  
346 of normal (95th percentile [90% CI]) HOMA-IR was 1.9 (1.8,  
347 2.0) in the FINRISK/DILGOM cohort and 2.0 (1.9, 2.2) in the  
348 FIN-D2D cohort (Fig. 1).

349 There was no sex difference among HOMA-IRs in either  
350 cohort (Fig. 1). Since there were slight differences in age and  
351 BMI between men and women in the two studies (Table 1) we  
352 also calculated age- and BMI-adjusted HOMA-IRs, which  
353 were very similar to the unadjusted values (Table 1). The  
354 *PNPLA3* genotype did not influence HOMA-IR in either co-  
355 hort (Fig. 1).

356 The 95th percentile of serum ALT in the FIN-D2D cohort  
357 was 31 U/l in women and 43 U/l in men. In the FINRISK/  
358 DILGOM cohort aged 25–74 years, age weakly inversely cor-  
359 related with HOMA-IR ( $\rho = -0.16$ ,  $p < 0.001$ ). No significant  
360 relationship was observed between age and HOMA-IR in the  
361 FIN-D2D cohort aged 45–74 years ( $\rho = 0.06$ ,  $p = 0.21$ ).

362 **Relationship between HOMA-IR and liver fat content**

363 Characteristics of the non-diabetic individuals in the liver fat  
364 cohort ( $n = 368$ ) are shown in Table 1. Of them, 35% had  
365 NAFLD as evaluated by <sup>1</sup>H-MRS. Liver fat percentage posi-  
366 tively correlated with HOMA-IR ( $r = 0.67$ ,  $p < 0.001$ )  
367 (Fig. 2a). Normal liver fat, defined as in the DHS (<5.56%),  
368 corresponded to a HOMA-IR of 2.0 (95% CI 1.9, 2.1)  
369 (Fig. 2a) in non-diabetic individuals, with a HOMA-IR of  
370 1.9 (1.8, 2.1) in women and 2.1 (1.9, 2.2) in men ( $p = 0.29$ ).  
371 The HOMA-IR corresponding to the normal liver fat content  
372 (<5.56%) was significantly higher in non-carriers (2.1 [2.0,  
373 2.2]) than carriers (1.8 [1.6, 1.9],  $p = 0.007$ ) of the *PNPLA3*  
374 I148M variant (Fig. 2b) (i.e. the variant allele carriers had a  
375 higher liver fat content for any given HOMA-IR than non-  
376 carriers). The upper 95th percentile for liver fat in the 96  
377 healthy individuals was 5.9%.

378 The discovery and validation groups for defining the  
379 HOMA-IR cut-off for NAFLD were similar with respect to  
380 clinical and biochemical characteristics (ESM Table 2). The  
381 AUROC for HOMA-IR was 0.88 (95% CI 0.84, 0.92) in the  
382 discovery group (Fig. 3). The optimal HOMA-IR cut-off for

NAFLD, based on the Youden index, the point of optimal 383  
sensitivity and specificity, was 1.9. This cut-off had a sensi- 384  
tivity of 87%, specificity of 79%, negative predictive value 385  
(NPV) of 92% and positive predictive value (PPV) of 67%. 386  
The results were similar for the validation group (AUROC 387  
0.80 [0.70, 0.88], sensitivity 68%, specificity 82%, NPV 388  
81% and PPV 70%) and for all individuals (AUROC 0.85 389  
[0.80, 0.89] sensitivity 80%, specificity 80%, NPV 88% and 390  
PPV 68%) (Fig. 3). The AUROC for bootstrap samples was 391  
0.88 (0.82, 0.92) and the overall estimate of optimism was 392  
0.00079. Neither sex ( $p = 0.22$ ) nor *PNPLA3* genotype 393  
( $p = 0.18$ ) significantly influenced the AUROC. 394

