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Time of day variations in novel cardiovascular disease risk factors measured in older men

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SCHOLARONE™
Manuscripts

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3 1 **Title:** Time of day variations in novel cardiovascular disease risk factors measured in older
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18 **Manuscript number of words:** 3897 words (including title page, abstract, manuscript,
19 tables).

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3 21 **Abstract (250 words, now 250)**
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6 22 **Objective:** We estimated associations of time of day with novel cardiovascular disease (CVD)
7 23 risk factors measured in older men.

8
9 24 **Methods:** Novel CVD risk factors (markers of inflammation and haemostasis, and cardiac
10 25 markers) were measured on one occasion between 08:00-19:00 hours in 4252 men aged 60-
11 26 79 years from the British Regional Heart Study. Linear models were used to estimate
12 27 associations between time of day and risk factors. When an association was found, we
13 28 examined whether the relationship between risk factors and cardiovascular mortality was
14 29 affected by the adjustment for time of day using survival analyses.

15
16 30 **Results:** N-terminal pro-brain natriuretic peptide (NT-ProBNP) levels increased by 3.3% per
17 31 hour [95% Confidence interval (CI) 1.9; 4.8], Interleukin-6 (IL-6) increased by 2.6% per hour
18 32 (95% CI 1.8; 3.4), while Tissue plasminogen activator (t-PA) decreased by 3.3% per hour
19 33 (95% CI 3.7; 2.9); these associations were unaffected by adjustment for possible
20 34 confounding factors. The percentages of variation in these risk factors attributable to time
21 35 of day were less than 2%. In survival analyses, the association of IL-6, NT-ProBNP, and t-PA
22 36 with cardiovascular mortality was not affected by the adjustment for time of day. C-Reactive
23 37 Protein, Fibrinogen, D-Dimer, von Willebrand Factor, and cardiac Troponin T showed no
24 38 associations with time of day.

25 39 **Conclusions:** In older men, markers of inflammation (IL-6), haemostasis (t-PA), and a cardiac
26 40 marker (NT-ProBNP) varied by time of day. The contribution of time of day to variations in
27 41 these markers was small, and did not appear to be relevant for the CVD risk prediction.
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3 42 **Strengths and limitations of this study (Max 5 points)**
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5 43 1) In older adults diurnal variations in novel CVD risk factors have not been yet estimated; to
6
7 44 our knowledge the findings from this study are novel
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9 45 2) Previous studies suggested that diurnal variations in CVD risk factors could be relevant for
10
11 46 cardiovascular risk prediction, but without testing the validity of this statement using
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13 47 statistical analysis. Our analysis answered this question and went beyond simple descriptive
14
15 48 diurnal patterns of CVD risk factors

16 49 3) The BRHS cohort benefits from using a large scale population-based sample of free-living
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18 50 older men and this increases statistical power and precision of estimates.

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20 51 4) However, the BRHS comprises male participants only, predominantly of white European
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22 52 ethnic origin, so findings may not be generalisable to women and non-white ethnic groups.
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53 Background

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55 Previous studies have reported time of day variation in both established and novel
56 cardiovascular disease (CVD) risk factors in middle aged adults, such as blood pressure,
57 lipids and some well-established inflammatory and haemostatic factors (e.g. white blood
58 cell, red blood cell, and platelets counts) ¹⁻³. However, the extent to which novel CVD risk
59 factors such as Interleukin-6, a marker of inflammation causally associated with CHD in a
60 recent study ⁴, and N-terminal pro-brain natriuretic peptide, a promising marker of heart
61 failure ⁵ vary by time of day have been less studied. Moreover, very little is known on time
62 of day variations in other emerging risk factors prospectively associated with CVD (e.g. t-PA,
63 D-Dimer, von Willebrand factor, and Cardiac Troponin T), although their causal association
64 with CVD remain debated or not yet tested.

65
66 We would expect that time of day variations in some novel CVD risk factors measured in
67 older adults may occur, consistent with findings in younger populations ⁶. However, in older
68 adults the degree of difference attributable to time of day has not been yet estimated;
69 establishing its importance and its effects on prediction of CVD risk is important given the
70 potentially wider use of N-terminal pro-brain natriuretic peptide in risk stratification (as
71 shown in a recent major meta-analysis in the general population ⁷), and potential causal link
72 between IL-6 and cardiovascular disease ⁴. Therefore, the aim of this study was to
73 investigate how novel CVD risk factors, including markers of inflammation, haemostasis and
74 myocardial function, varied by time of day in older British men.

76 Methods

77 *Participants*

78 The British Regional Heart Study (BRHS) is a prospective cohort study of cardiovascular
79 disease involving 7735 middle aged men (40-59 years) selected in 1978-80 from the age-sex
80 registers of one local primary care centre in 24 British towns ⁸. The 24 towns were selected
81 to represent the variation in cardiovascular disease across the UK ⁹. The National Research
82 Ethics Service (NRES) Committee for London provided ethical approval. Participants
83 provided informed written consent to the investigation, which was performed in accordance
84 with the Declaration of Helsinki ¹⁰.

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5 86 *Follow-up examination*
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7 87 In 1998-2000, an average of 20 years after the initial recruitment, 4252 surviving
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9 88 participants (77% response rate) aged 60-79 years who were resident in the UK attended a
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11 89 physical examination during which nurses took a fasting blood sample on one occasion for
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13 90 each participant.. The men were asked to fast for a minimum of 6 hours, during which they
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15 91 were instructed to drink only water, as previously reported ². The blood samples were
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17 92 collected between 08:00 h and 19:00 h and then assayed for a range of biochemical and
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19 93 haematological markers. The participants were also asked to complete a questionnaire
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21 94 which included questions on other established CVD risk factors, such as age, social class,
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23 95 smoking habits, alcohol consumption, and physical activity. Specifically, physical activity
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25 96 levels were self-reported ¹¹ and recently validated using accelerometers ¹². Incident CVD,
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27 97 including non-fatal stroke and non-fatal MI were recorded: their definitions have been
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29 98 reported elsewhere ¹³. Men were also asked whether a doctor had ever told them that they
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31 99 had heart failure ⁵. The number of blood samples collected and included in the analyses
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33 100 differ according to the risk factor measurements (the number of observations varied from
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35 101 3580 for N-terminal Pro-Brain Natriuretic Peptide to 3863 for von Willebrand Factor in
36
37 102 complete case analyses including all covariates of interest).
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39 103

104 **Novel risk factors**

105 Circulating levels of markers of inflammation (C-reactive protein [CRP], Interleukin 6 [IL-6],
106 Fibrinogen), cardiac markers (N-terminal pro-brain natriuretic peptide [NT-ProBNP], cardiac
107 Troponin T [cTnT], and markers of haemostasis (tissue plasminogen activator [t-PA] antigen,
108 fibrin D-dimer, von Willebrand factor [vWF]) were measured.

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110 Plasma D-dimer and t-PA levels were measured using an enzyme-linked immunosorbent
111 assay (ELISA; Biopool AB, Umeå, Sweden), as was VWF antigen (Dako, High Wycombe, UK).
112 C-reactive protein was assayed using ultrasensitive nephelometry (Dade Behring, Milton
113 Keynes, UK). IL-6 was assayed using a high-sensitivity ELISA (R & D Sys-tems, Oxford, UK).
114 Fibrinogen was assayed using an auto-mated Clauss assay in a coagulometer (MDA-180,
115 Organon Teknika, Cambridge, UK). NT-proBNP and hsTnT were measured in plasma samples
116 on an automated clinically validated immunoassay analyzer (e411, Roche Diagnostics,

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3 117 Burgess Hill, United Kingdom) using the manufacturers' calibrators and quality control
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5 118 reagents. Intra- and inter-assay Coefficient of Variations (CVs) were, respectively: 4.1% and
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7 119 6.6% for t-PA; 3.2% and 4.2% for vWF; 4.7% and 5.2% for D-dimer; 4.7% and 8.3% for CRP;
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9 120 7.5% and 8.9% for IL-6; 2.6% and 3.7% for Fibrinogen, and 4.4% and 7.7% for NT-ProBNP and
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11 121 cTnT.

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13 123 **Statistical methods**

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17 125 Firstly, the distributions of the outcomes were examined; the outcomes were log-
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19 126 transformed as the distributions were positively skewed. Therefore, analysis was carried out
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21 127 on their log-transformed values throughout. Unadjusted geometric means and 95%
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23 128 Confidence Intervals [CI] of the outcomes were plotted against hour of the day.

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25 26 130 *Adjusted associations between time of day and the outcomes*

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30 132 Associations between time of day (fitted as a continuous variable, range 8-18) and the
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32 133 outcomes were examined using linear multilevel random intercept models (level 1 =
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34 134 individual, level 2 = town of residence). The results can be interpreted as between-person
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36 135 variations over the course of the examination day; the estimates from the linear model
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38 136 were reported as the difference in the outcome levels per hour of sampling over the
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40 137 examination day. As the outcomes were log-transformed, the results were reported as
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42 138 percent difference in the outcome geometric mean per hour of sampling. All models were
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44 139 initially adjusted for age only. Next, the models were adjusted for age and other possible
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46 140 confounding factors: social class, BMI, previous stroke or myocardial infarction (MI), physical
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48 141 activity, smoking status, alcohol consumption, use of statin, and a seasonal term (fitted
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50 142 using a cosinor function, as in previous studies)¹⁴. As NT-ProBNP and cTnT are principally
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52 143 markers of heart failure, the association with time of day was adjusted for previous heart
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54 144 failure.

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56 146 When the association of time of the day with the outcomes was found to be statistically
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58 147 significant, the proportion of variance associated with time of the day was estimated using
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60 148 partial R-squared.

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3 149 *Sensitivity analyses*

4 150 Five sensitivity analyses were performed: (i) all models were additionally adjusted for fasting
5 151 time; (ii) interactions were fitted to test whether the time of day associations were modified
6 152 by age (fitted as continuous variable); (iii) as NT-ProBNP and cTnT were acknowledged as
7 153 specific cardiac markers ⁵, interactions were fitted to test whether the time of day
8 154 associations were modified by previous heart failure (yes/no); (iv) to explore the potential of
9 155 undiagnosed heart failure or cardiac damage influencing findings for NT-ProBNP and cTnT,
10 156 we repeated regression models after excluding men with NTproBNP > 400 pg/ml; (v) a
11 157 quadratic term for time of day was added to the models in order to check for non-linearity
12 158

13 159 As IL-6 has been causally associated with cardiovascular risk ⁴, and prospectively associated
14 160 with CVD mortality in the BRHS sample used here ¹⁵, we investigated the relevance of time
15 161 of day to the cardiovascular risk prediction by performing two survival analyses: in the first
16 162 analysis we used Cox models where unadjusted log IL-6 was used as the predictor and CVD
17 163 mortality as the clinical outcome; then, we repeated the same analysis using log IL-6
18 164 standardised by the time of day rather than unadjusted log IL-6. For completeness of
19 165 information, we repeated this sensitivity analysis for NT-ProBNP and t-PA.
20 166

21 167 **Results**

22 168
23 169 The characteristics of the study participants (mean age 68.7 years, standard deviation (SD) =
24 170 5.5) are reported in Table 1. The associations between time of day (by hour) and risk factors
25 171 are shown in Figure 1. Evidence of an increase over the course of the day was particularly
26 172 noticeable for IL-6, and for NT-ProBNP (Figure 1). Also, levels of t-PA were lower in the
27 173 afternoon in comparison with morning, while variations by time of day for other risk factors
28 174 were not clearly observable from the plots (Figure 1). The results of corresponding linear
29 175 regression analyses are shown in Table 2: statistically significant associations between time
30 176 of the day and some outcomes were found (Table 2): over the course of the examination
31 177 day NT-ProBNP levels increased by 3.3% per hour (95% CI 1.9; 4.8%), IL-6 increased by 2.6%
32 178 per hour (95% CI 1.8; 3.4%). Conversely, t-PA decreased by 3.3% per hour (95% CI 3.7; 2.9%).
33 179 The proportion of variance associated with time of the day from the fully adjusted models
34 180 was 0.5%, 1%, and 2% for NT-ProBNP, IL-6, and t-PA respectively.
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3 181 C-Reactive Protein, Fibrinogen, D-Dimer, von Willebrand Factor, and cardiac Troponin T
4 182 showed no consistent associations with time of day (Table 2).
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8 184 *Sensitivity analyses*

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10 185 Overall, we found that fasting time did not alter the magnitude of associations between
11 186 time of the day and the outcomes reported in Table 2. Only the association between time of
12 187 the day and t-PA was strongly attenuated after accounting for fasting time (fitted as
13 188 continuous variable): the decrease in t-PA levels was -3.3% (95%CI -3.7; -2.9) per hour
14 189 before the adjustment (Table 2) and -1.4% (95%CI -2.2; -0.1) after the adjustment for
15 190 fasting.
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22 192 For all outcomes, we also did not find evidence for an interaction between time of day
23 193 with age (results not shown).
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28 195 In stratified analysis, NT-ProBNP levels increased by 3.4% (95% CI 1.9; 4.8%, $p < 0.001$) per
29 196 hour in older men without heart failure. Although men who previously had heart failure had
30 197 increased NT-ProBNP levels, there was no evidence for an interaction between previous
31 198 heart failure with time of the day ($p = 0.954$). After excluding 466 men with NTproBNP levels
32 199 > 400 pg/ml (12% of the sample), associations between time of the day measures and
33 200 NTproBNP remained statistically significant and slightly increased in magnitude (3.9% [95%
34 201 CI 2.7; 5.1%], $p < 0.001$). As reported in the main analysis, no significant associations were
35 202 found between time of the day and cTnT in stratified analysis.
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43 204 When adding a quadratic term to the model, we found a significant improvement in model
44 205 fit for IL-6 only ($p = 0.030$ for the time of day squared term). The association of time of day
45 206 with IL-6 appeared to be slightly J-shaped, with a linear increase starting from 11:00 until
46 207 18:00 hours.
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52 209 We examined whether adjustment for hour of day affected the associations between risk
53 210 factors and CVD mortality. In survival analysis, higher levels of log IL-6 were associated with
54 211 increased CVD mortality (HR=1.70, 95%CI 1.54; 1.87). Standardising IL-6 by time of the day
55 212 did not change the relationship (HR=1.71, 95%CI 1.55; 1.88). Also, standardising NT-ProBNP
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3 213 levels by time of the day did not alter the magnitude of the effect on CVD mortality
4 214 (HR=1.92, 95%CI 1.81; 2.04). Finally, associations of t-PA levels with increased CVD mortality
5 215 did not change substantially before (HR=1.74, 95%CI 1.45; 2.09) and after standardising
6 216 (HR=1.77, 95%CI 1.47; 2.14) by time of the day.
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11 218 **Discussion**

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14 219 To our knowledge, this is the largest investigation of relationships between time of day and
15 220 novel CVD risk factors in older men. After adjusting our analysis for potential confounding
16 221 factors we demonstrated that some, but not all, novel CVD risk factors levels varied by time
17 222 of day. In particular, NT-ProBNP and IL-6 increased linearly over the course of the day.
18 223 Conversely, a decrease in t-PA was also observed; however, after accounting for fasting time
19 224 the relationship with time of the day was strongly attenuated (therefore fasting time could
20 225 partially explain the drop in t-PA levels observed in the afternoon vs morning). Also, we
21 226 observed a weak contribution of time of the day to the overall variation of these markers. In
22 227 sensitivity analyses, we observed that time of day did not have a sufficiently strong effect to
23 228 be taken into account when assessing the impact of IL-6, NT-ProBNP, and t-PA on CVD
24 229 mortality. Lastly, an association of time of day with other novel risk factors was not
25 230 observed.
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37 232 Literature on time of day variation in novel CVD markers of inflammation and haemostasis
38 233 in older adults is limited; to our knowledge this is the first time these findings have been
39 234 reported in older adults. Findings from earlier studies of younger adults were fairly
40 235 consistent with ours. For example a recent meta-analysis of several small studies which
41 236 analysed IL-6 proposed a diurnal pattern, with overall IL-6 levels increased between 08:00
42 237 and 18:00 hours as in our study¹⁶. However, in two previous very small studies of twelve¹⁷
43 238 and five¹⁸ participants, IL-6 peaked in the night-time. It is possible that peaks in IL-6 levels
44 239 may be associated with cognitive symptoms of depression¹⁹ and daily activities, although in
45 240 the BRHS population this has not yet been investigated. One previous study found that BRHS
46 241 men were more active in the morning and in early afternoon²⁰ when the main activities
47 242 were usually gardening, house works, shopping, or leisure walking. Whether IL-6 was
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3 243 implicated in this daily pattern remains uncertain and can potentially be explored in future
4 244 studies.

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8 246 Moreover, one previous study reported increased levels of NT-ProBNP over the course of
9 247 day ²¹ as we observed in our study. A decrease in t-PA over the examination day was also
10 248 reported in younger subjects (a 45-year-old UK population of 9377 men and women) ⁶;
11 249 however, t-PA did not vary by time of the day in a previous study large study of 1288
12 250 healthy 25 to 64-year-old men and women ²².

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19 252 In comparison to our study, findings regarding CRP, Fibrinogen, D-dimer, vWF and cTnT
20 253 reported in earlier studies of younger adults were similar: a few previous studies reported
21 254 that they did not find an association of time of day with CRP ²³, D-dimer ²⁴, and vWF ²⁵. In
22 255 one study, the variation in CRP, Fibrinogen, D-dimer, and vWF attributable to time of day
23 256 was minimal ⁶. Literature on cTnT is scarce: one small previous study of repeated measures
24 257 in 7 participants with type 2 diabetes reported a decrease in cTnT between 8am and 8pm ²⁶.

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31 259 Although one previous study suggested that diurnal variations in CVD risk factors could be
32 260 relevant for cardiovascular risk prediction ⁶, a prediction model like the one described in our
33 261 survival analysis was not performed. Our findings suggested the effect of time of the day
34 262 (from 08:00 h to 19:00 h) is not relevant for the CVD risk assessment. With this sensitivity
35 263 analysis we wanted to investigate time of day variations beyond simple descriptive diurnal
36 264 patterns; to our knowledge this is the first time this finding has been reported.

37 265

38 266 *Strengths and limitations*

39 267 The BRHS cohort benefits from using a large scale population-based sample of free-living
40 268 older men and this increases statistical power and precision of estimates. However, the
41 269 BRHS comprises male participants, predominantly of white European ethnic origin, so
42 270 findings may not be generalisable to women and non-white ethnic groups. The CVD risk
43 271 factors measurements were carried out on one occasion over an extended period of the day
44 272 (between 08:00 and 18:00 hours), offering only a partial understanding of the variations
45 273 over the 24 hours ^{27 28}. Therefore, in this study the relationship of the CVD risk factors to
46 274 time of day was explored using between-participant variation only. In future studies it may

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3 275 be advantageous to carry out repeated measurement of the risk factors over the 24 hours in
4 276 order to investigate within-person circadian variations. However, with repeated
5 277 measurements a possible and genuine diurnal variation may be disrupted and natural
6 278 sleeping patterns altered (repeated measures are usually taken every 1-2 hours during the
7 279 night)²⁹.

280

281 *Implications*

282 Variations of some CVD factors (in particular IL-6 and NT-ProBNP) over the course of the day
283 were observed, suggesting the role of time of the day as potential confounder during the
284 measurements. However, standardising these biological markers by time of day was not
285 particularly relevant for the cardiovascular risk prediction. Also, other sensitivity analyses
286 (stratified analysis and interaction tests) did not add relevant insights suggesting that time
287 of day variations may be not important for clinical risk stratification in general. Further
288 studies assessing both CVD risk factors levels and clinical outcomes (e.g. fatal or non-fatal
289 CVD events) during 24h are required to demonstrate whether a rapid increase of IL-6 over
290 the day may be relevant to the increased number of CVD events observed in early and late
291 morning³⁰, and whether the increased levels of NT-ProBNP over the day are related to the
292 afternoon peak in sudden death following heart failure³¹.

293

294 **Conclusions**

295 Variations in time of day were associated with variations of some, but not all, novel CVD risk
296 factors measured in older adults. The contribution of time of the day to the markers' overall
297 variation was small and unlikely to affect the CVD risk prediction or clinical risk stratification.

298

299 **AUTHORS CONTRIBUTIONS**

300 CS processed the data, performed statistical analyses, drafted and revised the manuscript,
301 and incorporated revisions of co-authors. PHW, SGW, BJ, and RM contributed to the design
302 of the study and revised the manuscript. LL enrolled participants, and collected data. PHW,
303 RM and SGW raised grant funding. All authors gave an intellectual contribution to the
304 manuscript and approved the final version.

305

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5
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7
8 310 collection, management, analysis, and interpretation of the data; preparation, review, or
9
10 311 approval of the manuscript; and the decision to submit the manuscript for publication. The
11
12 312 views expressed in this publication are those of the author(s) and not necessarily those of
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14 313 the BHF.

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17 **Conflict of interest statement**

18
19 316 The authors report no relationships that could be construed as a conflict of interest

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23 **Data sharing statement**

24 319 The collection and management of data over the last 39 years of the BRHS has been made
25
26 320 possible through grant funding from UK government agencies and charities. We welcome
27
28 321 proposals for collaborative projects and data sharing. For general data sharing enquiries,
29
30 322 please contact Lucy Lennon l.lennon@ucl.ac.uk

323 **Table 1** –Individual characteristics and risk factors levels in BRHS men who attended the
 324 examinations in 1998-2000

<i>Demographic and background characteristics</i>	
Age (years), mean (SD)	68.7 (5.5)
Social class (manual)	
Manual, n (%)	2166 (51.1)
Non-Manual, n (%)	1966 (46.3)
HMF, n (%)	112 (2.6)
<i>Physical health</i>	
BMI, mean (SD)	26.9 (3.7)
Prevalence of stroke/myocardial infarction, n (%)	153 (3.6)
Prevalence of heart failure, n (%)	390 (9.2)
<i>Behavioural factors</i>	
Smoking	
Never, n (%)	1233 (29.1)
Ex-smokers, n (%)	2464 (58.0)
Smokers, n (%)	548 (12.9)
Alcohol consumption	
None, n (%)	431 (10.3)
Occasional/light, n (%) ¹	2949 (70.5)
Moderate/Heavy, n (%) ²	779 (18.6)
Physical activity level	
Inactive, n (%)	471 (11.5)
Occasionally, n (%)	957 (23.4)
Light, n (%)	767 (18.7)
Moderate, n (%)	591 (14.4)
Moderate vigorous, n (%)	690 (16.8)
Vigorous, n (%)	621 (15.1)
<i>CVD risk factor, geometric mean (SD)</i>	
CRP, mg/L	1.74 (3.03)
IL-6, pg/mL	2.46 (1.94)
Fibrinogen, g/L	3.19 (1.25)
t-PA, ng/mL	10.23 (1.50)
vWF, IU/dL	132.41 (1.40)
D-dimer, ng/mL	84.32 (2.32)
NT-ProBNP, pg/mL	101.50 (3.32)
cTnT, pg/mL	12.07 (1.64)

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326 ¹ >=1 and <=15 units per week (1 unit is approximately 1 drink, such as one glass of wine)327 ² >=16 units per week (1 unit is approximately 1 drink, such as one glass of wine)

328

329 **Table 2** – Cross-sectional adjusted associations between time of day (fitted as continuous
 330 variable) and CVD risk factors measured in BRHS men (aged 60-79) attending the follow-up
 331 year 20 examination in 1998-2000. Associations are reported as percent difference in CVD
 332 risk factors levels per one hour of sampling over the examination day (08:00-19:00h). The
 333 statistically significant associations are marked in bold.
 334

CVD risk factor	Model 1: Age adjusted ¹		Model 2: Fully adjusted ²	
	Percent difference (95%CI) in the CVD risk factor levels per hour of sampling ³	p-value	Percent difference (95%CI) in the CVD risk factor levels per hour of sampling ³	p-value
NT-ProBNP	3.5 (2.0;5.0)	<0.001	3.3 (1.9; 4.8)	<0.001
IL-6	2.6 (1.7;3.4)	<0.001	2.6 (1.8; 3.4)	<0.001
t-PA	-3.3 (-3.8;-2.9)	<0.001	-3.3 (-3.7; -2.9)	<0.001
Fibrinogen	-0.2 (-0.5;0.0)	0.088	-0.2 (-0.5; 0.1)	0.104
cTnT	-0.4 (-0.9;0.2)	0.194	-0.4 (-1.0; 0.2)	0.174
CRP	-1.0 (-2.3;0.4)	0.151	-0.9 (-2.2; 0.4)	0.175
vWF	-0.2 (-0.6;0.2)	0.374	-0.2 (-0.6; 0.2)	0.380
D-Dimer	-0.0 (-1.0;1.0)	0.929	-0.1 (-1.0; 0.9)	0.890

335 ¹ Model 1: Two level linear models (level 1 = person, level 2 = town of residence during the
 336 BRHS recruitment) adjusted for age. Model 1 used the same number of observations of
 337 Model 2 (complete case analysis).