395 **Inter-laboratory variation in insulin assays**  
396 **and HOMA-IR**

397 The ten individuals (three men, seven women) recruited to 397  
investigate inter-laboratory variations ranged in age from 22 398  
to 62 years and in BMI from 21.3 to 42.4 kg/m<sup>2</sup>. Among the 399  
seven laboratories, the mean values ranged from 18.0 to 400  
91.2 pmol/l for insulin, from 4.7 to 6.1 mmol/l for glucose 401  
and from 0.69 to 4.0 for HOMA-IR. Freezing and thawing 402  
the serum on the same day had no impact on fasting insulin 403  
(52.8 ± 28.8 vs 54.0 ± 29.4 pmol/l,  $p = 0.077$ ). Serum insulin 404  
concentrations decreased over time when stored at -80°C de- 405  
grees for 2 weeks (54.0 ± 29.4 vs 45.6 ± 25.8 pmol/l, 406  
 $p = 0.005$ ). 407

408 The CV of fasting insulin measured in the seven participat- 408  
ing laboratories after 2 weeks of storage at -80°C averaged 409  
25.4%. The CV of fasting glucose was significantly lower and 410  
averaged 4.6%. The CV of HOMA-IR was 25.0%. The 411  
HOMA-IR value of 2.0, as measured in Helsinki, 412  
corresponded to HOMA-IRs of 1.3, 1.6, 1.8, 1.8, 2.0 and 2.1 413  
in the six other centres (Fig. 4). The relationships between 414  
insulin and glucose measurements in Helsinki vs the other 415  
centres are shown in ESM Fig. 2, 3. 416

417 The inter-laboratory CVs for the other analytes were as 417  
follows: total cholesterol 7.4%, LDL-cholesterol 12.8%, 418  
HDL-cholesterol 7.0%, triacylglycerol 8.3%, AST 11.7%, 419  
ALT 11.6%, GGT 11.3%, ferritin 19.1% and albumin 7.7%. 420  
All of these CVs, with the exception of ferritin, were signifi- 421  
cantly lower than the CV for fasting insulin ( $p < 0.01$ ). 422

423 **Discussion**

424 The present studies were undertaken to determine whether a 424  
single value of HOMA-IR could be used to clearly identify 425  
individuals with NAFLD, and how HOMA-IRs determined 426  
by different laboratories in European countries compare with 427  
each other. In two population-based cohorts, the upper limits 428  
of normal HOMA-IR were 1.9 and 2.0. In individuals whose 429  
liver fat content was determined using <sup>1</sup>H-MRS, a HOMA-IR 430