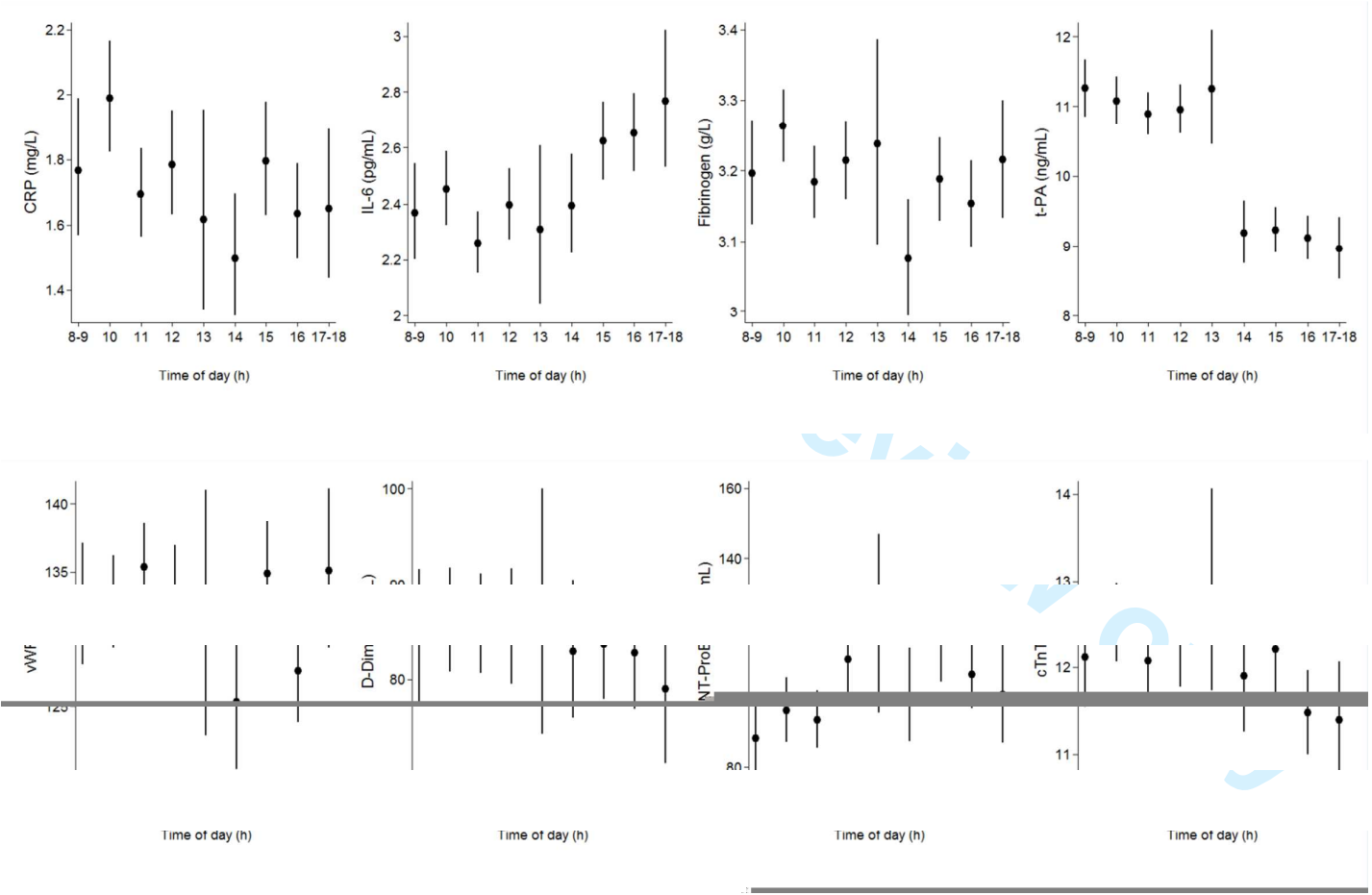
338 ² Model 2 = Model 1 additionally adjusted for social class, BMI, smoking status, alcohol
 339 consumption, physical activity, use of statin, and season. Associations with IL-6, t-PA,
 340 Fibrinogen, CRP, vWF, and D-Dimer were additionally adjusted for prevalence of stroke/MI,
 341 while association of time of the day with NT-ProBNP and cTnT models were additionally
 342 adjusted for prevalence of heart failure.

343 ³ Model 1 and Model 2 used the number of observations: 3580 for NT-ProBNP, 3832 for IL-6,
 344 3863 for t-PA, 3861 for Fibrinogen, 3827 for cTnT, 3838 for CRP, 3863 for vWF, 3859 for D-
 345 Dimer

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347 **Figure 1** – Unadjusted geometric means (95% CI) by time of the day¹ for CRP, IL-6, Fibrinogen t-PA (top), vWF, D-Dimer, NT-ProBNP and cTnT
348 (bottom) measured on one occasion in BRHS men aged 60-79 during the years 1998-2000.



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¹ Total number of men examined per hour was 33(0.7%) at 08:00-08:59, 363(8.5%) at 8:00-9:59h, 699(16%) at 10:00-10:59h, 771(18%) at 11:00-11:59h, 591(14%) at 12:00-12:59h, 99(2%) at 13:00-13:59h, 306(7%) at 14:00-14:59, 560(13%) at 15:00-15:59h, 566(13%) at 16:00-16:59, 260(6%) at 17:00-17:59, and 3(<0.1%) at 18:00-18:59

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STROBE Statement—Checklist of items that should be included in reports of *cross-sectional studies*

	Item No	Recommendation	
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	Abstract
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	Abstract
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Introduction, lines 66-68
Objectives	3	State specific objectives, including any prespecified hypotheses	Introduction, lines 66-67 72-74
Methods			
Study design	4	Present key elements of study design early in the paper	Methods, line 78-81
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Methods, lines 86-93
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants	Methods, lines 78-81 and 86-90
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	Methods, lines 104-121
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Methods, lines 91-121
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	Results, lines 86-90
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Methods, lines 104-121
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	Methods, lines 123-166
		(b) Describe any methods used to examine subgroups and interactions	Methods, lines 150-166
		(c) Explain how missing data were addressed	
		(d) If applicable, describe analytical methods taking account of sampling strategy	NA
		(e) Describe any sensitivity analyses	Methods, lines 150-166
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Methods, lines 78-81 and 86-90
		(b) Give reasons for non-participation at each stage	Methods, lines 78-81 and 86-90
		(c) Consider use of a flow diagram	Not necessary for this study, see Methods, lines 78-81 and 86-90

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2	Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
3			Table 1
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6			(b) Indicate number of participants with missing data for each variable of interest
7			Table 1
8	Outcome data	15*	Report numbers of outcome events or summary measures
9			Table 1, Figure 1
10	Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
11			Table 2
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13			(b) Report category boundaries when continuous variables were categorized
14			NA
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16			(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
17			NA
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19	Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
20			Results, lines 195-216
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22	Discussion		
23	Key results	18	Summarise key results with reference to study objectives
24			Results, lines 169-180 Discussion, lines 219-230
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27	Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
28			Discussion, lines 267-279
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30	Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
31			Discussion, lines 232-244 and 282 - 292
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34	Generalisability	21	Discuss the generalisability (external validity) of the study results
35			Discussion, lines 295-297
36			
37	Other information		
38	Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based
39			Acknowledgements, lines 306-314
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*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Time of day variations in cardiovascular disease risk factors measured in older men

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Secondary Subject Heading:	Public health
Keywords:	biological markers, older adults, cardiovascular disease, diurnal variations

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1 **Title:** Time of day variations in cardiovascular disease risk factors measured in older men

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17 **Manuscript number of words:** 4847 words (all included: title page, abstract, manuscript, all
18 tables and references).

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3 20 **Abstract (250 words, now 248)**
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5 21 **Objective:** We estimated associations of time of day with cardiovascular disease (CVD) risk
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7 22 factors measured in older men.

8
9 23 **Methods:** CVD risk factors (markers of inflammation and haemostasis, and cardiac markers)
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11 24 were measured on one occasion between 08:00-19:00 hours in 4252 men aged 60-79 years
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13 25 from the British Regional Heart Study. Linear models were used to estimate associations
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15 26 between time of day and risk factors. When an association was found, we examined
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17 27 whether the relationship between risk factors and cardiovascular mortality was affected by
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19 28 the adjustment for time of day using survival analyses.

20 29 **Results:** N-terminal pro-brain natriuretic peptide (NT-ProBNP) levels increased by 3.3% per
21
22 30 hour [95% Confidence interval (CI) 1.9; 4.8], Interleukin-6 (IL-6) increased by 2.6% per hour
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24 31 (95% CI 1.8; 3.4), while Tissue plasminogen activator (t-PA) decreased by 3.3% per hour
25
26 32 (95% CI 3.7; 2.9); these associations were unaffected by adjustment for possible
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28 33 confounding factors. The percentages of variation in these risk factors attributable to time
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30 34 of day were less than 2%. In survival analyses, the association of IL-6, NT-ProBNP, and t-PA
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32 35 with cardiovascular mortality was not affected by the adjustment for time of day. C-Reactive
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34 36 Protein, Fibrinogen, D-Dimer, von Willebrand Factor, and cardiac Troponin T showed no
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36 37 associations with time of day.

37 38 **Conclusions:** In older men, markers of inflammation (IL-6), haemostasis (t-PA), and a cardiac
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39 39 marker (NT-ProBNP) varied by time of day. The contribution of time of day to variations in
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41 40 these markers was small, and did not appear to be relevant for the CVD risk prediction.
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42 **Background**

43 Previous studies have reported time of day variation in both established and emerging
44 cardiovascular disease (CVD) risk factors in middle aged adults, such as blood pressure,
45 lipids and some well-established inflammatory and haemostatic factors (e.g. white blood
46 cell, red blood cell, and platelets counts) ¹⁻³. However, the extent to which emerging CVD
47 risk factors such as Interleukin-6, a marker of inflammation causally associated with CHD in
48 a recent study ⁴, and N-terminal pro-brain natriuretic peptide, a marker of heart failure ⁵
49 vary by time of day have been less studied. Moreover, very little is known on time of day
50 variations in other emerging risk factors prospectively associated with CVD (e.g. t-PA, D-
51 Dimer, von Willebrand factor, and Cardiac Troponin T), although their causal association
52 with CVD remain debated or not yet tested.

53
54 We would expect that time of day variations in some emerging CVD risk factors measured in
55 older adults may occur, consistent with findings in younger populations ⁶. However, in older
56 adults the degree of difference attributable to time of day has not been yet estimated;
57 establishing its importance and its effects on prediction of CVD risk is important given the
58 potentially wider use of N-terminal pro-brain natriuretic peptide in risk stratification (as
59 shown in a recent major meta-analysis in the general population ⁷), and potential causal link
60 between IL-6 and cardiovascular disease ⁴. Therefore, the aim of this study was to
61 investigate how emerging CVD risk factors, including markers of inflammation, haemostasis
62 and myocardial function, varied by time of day in older British men.

64 **Methods**

65 *Participants*

66 The British Regional Heart Study (BRHS) is a prospective cohort study of cardiovascular
67 disease involving 7735 middle aged men (40-59 years) selected in 1978-80 from the age-sex
68 registers of one local primary care centre in 24 British towns ⁸. The 24 towns were selected
69 to represent the variation in cardiovascular disease across the UK ⁹. The National Research
70 Ethics Service (NRES) Committee for London provided ethical approval. Participants
71 provided informed written consent to the investigation, which was performed in accordance
72 with the Declaration of Helsinki ¹⁰.

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3 74 *Follow-up examination*

4 75 In 1998-2000, an average of 20 years after the initial recruitment, 4252 surviving
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6 76 participants (77% response rate) aged 60-79 years who were resident in the UK attended a
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8 77 physical examination during which nurses took a fasting blood sample on one occasion for
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10 78 each participant. The men were asked to fast for a minimum of 6 hours, during which they
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12 79 were instructed to drink only water, as previously reported ². The blood samples were
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14 80 collected between 08:00 h and 19:00 h and then assayed for a range of biochemical and
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16 81 haematological markers. Participants' appointment times were non-systematically
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18 82 allocated. They were offered the opportunity to contact the BRHS team and change the time
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20 83 of examination, if unable to attend; a small proportion of participants did so.
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22 84
23 85 The participants were also asked to complete a questionnaire which included questions on
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25 86 other established CVD risk factors, such as age, social class, smoking habits, alcohol
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27 87 consumption, and physical activity. Specifically, physical activity levels were self-reported ¹¹
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29 88 and recently validated using accelerometers ¹². Incident CVD, including non-fatal stroke and
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31 89 non-fatal MI were recorded: their definitions have been reported elsewhere ¹³. Men were
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33 90 also asked whether a doctor had ever told them that they had heart failure ⁵. The number of
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35 91 blood samples collected and included in the analyses differ according to the risk factor
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37 92 measurements (the number of observations varied from 3580 for N-terminal Pro-Brain
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39 93 Natriuretic Peptide to 3863 for von Willebrand Factor in complete case analyses including all
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41 94 covariates of interest).

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43 96 *CVD Risk factors*

44 97 Circulating levels of markers of inflammation (C-reactive protein [CRP], Interleukin 6 [IL-6],
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46 98 Fibrinogen), cardiac markers (N-terminal pro-brain natriuretic peptide [NT-ProBNP], cardiac
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48 99 Troponin T [cTnT], and markers of haemostasis (tissue plasminogen activator [t-PA] antigen,
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50 100 fibrin D-dimer, von Willebrand factor [vWF]) were measured.

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52 102 D-dimer and t-PA levels were measured using an enzyme-linked immunosorbent assay
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54 103 (ELISA; Biopool AB, Umeå, Sweden), as was VWF antigen (Dako, High Wycombe, UK). C-
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56 104 reactive protein was assayed using ultrasensitive nephelometry (Dade Behring, Milton
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58 105 Keynes, UK). IL-6 was assayed using a high-sensitivity ELISA (R & D Sys-tems, Oxford, UK).

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3 106 Fibrinogen was assayed using an auto-mated Clauss assay in a coagulometer (MDA-180,
4 107 Organon Teknika, Cambridge, UK). NT-proBNP and hsTnT were measured in plasma samples
5 108 on an automated clinically validated immunoassay analyzer (e411, Roche Diagnostics,
6 109 Burgess Hill, United Kingdom) using the manufacturers' calibrators and quality control
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8 110 reagents. Intra- and inter-assay Coefficient of Variations (CVs) were, respectively: 4.1% and
9 111 6.6% for t-PA; 3.2% and 4.2% for vWF; 4.7% and 5.2% for D-dimer; 4.7% and 8.3% for CRP;
10 112 7.5% and 8.9% for IL-6; 2.6% and 3.7% for Fibrinogen, and 4.4% and 7.7% for NT-ProBNP and
11 113 cTnT.
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19 115 The samples were centrifuged and separated on the morning or afternoon of collection and
20 116 stored on site at -20°C until they were transferred to a central freezer storage location at -
21 117 70°C within 2 weeks of sample collection. Samples were then transferred on dry ice to a
22 118 single central laboratory and were thawed immediately before analysis. Plasma samples
23 119 were used for all the analyses reported here. The original sample collection took place
24 120 between January 1998 and March 2000. Most of the analyses described here were carried
25 121 out during 2000, after a maximum of 3 years storage; NT-ProBNP and cTnT were analysed in
26 122 2009.
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35 124 **Statistical methods**

36 125 Firstly, the distributions of the outcomes were examined; the outcomes were log-
37 126 transformed as the distributions were positively skewed. Therefore, analysis was carried out
38 127 on their log-transformed values throughout. Unadjusted geometric means and 95%
39 128 Confidence Intervals [CI] of the outcomes were plotted against hour of the day.
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45 130 *Adjusted associations between time of day and the outcomes*

46 131 Associations between time of day (fitted as a continuous variable, range 8-18) and the
47 132 outcomes were examined using linear multilevel random intercept models (level 1 =
48 133 individual, level 2 = town of residence). The results can be interpreted as between-person
49 134 variations over the course of the examination day; the estimates from the linear model
50 135 were reported as the difference in the outcome levels per hour of sampling over the
51 136 examination day. As the outcomes were log-transformed, the results were reported as
52 137 percent difference in the outcome geometric mean per hour of sampling. All models were
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3 138 initially adjusted for age only. Next, the models were adjusted for age and other possible
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5 139 confounding factors: social class, BMI, previous stroke or myocardial infarction (MI), physical
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7 140 activity, smoking status, alcohol consumption, use of statin, and a seasonal term (fitted
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9 141 using a cosinor function, as in previous studies)¹⁴. As NT-ProBNP and cTnT are principally
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11 142 markers of heart failure, the association with time of day was adjusted for previous heart
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13 143 failure.
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16 145 When the association of time of the day with the outcomes was found to be statistically
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18 146 significant, the proportion of variance associated with time of the day was estimated using
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20 147 partial R-squared.
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23 149 *Sensitivity analyses*

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25 150 Six sensitivity analyses were performed: (i) all models were additionally adjusted for fasting
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27 151 time and diabetes; (ii) all models were carried out excluding men with diabetes, (iii)
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29 152 interactions were fitted to test whether the time of day associations were modified by age
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31 153 (fitted as continuous variable); (iv) as NT-ProBNP and cTnT were acknowledged as specific
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33 154 cardiac markers⁵, interactions were fitted to test whether the time of day associations were
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35 155 modified by previous heart failure (yes/no); (v) to explore the potential of undiagnosed
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37 156 heart failure or cardiac damage influencing findings for NT-ProBNP and cTnT, we repeated
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39 157 regression models after excluding men with NTproBNP > 400 pg/ml; (vi) a quadratic term for
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41 158 time of day was added to the models in order to check for non-linearity
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45 160 As IL-6 has been causally associated with cardiovascular risk⁴, and prospectively associated
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47 161 with CVD mortality in the BRHS sample used here¹⁵, we investigated the relevance of time
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49 162 of day to the cardiovascular risk prediction by performing two survival analyses: in the first
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51 163 analysis we used Cox models where unadjusted log IL-6 was used as the predictor and CVD
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53 164 mortality as the clinical outcome; then, we repeated the same analysis using log IL-6
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55 165 standardised by the time of day rather than unadjusted log IL-6. For completeness of
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57 166 information, we repeated this sensitivity analysis for NT-ProBNP and t-PA.
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59 167

56 168 **Results**

58 169 The characteristics of the study participants (mean age 68.7 years, standard deviation (SD) =
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3 170 5.5) are reported in Table 1. The associations between time of day (by hour) and risk factors
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5 171 are shown in Figure 1. Evidence of an increase over the course of the day was particularly
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7 172 noticeable for IL-6, and for NT-ProBNP (Figure 1). Also, levels of t-PA were lower in the
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9 173 afternoon in comparison with morning, while variations by time of day for other risk factors
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11 174 were not clearly observable from the plots (Figure 1). The results of corresponding linear
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13 175 regression analyses are shown in Table 2: statistically significant associations between time
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15 176 of the day and some outcomes were found (Table 2): over the course of the examination
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17 177 day NT-ProBNP levels increased by 3.3% per hour (95% CI 1.9; 4.8%), IL-6 increased by 2.6%
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19 178 per hour (95% CI 1.8; 3.4%). Conversely, t-PA decreased by 3.3% per hour (95% CI 3.7; 2.9%).
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21 179 The proportion of variance associated with time of the day from the fully adjusted models
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23 180 was 0.5%, 1%, and 2% for NT-ProBNP, IL-6, and t-PA respectively.

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26 182 C-Reactive Protein, Fibrinogen, D-Dimer, von Willebrand Factor, and cardiac Troponin T
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28 183 showed no consistent associations with time of day (Table 2).
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30 184

31 185 *Sensitivity analyses*

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33 186 Overall, we found that fasting time did not alter the magnitude of associations between
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35 187 time of the day and the outcomes reported in Table 2. Only the association between time of
36
37 188 the day and t-PA was strongly attenuated after accounting for fasting time (fitted as
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39 189 continuous variable): the decrease in t-PA levels was -3.3% (95%CI -3.7; -2.9) per hour
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41 190 before the adjustment (Table 2) and -1.4% (95%CI -2.2; -0.1) after the adjustment for
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43 191 fasting. An additional adjustment for diabetes status did not alter the magnitude of the
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45 192 association between hour of the day and the outcomes. We also performed the analysis
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47 193 excluding men with diabetes completely (Table 2 – Model 3), but the association between
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49 194 time of day and the outcomes did not substantially change.

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51 195
52 196 For all outcomes, we also did not find evidence for an interaction between of time of day
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54 197 with age (results not shown).
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58 199 In stratified analysis, NT-ProBNP levels increased by 3.4% (95% CI 1.9; 4.8%, $p < 0.001$) per
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60 200 hour in older men without heart failure. Although men who previously had heart failure had
201 increased NT-ProBNP levels, there was no evidence for an interaction between previous

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3 202 heart failure with time of the day ($p=0.954$). After excluding 466 men with NTproBNP levels
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5 203 $>400\text{pg/ml}$ (12% of the sample), associations between time of the day measures and
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7 204 NTproBNP remained statistically significant and slightly increased in magnitude (3.9% [95%
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9 205 CI 2.7; 5.1%], $p<0.001$). As reported in the main analysis, no significant associations were
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11 206 found between time of the day and cTnT in stratified analysis.
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14 208 When adding a quadratic term to the model, we found a significant improvement in model
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16 209 fit for IL-6 only ($p=0.030$ for the time of day squared term). The association of time of day
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18 210 with IL-6 appeared to be slightly J-shaped, with a linear increase starting from 11:00 until
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20 211 19:00 hours.
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23 213 We examined whether adjustment for hour of day affected the associations between risk
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25 214 factors and CVD mortality. In survival analysis, higher levels of log IL-6 were associated with
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27 215 increased CVD mortality (HR=1.70, 95%CI 1.54; 1.87). Standardising IL-6 by time of the day
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29 216 did not change the relationship (HR=1.71, 95%CI 1.55; 1.88). Also, standardising NT-ProBNP
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31 217 levels by time of the day did not alter the magnitude of the effect on CVD mortality
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33 218 (HR=1.92, 95%CI 1.81; 2.04). Finally, associations of t-PA levels with increased CVD mortality
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35 219 did not change substantially before (HR=1.74, 95%CI 1.45; 2.09) and after standardising
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37 220 (HR=1.77, 95%CI 1.47; 2.14) by time of the day.
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222 Discussion

223 To our knowledge, this is the largest investigation of relationships between time of day and
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225 224 CVD risk factors in older men. After adjusting our analysis for potential confounding factors
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227 225 we demonstrated that some, but not all, CVD risk factors levels varied by time of day. In
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229 226 particular, NT-ProBNP and IL-6 increased linearly over the course of the day. Conversely, a
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231 227 decrease in t-PA was also observed; however, after accounting for fasting time the
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233 228 relationship with time of the day was strongly attenuated (therefore fasting time could
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235 229 partially explain the drop in t-PA levels observed in the afternoon vs morning). Our analyses
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237 230 showed that the contribution of time of the day to the overall variation of NT-ProBNP, IL-6,
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239 231 and t-PA was small and without clinical importance; we observed that time of day did not
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241 232 have a sufficiently strong effect to be taken into account when assessing the impact of IL-6,

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3 233 NT-ProBNP, and t-PA on CVD mortality. Lastly, an association of time of day with other risk
4 234 factors was not observed.