Springer **Table 1** Participant characteristics

Variable	FINRISK/DILGOM			FIN-D2D			Liver fat cohort		
	All ( <i>n</i> = 1167)	Women ( <i>n</i> = 798)	Men ( <i>n</i> = 369)	All ( <i>n</i> = 459)	Women ( <i>n</i> = 308)	Men ( <i>n</i> = 151)	All ( <i>n</i> = 368)	Women ( <i>n</i> = 221)	Men ( <i>n</i> = 147)
Age (years)	44 (35–56)	43 (33–53)	47 (36–56)**	55 (50–62)	55 (50–62)	57 (50–65)	42 (28–52)	42 (32–52)	41 (27–51)
Weight (kg)	63.4 (57.8–69.9)	60.2 (55.6–64.8)	72.2 (67.0–76.6)***	63.6 (58.3–70.0)	61.0 (55.7–64.9)	72.7 (67.9–76.7)***	83.4 (24.5–33.2)	81.4 (68.0–94.7)	86.3 (76–100.1)**
BMI (kg/m <sup>2</sup> )	22.7 (21.4–24.0)	22.5 (21.0–23.7)	23.4 (22.1–24.3)***	22.9 (21.6–24.1)	22.7 (21.3–23.9)	23.3 (22.1–24.3)**	28.8 (24.5–33.2)	29.8 ± 6.2	27.6 (24.5–31.1)*
Waist circumference (cm)	78.0 (73.0–83.0)	75.5 (71.5–80.0)	84.5 (80.0–88.5)***	81.0 ± 7.2	78.2 ± 5.9	87.1 ± 5.7***	96.9 ± 15.2	96.0 ± 16.3	98.4 ± 13.4
fS total cholesterol (mmol/l)	4.9 (4.4–5.6)	4.9 (4.3–5.5)	5.1 ± 0.9**	5.4 (4.8–6.0)	5.4 ± 0.9	5.3 ± 0.9	4.9 (4.3–5.6)	4.8 (4.3–5.7)	4.9 (4.4–5.5)
fS HDL-cholesterol (mmol/l)	1.5 (1.4–1.8)	1.6 (1.4–1.9)	1.4 (1.2–1.6)***	1.6 (1.4–1.8)	1.7 ± 0.3	1.4 (1.3–1.6) ***	1.4 (1.2–1.7)	1.5 (1.2–1.8)	1.3 (1.1–1.6)***
fS LDL-cholesterol (mmol/l)	3.0 (2.4–3.5)	2.8 (2.4–3.4)	3.2 ± 0.8***	3.3 (2.8–3.9)	3.3 (2.8–3.9)	3.5 ± 0.8	3.0 (2.4–3.6)	2.9 (2.3–3.5)	3.1 ± 0.9
fS triacylglycerol (mmol/l)	0.8 (0.6–1.0)	0.8 (0.6–1.0)	0.9 (0.7–1.1)***	0.9 (0.7–1.1)	0.9 (0.7–1.2)	0.9 (0.7–1.2)	1.1 (0.8–1.6)	1.0 (0.7–1.5)	1.2 (0.9–1.6)*
fP glucose (mmol/l)	5.5 (5.2–5.7)	5.4 ± 0.3	5.6 (5.4–5.8)***	5.6 (5.4–5.8)	5.6 (5.3–5.8)	5.7 ± 0.2***	5.4 (5.0–5.8)	5.4 ± 0.6	5.5 ± 0.5*
fS insulin (pmol/l)	24.6 (19.8–31.8)	25.2 (20.4–32.4)	24.0 (19.2–30.6)*	27.0 (20.4–33.6)	27.0 (21.6–33.6)	27.0 (19.8–33.6)	40.2 (22.2–66.0)	38.4 (21.6–66.0)	42.0 (22.8–72.0)
HOMA-IR	1.0 (0.8–1.3)	1.0 (0.8–1.3)	1.0 (0.8–1.3)	1.1 (0.8–1.4)	1.1 (0.8–1.4)	1.1 (0.8–1.4)	1.6 (0.8–2.7)	1.5 (0.8–2.5)	1.8 (0.9–3.2)
Adjusted HOMA-IR <sup>a</sup>	1.0 (0.9–1.1)	1.0 (0.9–1.1)	1.0 (1.0–1.1)**	1.1 (1.0–1.2)	1.1 (1.0–1.2)	1.1 (1.1–1.2)**	–	–	–
HbA <sub>1c</sub> (%)	–	–	–	5.1 (4.9–5.3)	5.1 (4.9–5.3)	5.2 (5.0–5.4)**	5.5 (5.3–5.7)	5.5 (5.3–5.7)	5.5 (5.2–5.7)
HbA <sub>1c</sub> (mmol/mol)	–	–	–	32 (30–34)	32 (30–34)	33 (31–36)**	37 (34–39)	37 (34–39)	37 (33–39)
fS ALT (U/l)	–	–	–	18 (15–23)	17 (14–22)	22 (16–28)***	25 (18–39)	22 (16–31)	34 (23–53)***
fS AST (U/l)	–	–	–	22 (18–26)	21 (17–25)	24 (20–28)***	26 (22–32)	24 (21–30)	29 (24–37)***
fS GGT (U/l)	–	–	–	19 (14–28)	17 (13–23)	23 (17–33)***	23 (15–40)	19 (14–33)	30 (20–50)***
PNPLA3 <sup>H48M</sup> (non-carrier/carrier, %)	59/41	59/41	60/40	60/40	63/37	53/47*	60/40	53/47	65/35
Liver fat (%)	–	–	–	–	–	–	2.7 (0.9–8.6)	2.4 (0.8–7.1)	3.3 (1.1–12.4)*

Data are means ± SD (variables with normal distribution) or median (25–75%) (variables with non-normal distribution), unless otherwise stated