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8 236 Literature on time of day variation in emerging CVD markers of inflammation and
9 237 haemostasis in older adults is limited; to our knowledge this is the first time these findings
10 238 have been reported in older adults. Findings from earlier studies of younger adults were
11 239 fairly consistent with ours. For example a recent meta-analysis of several small studies
12 240 which analysed IL-6 proposed a diurnal pattern, with overall IL-6 levels increased between
13 241 08:00 and 19:00 hours as in our study ¹⁶. However, in two previous very small studies of
14 242 twelve ¹⁷ and five ¹⁸ participants, IL-6 peaked in the night-time. It is possible that peaks in IL-
15 243 6 levels may be associated with cognitive symptoms of depression ¹⁹ and daily activities,
16 244 although in the BRHS population this has not yet been investigated. One previous study
17 245 found that BRHS men were more active in the morning and in early afternoon ²⁰ when the
18 246 main activities were usually gardening, house works, shopping, or leisure walking. Whether
19 247 IL-6 was implicated in this daily pattern remains uncertain and can potentially be explored in
20 248 future studies.

21 249

22 250 Moreover, one previous study reported increased levels of NT-ProBNP over the course of
23 251 day ²¹ as we observed in our study. A decrease in t-PA over the examination day was also
24 252 reported in younger subjects (a 45-year-old UK population of 9377 men and women) ⁶;
25 253 however, t-PA did not vary by time of the day in a previous study large study of 1288
26 254 healthy 25 to 64-year-old men and women ²².

27 255

28 256 In comparison to our study, findings regarding CRP, Fibrinogen, D-dimer, vWF and cTnT
29 257 reported in earlier studies of younger adults were similar: a few previous studies reported
30 258 that they did not find an association of time of day with CRP ²³, D-dimer ²⁴, and vWF ²⁵. In
31 259 one study, the variation in CRP, Fibrinogen, D-dimer, and vWF attributable to time of day
32 260 was minimal ⁶. Literature on cTnT is scarce: one small previous study of repeated measures
33 261 in 7 participants with type 2 diabetes reported a decrease in cTnT between 8am and 8pm ²⁶.

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35 263 Although one previous study suggested that diurnal variations in CVD risk factors could be
36 264 relevant for cardiovascular risk prediction ⁶, a prediction model like the one described in our

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3 265 survival analysis was not performed. Our findings suggested the effect of time of the day
4 266 (from 08:00 h to 19:00 h) is not relevant for the CVD risk assessment. With this sensitivity
5 267 analysis we wanted to investigate time of day variations beyond simple descriptive diurnal
6 268 patterns; to our knowledge this is the first time this finding has been reported.
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11 270 *Strengths and limitations*

12 271 The BRHS cohort benefits from using a large scale population-based sample of free-living
13 272 older men and this increases statistical power and precision of estimates. However, the
14 273 BRHS comprises male participants, predominantly of white European ethnic origin, so
15 274 findings may not be generalisable to women and non-white ethnic groups. The CVD risk
16 275 factors measurements were carried out on one occasion over an extended period of the day
17 276 (between 08:00 and 19:00 hours), offering only a partial understanding of the variations
18 277 over the 24 hours^{27 28}. Therefore, in this study the relationship of the CVD risk factors to
19 278 time of day was explored using between-participant variation only. In future studies it may
20 279 be advantageous to carry out repeated measurement of the risk factors over the 24 hours in
21 280 order to investigate within-person circadian variations. However, with repeated
22 281 measurements a possible and genuine diurnal variation may be disrupted and natural
23 282 sleeping patterns altered (repeated measures are usually taken every 1-2 hours during the
24 283 night)²⁹.
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41 285 *Implications*

42 286 Variations of some CVD factors (in particular IL-6 and NT-ProBNP) over the course of the day
43 287 were observed, suggesting the role of time of the day as potential confounder during the
44 288 measurements. However, standardising these biological markers by time of day was not
45 289 particularly relevant for the cardiovascular risk prediction. Also, other sensitivity analyses
46 290 (stratified analysis and interaction tests) did not add relevant insights suggesting that time
47 291 of day variations may be not important for clinical risk stratification in general. Further
48 292 studies assessing both CVD risk factors levels and clinical outcomes (e.g. fatal or non-fatal
49 293 CVD events) during 24h are required to demonstrate whether a rapid increase of IL-6 over
50 294 the day may be relevant to the increased number of CVD events observed in early and late
51 295 morning³⁰, and whether the increased levels of NT-ProBNP over the day are related to the
52 296 afternoon peak in sudden death following heart failure³¹.
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5 298 **Conclusions**6 299 Variations in time of day were associated with variations of some, but not all, CVD risk
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8 300 factors measured in older adults. The contribution of time of the day to the markers' overall
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10 301 variation was small and unlikely to affect the CVD risk prediction or clinical risk stratification.
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14 303 **AUTHORS CONTRIBUTIONS**15 304 CS processed the data, performed statistical analyses, drafted and revised the manuscript,
16
17 305 and incorporated revisions of co-authors. PHW, SGW, BJ, and RM contributed to the design
18
19 306 of the study and revised the manuscript. LL enrolled participants, and collected data. PHW,
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21 307 RM and SGW raised grant funding. All authors gave an intellectual contribution to the
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23 308 manuscript and approved the final version.24 309 **Acknowledgements**25
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27
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29
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32 313 collection, management, analysis, and interpretation of the data; preparation, review, or
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34 314 approval of the manuscript; and the decision to submit the manuscript for publication. The
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36 315 views expressed in this publication are those of the author(s) and not necessarily those of
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38 316 the BHF.39 317 **Conflict of interest statement**

40 318 The authors report no relationships that could be construed as a conflict of interest

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42 319 **Data Sharing statement**43
44 320 The collection and management of data over the last 39 years of the BRHS has been made
45
46 321 possible through grant funding from UK government agencies and charities. We welcome
47
48 322 proposals for collaborative projects and data sharing. For general data sharing enquiries,
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50 323 please contact Lucy Lennon (l.lennon@ucl.ac.uk).
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324 **Table 1** –Individual characteristics and risk factors levels in the British Regional Heart Study
 325 (BRHS) men who attended the examinations in 1998-2000

<i>Demographic and background characteristics</i>	
Age (years), mean (Standard Deviation, SD)	68.7 (5.5)
Social class (manual)	
Manual, n (%)	2166 (51.1)
Non-Manual, n (%)	1966 (46.3)
Armed Forces, n (%)	112 (2.6)
<i>Physical health</i>	
Body Mass Index, mean (SD)	26.9 (3.7)
Prevalence of stroke/myocardial infarction, n (%)	153 (3.6)
Prevalence of heart failure, n (%)	390 (9.2)
Diabetes, n (%)	478 (11.2)
<i>Behavioural factors</i>	
Smoking	
Never, n (%)	1233 (29.1)
Ex-smokers, n (%)	2464 (58.0)
Smokers, n (%)	548 (12.9)
Alcohol consumption	
None, n (%)	431 (10.3)
Occasional/light, n (%) ¹	2949 (70.5)
Moderate/Heavy, n (%) ²	779 (18.6)
Physical activity level	
Inactive, n (%)	471 (11.5)
Occasionally, n (%)	957 (23.4)
Light, n (%)	767 (18.7)
Moderate, n (%)	591 (14.4)
Moderate vigorous, n (%)	690 (16.8)
Vigorous, n (%)	621 (15.1)
<i>CVD risk factor, geometric mean (SD) †</i>	
CRP, mg/L	1.74 (3.03)
IL-6, pg/mL	2.46 (1.94)
Fibrinogen, g/L	3.19 (1.25)
t-PA, ng/mL	10.23 (1.50)
vWF, IU/dL	132.41 (1.40)
D-dimer, ng/mL	84.32 (2.32)
NT-ProBNP, pg/mL	101.50 (3.32)
cTnT, pg/mL	12.07 (1.64)

326 ¹ >=1 and <=15 units per week (1 unit is approximately 1 drink, such as one glass of wine)

327 ² >=16 units per week (1 unit is approximately 1 drink, such as one glass of wine)

328 † Abbreviations: C-Reactive Protein (CRP), Interleukin-6 (IL-6), Fibrinogen, tissue plasminogen activator (t-PA) ,
 329 von Willebrand factor (vWF), fibrin D-dimer, N-terminal pro-brain natriuretic peptide (NT-ProBNP), and cardiac
 330 Troponin T (cTnT)

331 **Table 2** – Cross-sectional adjusted associations between time of day (fitted as continuous variable) and cardiovascular disease (CVD) risk
 332 factors measured in the British Regional Heart Study (BRHS) men (aged 60-79) attending the follow-up year 20 examination in 1998-2000.
 333 Associations are reported as percent difference in CVD risk factors levels per one hour of sampling over the examination day (08:00-19:00h).
 334 The statistically significant associations are marked in bold.

CVD risk factor [†]	Model 1: Age adjusted ¹		Model 2: Fully adjusted ²		Model 3: Fully adjusted ² excluding men with diabetes	
	Percent difference (95%CI) in the CVD risk factor levels per hour of sampling ³	p-value	Percent difference (95%CI) in the CVD risk factor levels per hour of sampling ³	p-value	Percent difference (95%CI) in the CVD risk factor levels per hour of sampling ⁴	p-value
NT-ProBNP	3.5 (2.0;5.0)	<0.001	3.3 (1.9; 4.8)	<0.001	3.5 (2.0; 5.0)	<0.001
IL-6	2.6 (1.7;3.4)	<0.001	2.6 (1.8; 3.4)	<0.001	2.4 (1.6; 3.3)	<0.001
t-PA	-3.3 (-3.8;-2.9)	<0.001	-3.3 (-3.7; -2.9)	<0.001	-3.2 (-3.6; -2.7)	<0.001
Fibrinogen	-0.2 (-0.5;0.0)	0.088	-0.2 (-0.5; 0.1)	0.104	-0.2 (-0.5; 0.1)	0.149
cTnT	-0.4 (-0.9;0.2)	0.194	-0.4 (-1.0; 0.2)	0.174	-0.4 (-1.0; 0.2)	0.165
CRP	-1.0 (-2.3;0.4)	0.151	-0.9 (-2.2; 0.4)	0.175	-0.9 (-2.2; 0.5)	0.191
vWF	-0.2 (-0.6;0.2)	0.374	-0.2 (-0.6; 0.2)	0.380	-0.1 (-0.5; 0.3)	0.703
D-Dimer	-0.0 (-1.0;1.0)	0.929	-0.1 (-1.0; 0.9)	0.890	-0.1 (-1.2; 0.9)	0.801

335 ¹ Model 1: Two level linear models (level 1 = person, level 2 = town of residence during the BRHS recruitment) adjusted for age. Model 1 used the same number of
 336 observations of Model 2 (complete case analysis).

337 ² Model 1 additionally adjusted for social class, Body Mass Index, smoking status, alcohol consumption, physical activity, use of statin, and season. Associations with IL-6, t-
 338 PA, Fibrinogen, CRP, vWF, and D-Dimer were additionally adjusted for prevalence of stroke/MI, while association of time of the day with NT-ProBNP and cTnT models were
 339 additionally adjusted for prevalence of heart failure.

340 ³ Model 1 and Model 2 used the same number of observations: 3580 for NT-ProBNP, 3832 for IL-6, 3863 for t-PA, 3861 for Fibrinogen, 3827 for cTnT, 3838 for CRP, 3863 for
 341 vWF, 3859 for D-Dimer

342 ⁴ Model 3 number of observations: 3176 for NT-ProBNP, 3398 for IL-6, 3429 for t-PA, 3427 for Fibrinogen, 3398 for cTnT, 3405 for CRP, 3429 for vWF, 3425 for D-Dimer

343 [†] Abbreviations: C-Reactive Protein (CRP), Interleukin-6 (IL-6), Fibrinogen, tissue plasminogen activator (t-PA), von Willebrand factor (vWF), fibrin D-dimer, N-terminal pro-
 344 brain natriuretic peptide (NT-ProBNP), and cardiac Troponin T (cTnT)

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3 433 **Figure 1** – Unadjusted geometric means (95% CI) by time of the day¹ for C-Reactive Protein (CRP), Interleukin-6
4 434 (IL-6), Fibrinogen, tissue plasminogen activator (t-PA), von Willebrand factor (vWF), fibrin D-dimer, N-terminal
5 435 pro-brain natriuretic peptide (NT-ProBNP), and cardiac Troponin T (cTnT) measured on one occasion in BRHS
6 436 men aged 60-79 during the years 1998-2000.
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10 439 ¹Total number of men examined per hour was 33(0.7%) at 08:00-08:59, 363(8.5%) at 8:00-9:59h, 699(16%) at
11 440 10:00-10:59h, 771(18%) at 11:00-11:59h, 591(14%) at 12:00-12:59h, 99(2%) at 13:00-13:59h, 306(7%) at
12 441 14:00-14:59), 560(13%) at 15:00-15:59h, 566(13%) at 16:00-16:59, 260(6%) at 17:00-17:59, and 3(<0.1%) at
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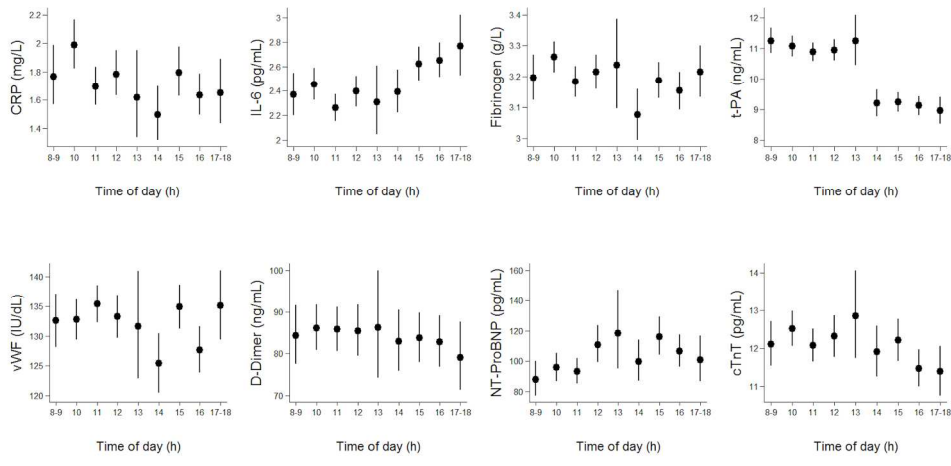


Figure 1 – Unadjusted geometric means (95% CI) by time of the day

186x99mm (300 x 300 DPI)

review only

STROBE Statement—Checklist of items that should be included in reports of *cross-sectional studies*

	Item No	Recommendation	
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	Abstract
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	Abstract
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Introduction, lines 54-62
Objectives	3	State specific objectives, including any prespecified hypotheses	Introduction, lines 54-56 and 60-62
Methods			
Study design	4	Present key elements of study design early in the paper	Methods, line 66-72
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Methods, lines 75-84
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants	Methods, lines 66-72 and 75-84
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	Methods, lines 97-123
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Methods, lines 75-123
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	Results, lines 75-78
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Methods, lines 91-114
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	Methods, lines 125-159
		(b) Describe any methods used to examine subgroups and interactions	Methods, lines 152 and 156-158
		(c) Explain how missing data were addressed	
		(d) If applicable, describe analytical methods taking account of sampling strategy	NA
		(e) Describe any sensitivity analyses	Methods, lines 151-159
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Methods, lines 66-68 and 91-95
		(b) Give reasons for non-participation at each stage	Methods, lines 66-76
		(c) Consider use of a flow diagram	Not necessary for this study, see Methods, lines 66-68 and 91-95

1			
2	Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
3			Table 1
4			
5			
6			(b) Indicate number of participants with missing data for each variable of interest
7			Table 1
8	Outcome data	15*	Report numbers of outcome events or summary measures
9			Table 1, Figure 1
10	Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
11			Table 2
12			
13			(b) Report category boundaries when continuous variables were categorized
14			NA
15			
16			(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
17			NA
18			
19	Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
20			Results, lines 187-195
21			
22	Discussion		
23	Key results	18	Summarise key results with reference to study objectives
24			Results, lines 170-181 Discussion, lines 224-235
25			
26			
27	Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
28			Discussion, lines 272-284
29			
30	Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
31			Discussion, lines 230-234 and 272 - 284
32			
33			
34	Generalisability	21	Discuss the generalisability (external validity) of the study results
35			Discussion, lines 287-289
36			
37	Other information		
38	Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based
39			Acknowledgements, lines 287-302
40			
41			
42			

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Associations of time of day with cardiovascular disease risk factors measured in older men: results from the British Regional Heart Study

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1 **Title:** Associations of time of day with cardiovascular disease risk factors measured in older
2 men: results from the British Regional Heart Study

3
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18

19 **Manuscript number of words:** 4847 words (all included: title page, abstract, manuscript, all
20 tables and references).

1
2
3 21 **Abstract (250 words, now 248)**
4

5 22 **Objective:** We estimated associations of time of day with cardiovascular disease (CVD) risk
6
7 23 factors measured in older men.

8
9 24 **Methods:** CVD risk factors (markers of inflammation and haemostasis, and cardiac markers)
10
11 25 were measured on one occasion between 08:00-19:00 hours in 4252 men aged 60-79 years
12
13 26 from the British Regional Heart Study. Linear models were used to estimate associations
14
15 27 between time of day and risk factors. When an association was found, we examined
16
17 28 whether the relationship between risk factors and cardiovascular mortality was affected by
18
19 29 the adjustment for time of day using survival analyses.

20 30 **Results:** N-terminal pro-brain natriuretic peptide (NT-ProBNP) levels increased by 3.3% per
21
22 31 hour [95% Confidence interval (CI) 1.9; 4.8], Interleukin-6 (IL-6) increased by 2.6% per hour
23
24 32 (95% CI 1.8; 3.4), while Tissue plasminogen activator (t-PA) decreased by 3.3% per hour
25
26 33 (95% CI 3.7; 2.9); these associations were unaffected by adjustment for possible
27
28 34 confounding factors. The percentages of variation in these risk factors attributable to time
29
30 35 of day were less than 2%. In survival analyses, the association of IL-6, NT-ProBNP, and t-PA
31
32 36 with cardiovascular mortality was not affected by the adjustment for time of day. C-Reactive
33
34 37 Protein, Fibrinogen, D-Dimer, von Willebrand Factor, and cardiac Troponin T showed no
35
36 38 associations with time of day.

37 39 **Conclusions:** In older men, markers of inflammation (IL-6), haemostasis (t-PA), and a cardiac
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39 40 marker (NT-ProBNP) varied by time of day. The contribution of time of day to variations in
40
41 41 these markers was small, and did not appear to be relevant for the CVD risk prediction.
42

43 **Background**

44 Previous studies have reported time of day variation in both established and emerging
45 cardiovascular disease (CVD) risk factors in middle aged adults, such as blood pressure,
46 lipids and some well-established inflammatory and haemostatic factors (e.g. white blood
47 cell, red blood cell, and platelets counts) ¹⁻³. However, the extent to which emerging CVD
48 risk factors such as Interleukin-6, a marker of inflammation causally associated with CHD in
49 a recent study ⁴, and N-terminal pro-brain natriuretic peptide, a marker of heart failure ⁵
50 vary by time of day have been less studied. Moreover, very little is known on time of day
51 variations in other emerging risk factors prospectively associated with CVD (e.g. t-PA, D-
52 Dimer, von Willebrand factor, and Cardiac Troponin T), although their causal association
53 with CVD remain debated or not yet tested.

54
55 We would expect that time of day variations in some emerging CVD risk factors measured in
56 older adults may occur, consistent with findings in younger populations ⁶. However, in older
57 adults the degree of difference attributable to time of day has not been yet estimated;
58 establishing its importance and its effects on prediction of CVD risk is important given the
59 potentially wider use of N-terminal pro-brain natriuretic peptide in risk stratification (as
60 shown in a recent major meta-analysis in the general population ⁷), and potential causal link
61 between IL-6 and cardiovascular disease ⁴. Therefore, the aim of this study was to
62 investigate how emerging CVD risk factors, including markers of inflammation, haemostasis
63 and myocardial function, varied by time of day in older British men.

65 **Methods**

66 *Participants*

67 The British Regional Heart Study (BRHS) is a prospective cohort study of cardiovascular
68 disease involving 7735 middle aged men (40-59 years) selected in 1978-80 from the age-sex
69 registers of one local primary care centre in 24 British towns ⁸. The 24 towns were selected
70 to represent the variation in cardiovascular disease across the UK ⁹. The National Research
71 Ethics Service (NRES) Committee for London provided ethical approval. Participants
72 provided informed written consent to the investigation, which was performed in accordance
73 with the Declaration of Helsinki ¹⁰.

74

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3 75 *Follow-up examination*

4 76 In 1998-2000, an average of 20 years after the initial recruitment, 4252 surviving
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6 77 participants (77% response rate) aged 60-79 years who were resident in the UK attended a
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8 78 physical examination during which nurses took a fasting blood sample on one occasion for
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10 79 each participant. The men were asked to fast for a minimum of 6 hours, during which they
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12 80 were instructed to drink only water, as previously reported ². The blood samples were
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14 81 collected between 08:00 h and 19:00 h and then assayed for a range of biochemical and
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16 82 haematological markers. Participants' appointment times were non-systematically
17
18 83 allocated. They were offered the opportunity to contact the BRHS team and change the time
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20 84 of examination, if unable to attend; a small proportion of participants did so.
21

22 85
23 86 The participants were also asked to complete a questionnaire which included questions on
24
25 87 other established CVD risk factors, such as age, social class, smoking habits, alcohol
26
27 88 consumption, and physical activity. Specifically, physical activity levels were self-reported ¹¹
28
29 89 and recently validated using accelerometers ¹². Incident CVD, including non-fatal stroke and
30
31 90 non-fatal MI were recorded: their definitions have been reported elsewhere ¹³. Men were
32
33 91 also asked whether a doctor had ever told them that they had heart failure ⁵. The number of
34
35 92 blood samples collected and included in the analyses differ according to the risk factor
36
37 93 measurements (the number of observations varied from 3580 for N-terminal Pro-Brain
38
39 94 Natriuretic Peptide to 3863 for von Willebrand Factor in complete case analyses including all
40
41 95 covariates of interest).
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43 96

44 97 *CVD Risk factors*

45 98 Circulating levels of markers of inflammation (C-reactive protein [CRP], Interleukin 6 [IL-6],
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47 99 Fibrinogen), cardiac markers (N-terminal pro-brain natriuretic peptide [NT-ProBNP], cardiac
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49 100 Troponin T [cTnT], and markers of haemostasis (tissue plasminogen activator [t-PA] antigen,
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51 101 fibrin D-dimer, von Willebrand factor [vWF]) were measured.

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53 103 D-dimer and t-PA levels were measured using an enzyme-linked immunosorbent assay
54
55 104 (ELISA; Biopool AB, Umeå, Sweden), as was VWF antigen (Dako, High Wycombe, UK). C-
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57 105 reactive protein was assayed using ultrasensitive nephelometry (Dade Behring, Milton
58
59 106 Keynes, UK). IL-6 was assayed using a high-sensitivity ELISA (R & D Systems, Oxford, UK).
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3 107 Fibrinogen was assayed using an auto-mated Clauss assay in a coagulometer (MDA-180,
4 108 Organon Teknika, Cambridge, UK). NT-proBNP and hsTnT were measured in plasma samples
5 109 on an automated clinically validated immunoassay analyzer (e411, Roche Diagnostics,
6 110 Burgess Hill, United Kingdom) using the manufacturers' calibrators and quality control
7
8 111 reagents. Intra- and inter-assay Coefficient of Variations (CVs) were, respectively: 4.1% and
9 112 6.6% for t-PA; 3.2% and 4.2% for vWF; 4.7% and 5.2% for D-dimer; 4.7% and 8.3% for CRP;
10 113 7.5% and 8.9% for IL-6; 2.6% and 3.7% for Fibrinogen, and 4.4% and 7.7% for NT-ProBNP and
11 114 cTnT.

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116 The samples were centrifuged and separated on the morning or afternoon of collection and
117 stored on site at -20°C until they were transferred to a central freezer storage location at -
118 70°C within 2 weeks of sample collection. Samples were then transferred on dry ice to a
119 single central laboratory and were thawed immediately before analysis. Plasma samples
120 were used for all the analyses reported here. The original sample collection took place
121 between January 1998 and March 2000. Most of the analyses described here were carried
122 out during 2000, after a maximum of 3 years storage; NT-ProBNP and cTnT were analysed in
123 2009.