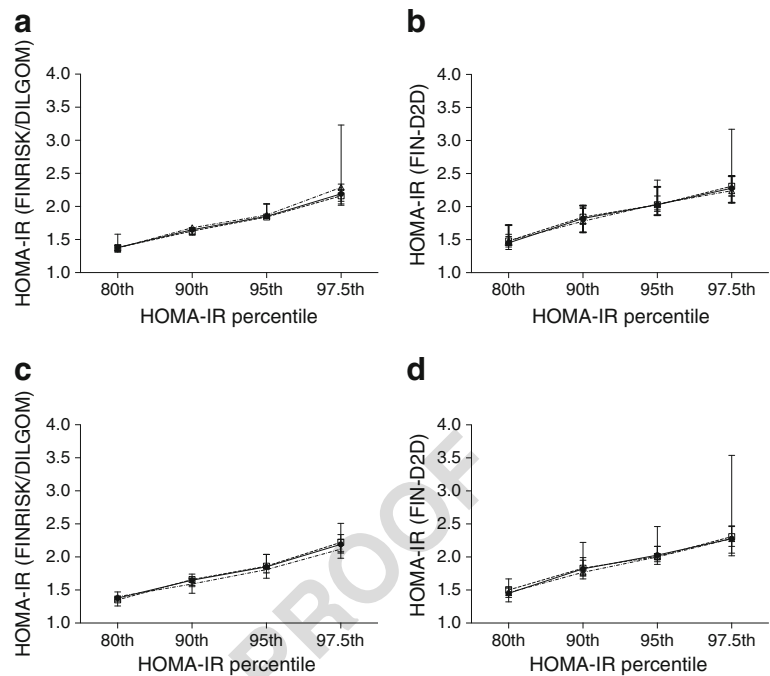
<sup>a</sup> Adjusted for BMI and age

\**p* < 0.05, \*\**p* < 0.005, \*\*\**p* < 0.001 for men vs women within cohorts

fP, fasting plasma; fS, fasting serum



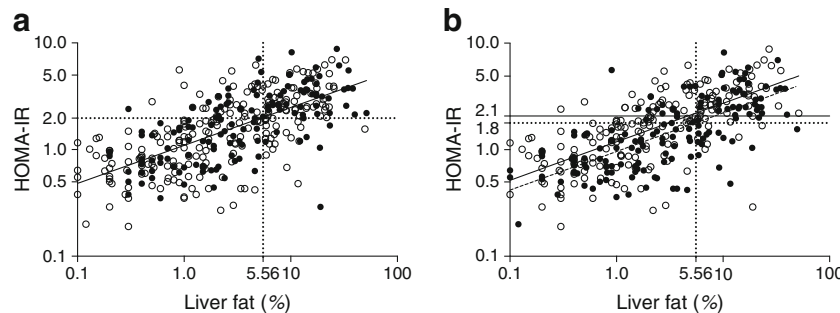
**Fig. 1** Percentiles (90% CI) of HOMA-IR in healthy men (triangles and dotted/dashed lines), women (squares and dashed lines) and all individuals (circles and solid lines) in the population-based FINRISK/DILGOM (a) and FIN-D2D cohorts (b), and in I148M variant non-carriers (PNPLA3<sup>I148II</sup>, squares and dashed lines) and carriers (PNPLA3<sup>I148M/MM</sup>, triangles and dotted/dashed lines) and all individuals (circles and solid lines) in the population-based FINRISK/DILGOM (c) and FIN-D2D cohorts (d)



431 cut-off of 1.9 was optimal for diagnosing NAFLD based on  
 432 the Youden index. A HOMA-IR of 2.0 corresponded to the  
 433 upper limit of normal liver fat content of 5.56%, as defined in  
 434 the DHS. A HOMA-IR value of 2.0 corresponded to HOMA-  
 435 IRs between 1.3 and 2.1 in six other laboratories, with an inter-  
 436 laboratory CV of 25%. These data show that the upper limit of  
 437 normal HOMA-IR closely corresponds to the upper limit of  
 438 liver fat defined as in the DHS, and that there is large inter-  
 439 laboratory variation in insulin measurements.

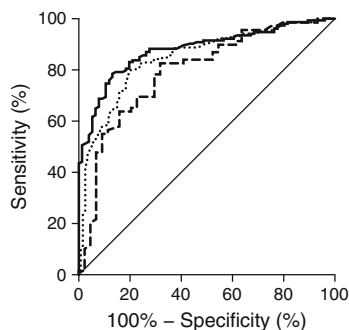
440 The upper limit of a reference value is usually defined in  
 441 population-based samples of healthy individuals as the mean +  
 442 2 SD in normally distributed samples and as the 95th percent-  
 443 ile in non-normally distributed samples [12]. In this study, the  
 444 95th percentiles were 1.9 and 2.0 in the healthy individuals of  
 445 two population-based cohorts. Three previous studies have  
 446 been performed in healthy individuals. These studies were

447 smaller (161 Japanese, 161 Italian and 312 Brazilian individ- 447  
 448 uals) than the present study (459–1167 Finnish individuals) 448  
 449 [14–16]. In the Japanese study [14], the 90th percentile of 449  
 450 HOMA-IR was 1.7, which is comparable with that found in 450  
 451 present study. In the Italian study, however, the participants 451  
 452 were not healthy as they included diabetic and hypertensive 452  
 453 individuals. The 80th percentile of HOMA-IR was 2.77 [16]. 453  
 454 This study used a non-specific RIA from Linco Research (St. 454  
 455 Charles, MO, USA), which has produced the highest insulin 455  
 456 concentrations of several insulin assays tested [17, 29]. 456  
 457 Similarly, the Brazilian study also used this RIA, and the 457  
 458 90th percentile of HOMA-IR was equally high (2.71) [15]. 458  
 459 Thus, the higher HOMA-IR in these studies compared with 459  
 460 the present study could be due to the inclusion of diabetic and 460  
 461 hypertensive individuals in the Italian study, and to the use of 461  
 462 an RIA that is no longer used in most laboratories [17]. 462



**Fig. 2** Relationship between liver fat measured by <sup>1</sup>H-MRS (log<sub>10</sub>) and HOMA-IR (log<sub>10</sub>). (a) The relationship was similar (slopes  $p = 0.79$ , elevations  $p = 0.75$ ) in men (black circles) ( $r = 0.67$ ,  $p < 0.001$ ) and in women (white circles) ( $r = 0.66$ ,  $p < 0.001$ ). The HOMA-IR corresponding to normal liver fat (<5.56%), as defined in the DHS [18], was 2.0. (b) There was a significant difference in the intercepts of the regression lines

( $p = 0.007$ ) between carriers (PNPLA3<sup>I148M/MM</sup>) ( $r = 0.69$ ,  $p < 0.001$ ) (black circles) and non-carriers (PNPLA3<sup>I148II</sup>) ( $r = 0.68$ ,  $p < 0.001$ ) (white circles) of the I148M variant, showing that HOMA-IR was lower for any given liver fat content in carriers than non-carriers. No significant difference between the slopes was observed ( $p = 0.99$ )



**Fig. 3** AUROC for HOMA-IR and NAFLD. The AUROC for HOMA-IR was 0.88 (95% CI 0.84, 0.92) in the discovery group (solid line), 0.80 (0.70, 0.88) in the validation group (dashed line) and 0.85 (0.80, 0.89) in all individuals (dotted line)

463 We found no significant differences in HOMA-IR percentiles between men and women among the healthy individuals  
 464 in either population-based cohort (Fig. 1). The men were, however, slightly more obese and older than the women,  
 465 and therefore we also calculated age- and BMI-adjusted HOMA-IRs. After adjustment, men had slightly higher HOMA-IRs  
 466 than women in both studies, but the differences in absolute units were trivial (0.02 in FINRISK/DILGOM and 0.05 in the  
 467 FIN-D2D study; Table 1). Previous population-based studies including healthy individuals have not reported HOMA-IRs  
 468 separately for men and women [14–16].