124

125 **Statistical methods**

126 Firstly, the distributions of the outcomes were examined; the outcomes were log-
127 transformed as the distributions were positively skewed. Therefore, analysis was carried out
128 on their log-transformed values throughout. Unadjusted geometric means and 95%
129 Confidence Intervals [CI] of the outcomes were plotted against hour of the day.

130

131 *Adjusted associations between time of day and the outcomes*

132 Associations between time of day (fitted as a continuous variable, range 8-18) and the
133 outcomes were examined using linear multilevel random intercept models (level 1 =
134 individual, level 2 = town of residence). The results can be interpreted as between-person
135 variations over the course of the examination day; the estimates from the linear model
136 were reported as the difference in the outcome levels per hour of sampling over the
137 examination day. As the outcomes were log-transformed, the results were reported as
138 percent difference in the outcome geometric mean per hour of sampling. All models were

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3 139 initially adjusted for age only. Next, the models were adjusted for age and other possible
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5 140 confounding factors: social class, BMI, previous stroke or myocardial infarction (MI), physical
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7 141 activity, smoking status, alcohol consumption, use of statin, and a seasonal term (fitted
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9 142 using a cosinor function, as in previous studies)¹⁴. As NT-ProBNP and cTnT are principally
10
11 143 markers of heart failure, the association with time of day was adjusted for previous heart
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13 144 failure.
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16 146 When the association of time of the day with the outcomes was found to be statistically
17
18 147 significant, the proportion of variance associated with time of the day was estimated using
19
20 148 partial R-squared.
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22 149

23 150 *Sensitivity analyses*

24 151 Six sensitivity analyses were performed: (i) all models were additionally adjusted for fasting
25
26 152 time and diabetes; (ii) all models were carried out excluding men with diabetes, (iii)
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28 153 interactions were fitted to test whether the time of day associations were modified by age
29
30 154 (fitted as continuous variable); (iv) as NT-ProBNP and cTnT were acknowledged as specific
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32 155 cardiac markers⁵, interactions were fitted to test whether the time of day associations were
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34 156 modified by previous heart failure (yes/no); (v) to explore the potential of undiagnosed
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36 157 heart failure or cardiac damage influencing findings for NT-ProBNP and cTnT, we repeated
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38 158 regression models after excluding men with NTproBNP > 400 pg/ml; (vi) a quadratic term for
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40 159 time of day was added to the models in order to check for non-linearity
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43 161 As IL-6 has been causally associated with cardiovascular risk⁴, and prospectively associated
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45 162 with CVD mortality in the BRHS sample used here¹⁵, we investigated the relevance of time
46
47 163 of day to the cardiovascular risk prediction by performing two survival analyses: in the first
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49 164 analysis we used Cox models where unadjusted log IL-6 was used as the predictor and CVD
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51 165 mortality as the clinical outcome; then, we repeated the same analysis using log IL-6
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53 166 standardised by the time of day rather than unadjusted log IL-6. For completeness of
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55 167 information, we repeated this sensitivity analysis for NT-ProBNP and t-PA.
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57 168

58 169 **Results**

59 170 The characteristics of the study participants (mean age 68.7 years, standard deviation (SD) =
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3 171 5.5) are reported in Table 1. The associations between time of day (by hour) and risk factors
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5 172 are shown in Figure 1. Evidence of an increase over the course of the day was particularly
6
7 173 noticeable for IL-6, and for NT-ProBNP (Figure 1). Also, levels of t-PA were lower in the
8
9 174 afternoon in comparison with morning, while variations by time of day for other risk factors
10
11 175 were not clearly observable from the plots (Figure 1). The results of corresponding linear
12
13 176 regression analyses are shown in Table 2: statistically significant associations between time
14
15 177 of the day and some outcomes were found (Table 2): over the course of the examination
16
17 178 day NT-ProBNP levels increased by 3.3% per hour (95% CI 1.9; 4.8%), IL-6 increased by 2.6%
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19 179 per hour (95% CI 1.8; 3.4%). Conversely, t-PA decreased by 3.3% per hour (95% CI 3.7; 2.9%).
20
21 180 The proportion of variance associated with time of the day from the fully adjusted models
22
23 181 was 0.5%, 1%, and 2% for NT-ProBNP, IL-6, and t-PA respectively.

24
25 183 C-Reactive Protein, Fibrinogen, D-Dimer, von Willebrand Factor, and cardiac Troponin T
26
27 184 showed no consistent associations with time of day (Table 2).

28 29 30 186 *Sensitivity analyses*

31
32 187 Overall, we found that fasting time did not alter the magnitude of associations between
33
34 188 time of the day and the outcomes reported in Table 2. Only the association between time of
35
36 189 the day and t-PA was strongly attenuated after accounting for fasting time (fitted as
37
38 190 continuous variable): the decrease in t-PA levels was -3.3% (95%CI -3.7; -2.9) per hour
39
40 191 before the adjustment (Table 2) and -1.4% (95%CI -2.2; -0.1) after the adjustment for
41
42 192 fasting. An additional adjustment for diabetes status did not alter the magnitude of the
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44 193 association between hour of the day and the outcomes. We also performed the analysis
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46 194 excluding men with diabetes completely (Table 2 – Model 3), but the association between
47
48 195 time of day and the outcomes did not substantially change.

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50 197 For all outcomes, we also did not find evidence for an interaction between of time of day
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52 198 with age (results not shown).

53
54
55 200 In stratified analysis, NT-ProBNP levels increased by 3.4% (95% CI 1.9; 4.8%, $p < 0.001$) per
56
57 201 hour in older men without heart failure. Although men who previously had heart failure had
58
59 202 increased NT-ProBNP levels, there was no evidence for an interaction between previous
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3 203 heart failure with time of the day ($p=0.954$). After excluding 466 men with NTproBNP levels
4 204 $>400\text{pg/ml}$ (12% of the sample), associations between time of the day measures and
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6 205 NTproBNP remained statistically significant and slightly increased in magnitude (3.9% [95%
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8 206 CI 2.7; 5.1%], $p<0.001$). As reported in the main analysis, no significant associations were
9
10 207 found between time of the day and cTnT in stratified analysis.
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12 208
13 209 When adding a quadratic term to the model, we found a significant improvement in model
14 210 fit for IL-6 only ($p=0.030$ for the time of day squared term). The association of time of day
15 211 with IL-6 appeared to be slightly J-shaped, with a linear increase starting from 11:00 until
16 212 19:00 hours.
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21 213
22 214 We examined whether adjustment for hour of day affected the associations between risk
23 215 factors and CVD mortality. In survival analysis, higher levels of log IL-6 were associated with
24 216 increased CVD mortality (HR=1.70, 95%CI 1.54; 1.87). Standardising IL-6 by time of the day
25 217 did not change the relationship (HR=1.71, 95%CI 1.55; 1.88). Also, standardising NT-ProBNP
26 218 levels by time of the day did not alter the magnitude of the effect on CVD mortality
27 219 (HR=1.92, 95%CI 1.81; 2.04). Finally, associations of t-PA levels with increased CVD mortality
28 220 did not change substantially before (HR=1.74, 95%CI 1.45; 2.09) and after standardising
29 221 (HR=1.77, 95%CI 1.47; 2.14) by time of the day.
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38 222

39 223 **Discussion**

40 224 To our knowledge, this is the largest investigation of relationships between time of day and
41 225 CVD risk factors in older men. After adjusting our analysis for potential confounding factors
42 226 we demonstrated that some, but not all, CVD risk factors levels varied by time of day. In
43 227 particular, NT-ProBNP and IL-6 increased linearly over the course of the day. Conversely, a
44 228 decrease in t-PA was also observed; however, after accounting for fasting time the
45 229 relationship with time of the day was strongly attenuated (therefore fasting time could
46 230 partially explain the drop in t-PA levels observed in the afternoon vs morning). Our analyses
47 231 showed that the contribution of time of the day to the overall variation of NT-ProBNP, IL-6,
48 232 and t-PA was small and without clinical importance; we observed that time of day did not
49 233 have a sufficiently strong effect to be taken into account when assessing the impact of IL-6,
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3 234 NT-ProBNP, and t-PA on CVD mortality. Lastly, an association of time of day with other risk
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5 235 factors was not observed.

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8 237 Literature on time of day variation in emerging CVD markers of inflammation and
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10 238 haemostasis in older adults is limited; to our knowledge this is the first time these findings
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12 239 have been reported in older adults. Findings from earlier studies of younger adults were
13
14 240 fairly consistent with ours. For example a recent meta-analysis of several small studies
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16 241 which analysed IL-6 proposed a diurnal pattern, with overall IL-6 levels increased between
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18 242 08:00 and 19:00 hours as in our study ¹⁶. However, in two previous very small studies of
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20 243 twelve ¹⁷ and five ¹⁸ participants, IL-6 peaked in the night-time. It is possible that peaks in IL-
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22 244 6 levels may be associated with cognitive symptoms of depression ¹⁹ and daily activities,
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24 245 although in the BRHS population this has not yet been investigated. One previous study
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26 246 found that BRHS men were more active in the morning and in early afternoon ²⁰ when the
27
28 247 main activities were usually gardening, house works, shopping, or leisure walking. Whether
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30 248 IL-6 was implicated in this daily pattern remains uncertain and can potentially be explored in
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32 249 future studies.

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34 251 Moreover, one previous study reported increased levels of NT-ProBNP over the course of
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36 252 day ²¹ as we observed in our study. A decrease in t-PA over the examination day was also
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38 253 reported in younger subjects (a 45-year-old UK population of 9377 men and women) ⁶;
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40 254 however, t-PA did not vary by time of the day in a previous study large study of 1288
41
42 255 healthy 25 to 64-year-old men and women ²².

43 256

44 257 In comparison to our study, findings regarding CRP, Fibrinogen, D-dimer, vWF and cTnT
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46 258 reported in earlier studies of younger adults were similar: a few previous studies reported
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48 259 that they did not find an association of time of day with CRP ²³, D-dimer ²⁴, and vWF ²⁵. In
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50 260 one study, the variation in CRP, Fibrinogen, D-dimer, and vWF attributable to time of day
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52 261 was minimal ⁶. Literature on cTnT is scarce: one small previous study of repeated measures
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54 262 in 7 participants with type 2 diabetes reported a decrease in cTnT between 8am and 8pm ²⁶.

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56 264 Although one previous study suggested that diurnal variations in CVD risk factors could be
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58 265 relevant for cardiovascular risk prediction ⁶, a prediction model like the one described in our
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3 266 survival analysis was not performed. Our findings suggested the effect of time of the day
4 267 (from 08:00 h to 19:00 h) is not relevant for the CVD risk assessment. With this sensitivity
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6 268 analysis we wanted to investigate time of day variations beyond simple descriptive diurnal
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8 269 patterns; to our knowledge this is the first time this finding has been reported.
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10 270

11 271 *Strengths and limitations*

12 272 The BRHS cohort benefits from using a large scale population-based sample of free-living
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14 273 older men and this increases statistical power and precision of estimates. However, the
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16 274 BRHS comprises male participants, predominantly of white European ethnic origin, so
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18 275 findings may not be generalisable to women and non-white ethnic groups. The CVD risk
19
20 276 factors measurements were carried out on one occasion over an extended period of the day
21
22 277 (between 08:00 and 19:00 hours), offering only a partial understanding of the variations
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24 278 over the 24 hours^{27 28}. Therefore, in this study the relationship of the CVD risk factors to
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26 279 time of day was explored using between-participant variation only. In future studies it may
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28 280 be advantageous to carry out repeated measurement of the risk factors over the 24 hours in
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30 281 order to investigate within-person circadian variations. However, with repeated
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32 282 measurements a possible and genuine diurnal variation may be disrupted and natural
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34 283 sleeping patterns altered (repeated measures are usually taken every 1-2 hours during the
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36 284 night)²⁹.

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38 286 *Implications*

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40 287 Variations of some CVD factors (in particular IL-6 and NT-ProBNP) over the course of the day
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42 288 were observed, suggesting the role of time of the day as potential confounder during the
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44 289 measurements. However, standardising these biological markers by time of day was not
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46 290 particularly relevant for the cardiovascular risk prediction. Also, other sensitivity analyses
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48 291 (stratified analysis and interaction tests) did not add relevant insights suggesting that time
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50 292 of day variations may be not important for clinical risk stratification in general. Further
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52 293 studies assessing both CVD risk factors levels and clinical outcomes (e.g. fatal or non-fatal
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54 294 CVD events) during 24h are required to demonstrate whether a rapid increase of IL-6 over
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56 295 the day may be relevant to the increased number of CVD events observed in early and late
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58 296 morning³⁰, and whether the increased levels of NT-ProBNP over the day are related to the
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60 297 afternoon peak in sudden death following heart failure³¹.