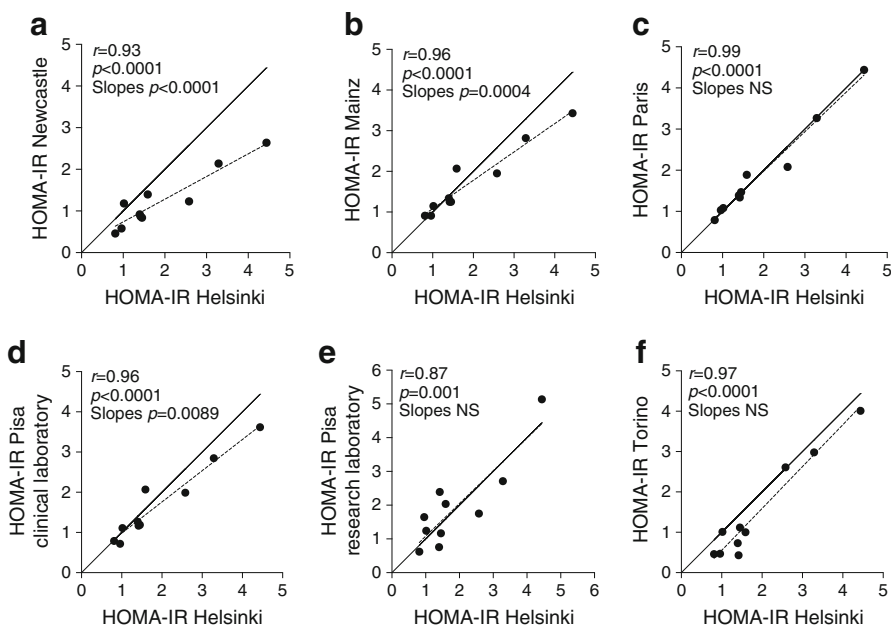
474 In keeping with the 95th percentile in healthy individuals in the population-based cohorts, we found a HOMA-IR of 1.9 to  
 475 best distinguish non-diabetic individuals with and without NAFLD. This value is similar to that found in 204 Brazilian  
 476 individuals [30]. As in the present study, in ROC analysis, a HOMA-IR of 2.0 (AUROC 0.84) best distinguished between  
 477 NAFLD and non-NAFLD diagnosed by ultrasound or biopsy.

In keeping with these data, a study comprising 263 Columbian men found a HOMA-IR of 1.7 (AUROC 0.78) to be the cut-off for NAFLD diagnosed by ultrasound [31]. In a recent population-based study in Iran, the best cut-off for NAFLD diagnosed by ultrasound was 2.0 in women and 1.8 in men [32]. Even though these results in different ethnic groups seem consistent, it will be important to perform studies in ethnic groups other than Finns.

In linear regression analysis (Fig. 2), HOMA-IRs of 1.9 and 2.0 corresponded to liver fat contents of 5.0% and 5.56%. The latter value is identical to that defined as the upper limit of normal liver fat measured by <sup>1</sup>H-MRS in the DHS [18]. The prevalence of NAFLD in the population-based DHS was 31% [33], which is comparable with that in our cohort of research volunteers (35%). In our cohort, the 95th percentile of liver fat in healthy individuals was 5.9%. This value is close to the 5.56% in the DHS [18]. However, as our liver fat cohort was not population-based, the 5.56% in the DHS can be considered more accurate than our estimate of 5.9%.

The PNPLA3 I148M variant predisposes to NAFLD but not to features of insulin resistance [34, 35]. Thus, despite an increased liver fat content in PNPLA3 I148M variant carriers, HOMA-IR has been reported to be similar in carriers and non-carriers of similar age, sex and BMI [36–39]. Consistent with these data, in the present two healthy population-based cohorts, no difference existed in clinical characteristics between carriers and non-carriers of the PNPLA3 I148M variant. The upper limit of normal HOMA-IR was the same for both groups. In the liver fat cohort, in which 35% of individuals had NAFLD, the optimal cut-off for distinguishing NAFLD from non-NAFLD was also not affected by genotype.

**Fig. 4** Linear regression between HOMA-IR measured in Helsinki by chemiluminescent immunoassay using Liaison XL (DiaSorin, Saluggia, Italy) for insulin measurement and HOMA-IR measured in six other laboratories (a–f) using insulin assays as described in ESM Fig. 2. (f) Intercepts  $p = 0.0005$



513 However, when comparing carriers and non-carriers at a sim- 566  
 514 ilar liver fat content, carriers were found to have lower 567  
 515 HOMA-IR than non-carriers (Fig. 2b). These data imply that 568  
 516 HOMA-IR cannot be used to diagnose individuals with 569  
 517 NAFLD due to the PNPLA3 I148M variant, and that they 570  
 518 can only be identified by genotyping for this gene variant [10].