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299 Conclusions

300 Variations in time of day were associated with variations of some, but not all, CVD risk
301 factors measured in older adults. The contribution of time of the day to the markers' overall
302 variation was small and unlikely to affect the CVD risk prediction or clinical risk stratification.

303

304 AUTHORS CONTRIBUTIONS

305 CS processed the data, performed statistical analyses, drafted and revised the manuscript,
306 and incorporated revisions of co-authors. PHW, SGW, BJ, and RM contributed to the design
307 of the study and revised the manuscript. LL enrolled participants, and collected data. PHW,
308 RM and SGW raised grant funding. All authors gave an intellectual contribution to the
309 manuscript and approved the final version.

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314 collection, management, analysis, and interpretation of the data; preparation, review, or
315 approval of the manuscript; and the decision to submit the manuscript for publication. The
316 views expressed in this publication are those of the author(s) and not necessarily those of
317 the BHF.

318 Conflict of interest statement

319 The authors report no relationships that could be construed as a conflict of interest

320 Data Sharing statement

321 The collection and management of data over the last 39 years of the BRHS has been made
322 possible through grant funding from UK government agencies and charities. We welcome
323 proposals for collaborative projects and data sharing. For general data sharing enquiries,
324 please contact Lucy Lennon (l.lennon@ucl.ac.uk).

325 **Table 1** –Individual characteristics and risk factors levels in the British Regional Heart Study
 326 (BRHS) men who attended the examinations in 1998-2000

<i>Demographic and background characteristics</i>	
Age (years), mean (Standard Deviation, SD)	68.7 (5.5)
Social class (manual)	
Manual, n (%)	2166 (51.1)
Non-Manual, n (%)	1966 (46.3)
Armed Forces, n (%)	112 (2.6)
<i>Physical health</i>	
Body Mass Index, mean (SD)	26.9 (3.7)
Prevalence of stroke/myocardial infarction, n (%)	153 (3.6)
Prevalence of heart failure, n (%)	390 (9.2)
Diabetes, n (%)	478 (11.2)
<i>Behavioural factors</i>	
Smoking	
Never, n (%)	1233 (29.1)
Ex-smokers, n (%)	2464 (58.0)
Smokers, n (%)	548 (12.9)
Alcohol consumption	
None, n (%)	431 (10.3)
Occasional/light, n (%) ¹	2949 (70.5)
Moderate/Heavy, n (%) ²	779 (18.6)
Physical activity level	
Inactive, n (%)	471 (11.5)
Occasionally, n (%)	957 (23.4)
Light, n (%)	767 (18.7)
Moderate, n (%)	591 (14.4)
Moderate vigorous, n (%)	690 (16.8)
Vigorous, n (%)	621 (15.1)
<i>CVD risk factor, geometric mean (SD) †</i>	
CRP, mg/L	1.74 (3.03)
IL-6, pg/mL	2.46 (1.94)
Fibrinogen, g/L	3.19 (1.25)
t-PA, ng/mL	10.23 (1.50)
vWF, IU/dL	132.41 (1.40)
D-dimer, ng/mL	84.32 (2.32)
NT-ProBNP, pg/mL	101.50 (3.32)
cTnT, pg/mL	12.07 (1.64)

¹ >=1 and <=15 units per week (1 unit is approximately 1 drink, such as one glass of wine)

² >=16 units per week (1 unit is approximately 1 drink, such as one glass of wine)

[†] Abbreviations: C-Reactive Protein (CRP), Interleukin-6 (IL-6), Fibrinogen, tissue plasminogen activator (t-PA) , von Willebrand factor (vWF), fibrin D-dimer, N-terminal pro-brain natriuretic peptide (NT-ProBNP), and cardiac Troponin T (cTnT)

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332 **Table 2** – Cross-sectional adjusted associations between time of day (fitted as continuous variable) and cardiovascular disease (CVD) risk
333 factors measured in the British Regional Heart Study (BRHS) men (aged 60-79) attending the follow-up year 20 examination in 1998-2000.
334 Associations are reported as percent difference in CVD risk factors levels per one hour of sampling over the examination day (08:00-19:00h).
335 The statistically significant associations are marked in bold.

CVD risk factor †	Model 1: Age adjusted ¹		Model 2: Fully adjusted ²		Model 3: Fully adjusted ² excluding men with diabetes	
	Percent difference (95%CI) in the CVD risk factor levels per hour of sampling ³	p-value	Percent difference (95%CI) in the CVD risk factor levels per hour of sampling ³	p-value	Percent difference (95%CI) in the CVD risk factor levels per hour of sampling ⁴	p-value
NT-ProBNP	3.5 (2.0;5.0)	<0.001	3.3 (1.9; 4.8)	<0.001	3.5 (2.0; 5.0)	<0.001
IL-6	2.6 (1.7;3.4)	<0.001	2.6 (1.8; 3.4)	<0.001	2.4 (1.6; 3.3)	<0.001
t-PA	-3.3 (-3.8;-2.9)	<0.001	-3.3 (-3.7; -2.9)	<0.001	-3.2 (-3.6; -2.7)	<0.001
Fibrinogen	-0.2 (-0.5;0.0)	0.088	-0.2 (-0.5; 0.1)	0.104	-0.2 (-0.5; 0.1)	0.149
cTnT	-0.4 (-0.9;0.2)	0.194	-0.4 (-1.0; 0.2)	0.174	-0.4 (-1.0; 0.2)	0.165
CRP	-1.0 (-2.3;0.4)	0.151	-0.9 (-2.2; 0.4)	0.175	-0.9 (-2.2; 0.5)	0.191
vWF	-0.2 (-0.6;0.2)	0.374	-0.2 (-0.6; 0.2)	0.380	-0.1 (-0.5; 0.3)	0.703
D-Dimer	-0.0 (-1.0;1.0)	0.929	-0.1 (-1.0; 0.9)	0.890	-0.1 (-1.2; 0.9)	0.801

¹ Model 1: Two level linear models (level 1 = person, level 2 = town of residence during the BRHS recruitment) adjusted for age. Model 1 used the same number of observations of Model 2 (complete case analysis).

² Model 1 additionally adjusted for social class, Body Mass Index, smoking status, alcohol consumption, physical activity, use of statin, and season. Associations with IL-6, t-PA, Fibrinogen, CRP, vWF, and D-Dimer were additionally adjusted for prevalence of stroke/MI, while association of time of the day with NT-ProBNP and cTnT models were additionally adjusted for prevalence of heart failure.

³ Model 1 and Model 2 used the same number of observations: 3580 for NT-ProBNP, 3832 for IL-6, 3863 for t-PA, 3861 for Fibrinogen, 3827 for cTnT, 3838 for CRP, 3863 for vWF, 3859 for D-Dimer

⁴ Model 3 number of observations: 3176 for NT-ProBNP, 3398 for IL-6, 3429 for t-PA, 3427 for Fibrinogen, 3398 for cTnT, 3405 for CRP, 3429 for vWF, 3425 for D-Dimer

† Abbreviations: C-Reactive Protein (CRP), Interleukin-6 (IL-6), Fibrinogen, tissue plasminogen activator (t-PA) , von Willebrand factor (vWF), fibrin D-dimer, N-terminal pro-brain natriuretic peptide (NT-ProBNP), and cardiac Troponin T (cTnT)

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3 434 **Figure 1** – Unadjusted geometric means (95% CI) by time of the day¹ for C-Reactive Protein (CRP), Interleukin-6
4 435 (IL-6), Fibrinogen, tissue plasminogen activator (t-PA), von Willebrand factor (vWF), fibrin D-dimer, N-terminal
5 436 pro-brain natriuretic peptide (NT-ProBNP), and cardiac Troponin T (cTnT) measured on one occasion in BRHS
6 437 men aged 60-79 during the years 1998-2000.
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10 440 ¹Total number of men examined per hour was 33(0.7%) at 08:00-08:59, 363(8.5%) at 8:00-9:59h, 699(16%) at
11 441 10:00-10:59h, 771(18%) at 11:00-11:59h, 591(14%) at 12:00-12:59h, 99(2%) at 13:00-13:59h, 306(7%) at
12 442 14:00-14:59), 560(13%) at 15:00-15:59h, 566(13%) at 16:00-16:59, 260(6%) at 17:00-17:59, and 3(<0.1%) at
13 443 18:00-18:59
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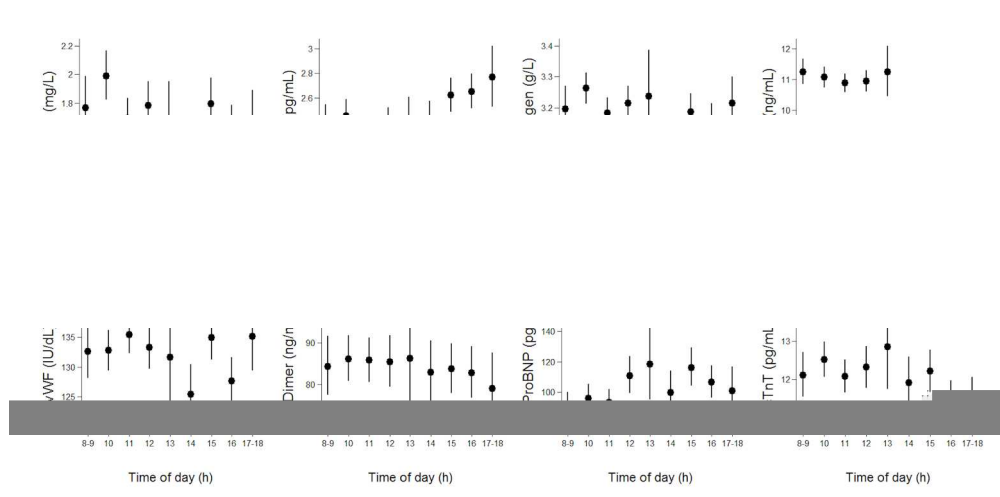


Figure 1 – Unadjusted geometric means (95% CI) by time of the day

186x99mm (300 x 300 DPI)

review only

STROBE Statement—Checklist of items that should be included in reports of *cross-sectional studies*

	Item No	Recommendation	
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	Abstract
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	Abstract
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Introduction, lines 54-62
Objectives	3	State specific objectives, including any prespecified hypotheses	Introduction, lines 54-56 and 60-62
Methods			
Study design	4	Present key elements of study design early in the paper	Methods, line 66-72
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Methods, lines 75-84
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants	Methods, lines 66-72 and 75-84
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	Methods, lines 97-123
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Methods, lines 75-123
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	Results, lines 75-78
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Methods, lines 91-114
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	Methods, lines 125-159
		(b) Describe any methods used to examine subgroups and interactions	Methods, lines 152 and 156-158
		(c) Explain how missing data were addressed	
		(d) If applicable, describe analytical methods taking account of sampling strategy	NA
		(e) Describe any sensitivity analyses	Methods, lines 151-159
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Methods, lines 66-68 and 91-95
		(b) Give reasons for non-participation at each stage	Methods, lines 66-76
		(c) Consider use of a flow diagram	Not necessary for this study, see Methods, lines 66-68 and 91-95

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2	Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
3			Table 1
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6			(b) Indicate number of participants with missing data for each variable of interest
7			Table 1
8	Outcome data	15*	Report numbers of outcome events or summary measures
9			Table 1, Figure 1
10	Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
11			Table 2
12			
13			(b) Report category boundaries when continuous variables were categorized
14			NA
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16			(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
17			NA
18			
19	Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
20			Results, lines 187-195
21			
22	Discussion		
23	Key results	18	Summarise key results with reference to study objectives
24			Results, lines 170-181 Discussion, lines 224-235
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27	Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
28			Discussion, lines 272-284
29			
30	Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
31			Discussion, lines 230-234 and 272 - 284
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34	Generalisability	21	Discuss the generalisability (external validity) of the study results
35			Discussion, lines 287-289
36			
37	Other information		
38	Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based
39			Acknowledgements, lines 287-302
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*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.