519 A limitation of HOMA-IR is that it is valid only as long as 571  
 520 serum insulin concentrations reflect merely insulin sensitivity, 572  
 521 not secretion [40–42]. In individuals with non-diabetic glu- 573  
 522 cose tolerance, fasting glucose and insulin concentrations are 574  
 523 closely positively correlated [43]. Once glucose tolerance be- 575  
 524 comes diabetic, insulin concentrations start to decline and 576  
 525 their relationship to glucose is inverse rather than positive 577  
 526 [6]. Under such conditions HOMA-IR underestimates insulin 578  
 527 resistance-associated NAFLD, although in a recent study in- 579  
 528 cluding 56 participants with type 2 diabetes, a HOMA-IR of 580  
 529 4.5 was estimated to be the optimal threshold for 581  
 530 distinguishing NAFLD diagnosed by ultrasound or computed 582  
 531 tomography [44]. The method used to measure insulin con- 583  
 532 centrations was not specified. The extreme example is type 1 584  
 533 diabetes, where there is no endogenous insulin. HOMA-IR is 585  
 534 also influenced by insulin clearance, unlike direct measure- 586  
 535 ments of insulin sensitivity. However, this may not be a prob- 587  
 536 lem as the decrease in insulin clearance closely parallels that in 588  
 537 hepatic insulin sensitivity [45]. 589

538 Use of HOMA-IR in the clinic assumes the degree of inter- 590  
 539 laboratory variation in insulin assays is known [29]. In the 591  
 540 present study, we analysed fasting blood samples obtained 592  
 541 from ten individuals covering a wide range of HOMA-IRs 593  
 542 after a similar period of freezing and thawing and time of 594  
 543 storage. From the regression lines relating assay results be- 595  
 544 tween two laboratories (Fig. 4), the upper limit of normal 596  
 545 HOMA-IR was similar in Helsinki and Paris using the same 597  
 546 insulin assay (2.0), but was 1.3, 1.6, 1.8, 1.8 and 2.1 in the five 598  
 547 other laboratories using different assays. The inter-laboratory 599  
 548 CV was 25%. In contrast, the inter-laboratory CVs for other 600  
 549 analytes, with the exception of ferritin, were much lower and 601  
 550 ranged from 5% to 13%. This implies that every laboratory 602  
 551 should establish its own reference value for HOMA-IR, or at 603  
 552 least understand how its insulin assay compares with other 604  
 553 laboratories. Furthermore, reference values for HOMA-IR, 605  
 554 even in healthy individuals, and the relationship between 606  
 555 HOMA-IR and liver fat may be population-specific.

556 We conclude that the upper limit of HOMA-IR, defined 607  
 557 based on the identification of healthy individuals in two 608  
 558 population-based Finnish cohorts, closely corresponds to the 609  
 559 upper limit of normal liver fat content (<5.56%) found in the 610  
 560 DHS. This finding supports the use of HOMA-IR in identify- 611  
 561 ing individuals with ‘metabolic NAFLD’. The use of HOMA- 612  
 562 IR has, however, several limitations. HOMA-IR varies con- 613  
 563 siderably and more than other routine analytes among labora- 614  
 564 tories, particularly due to the use of different insulin assays. If 615  
 565 HOMA-IR were to be used as a surrogate for insulin 616

resistance and NAFLD, insulin assays would need to be 566  
 standardised. In addition, HOMA-IR underestimates liver fat 567  
 content in individuals with NAFLD associated with the 568  
 PNPLA3 I148M variant and, although not examined in this 569  
 study, in individuals with defective insulin secretion. 570

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 interpreted the data and wrote the main text. EI, HY-J, EB, KC, AG, 593  
 DS and QMA designed and EI, HY-J, EB, KC, DB-R, J-ML, AG, DS, 594  
 JMS and QMA acquired data in and supervised the inter-laboratory insu- 595  
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 this work. 602

**Data availability** The data that support the findings of this study are 603  
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