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Biomarkers and allostatic load as predictors of mortality: Lolland-Falster Health Study

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Nykøbing Falster Hospital,

6 September 2021

University of Copenhagen, Denmark

Biomarkers and allostatic load as predictors of mortality: Lolland-Falster Health Study

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Abstract

Objectives: The purposes of the present study were to determine the association between 1) 10 individual biomarkers and all-cause mortality; and between 2) allostatic load, across three physiological systems (cardiovascular, inflammatory, metabolic), and all-cause mortality. **Design:** Prospective cohort study. **Setting:** We used data from the Lolland-Falster Health Study undertaken in Denmark in 2016-2020 and used data on systolic (SBP) and diastolic blood pressure (DBP), pulse rate, waist-hip ratio (WHR), and levels of low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), triglycerides, glycated haemoglobin (HbA1c), c-reactive protein (CRP), and serum albumin. All biomarkers were divided into quartiles with high-risk values defined as those in the highest (pulse rate, WHR, triglycerides, HbA1c, CRP) or lowest (HDL-c, albumin) quartile, or a combination hereof (LDL-c, SBP, DBP). The ten biomarkers were combined into a summary measure of allostatic load (AL) index. Participants were followed up for death for an average of 2.6 years. **Participants:** We examined a total of 13,725 individuals aged 18+ years. **Primary outcome measure:** Cox proportional hazard regression (HR) analysis were performed to examine the association between AL index and mortality in men and women. **Results:** All-cause mortality increased with increasing AL index. With low AL index as reference, the HR was 1.76 (95% confidence interval (CI) 0.90-3.44) for mid AL, and HR 2.94 (95% CI: 1.53-5.66) for high AL. **Conclusions:** Elevated physiological burden measured by mid and high AL index was associated with a steeper increase of mortality than individual biomarkers.

Abstract word count: 242

Keywords: Biomarker, Allostatic Load, Blood, Mortality, population-based, LOFUS.

Manuscript word count: 3445

Article Summary

Strengths and limitations

- Analysis based on a large population-based health study.
- Complete follow-up for death via linkage with Danish Civil Registration System.
- Biomarkers from only one point in time.
- No biomarker from neuroendocrine system available.

Introduction

Biological markers (biomarkers) were originally defined as “cellular, biochemical or molecular alterations that are measurable in biological media such as cells, human tissues or fluids” [1]. Later the definition was extended to include “indicators of normal biological processes, pathogenic processes and pharmacological responses to therapeutic interventions” [2]. In clinical settings, measurement of biomarkers in blood samples is used to detect and diagnose medical conditions. Biomarkers as independent predictors of all-cause mortality are therefore of considerable clinical and research interest [3]; dyslipidaemia including high levels of triglycerides and low-density lipoprotein cholesterol (LDL-c), and low levels of high-density lipoprotein cholesterol (HDL-c), have been reported to be independent risk factors for all-cause mortality [4-6]. Lower levels of albumin [7] and higher levels of C-reactive protein (CRP), [8] and haemoglobin A1c (HbA1c) [9] have likewise been linked to mortality. Also, there is some evidence that the relationship between some of these biomarkers and all-cause mortality varies across sex and age-groups [10,11].

The concept of allostatic load (AL) refers to the “wear and tear” of the body resulting from repeated stimulation of stress responses via the hypothalamic–pituitary–adrenal axis and the sympathetic-adrenal-medullary system [12]. As a latent variable, AL cannot be directly measured but it can be estimated using an AL index, which is composite of biomarkers from multiple organ systems integrated into a single score. The most widely used construct of AL was developed by Seeman et al. in 1997 and includes 10 biomarkers monitoring various physiological systems [13]. It has been suggested that in the calculation of AL, the threshold of risk for each biomarker should be obtained by the quartiles or quintiles of the values of the biomarker [14]. AL has been reported to be a better predictor

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4 of mortality than individual biomarkers, however, there are still gaps in the understanding
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6 of the associations [15-16].
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10 This study provides data from the Lolland-Falster Health Study (LOFUS) [17],
11 a population-based survey undertaken in 2016-2020 in Lolland-Falster, a rural-provincial
12 region in Denmark with a life expectancy much below the national average [18], and with
13 health problems reported more frequently than in the rest of the country [19]. Using the
14 LOFUS data, the purposes of the present study were 1) to determine the association
15 between 10 individual biomarkers and all-cause mortality; and 2) to examine the
16 association between AL, across three physiological systems (cardiovascular, inflammatory,
17 metabolic system), and all-cause mortality.
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28 **Methods**

29 Study population

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31 We undertook a prospective cohort study of participants from LOFUS; a household-based
32 population study with data collected between February 2016 and February 2020. Persons
33 aged 18 years and above were randomly sampled from the Danish Civil Registration
34 System and invited to participate together with the rest of their households. A detailed
35 description of the study protocol [17] and information on the socio-economic determinants
36 of participation [20] have been published previously. Persons below 18 years, and
37 pregnant women were excluded from the present study.
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50 Self-reported data

51 From questionnaires, we used data on smoking (never, former, current), and presence of
52 chronic conditions (cardiovascular disease, diabetes, cancer) at the time of participation in
53 LOFUS.
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Biomarkers

Non-fasting blood samples were collected in vacutainer blood collection tubes (Becton, Dickinson and Company; Franklin Lakes, NJ, USA) and kept at room temperature until same day analysis at the Department of Clinical Biochemistry at Nykøbing Falster Hospital, accredited by the standard ISO 15189. We used data on HDL-c, LDL-c, triglycerides, albumin, CRP, and HbA1c. LDL was calculated by using Friedewald formula [21] when the plasma triglyceride concentration was below 4.5 mmol/L. Systolic and diastolic blood pressure were based on three consecutive digital measurements on the upper left arm (apparatus type Welch Allyn Connex pro BPO 3400). The mean values of the second and third measurement were used in this study (only one measurement was used if the other was missing). Waist-hip ratio (WHR) was calculated by waist-circumference divided by hip-circumference. Body mass index (BMI) was defined as weight in kilograms divided by height in meters squared (kg/m²).

In the calculation of AL, biomarkers are most often dichotomized into low and high values based on either a percentile or a predetermined cut-off value [14]. However, before doing so, we mapped for each biomarker the association between level of the marker and all-cause mortality, see method below. For most biomarkers the association was monotonic, see Supplementary Figure 1. These biomarkers were then dichotomized according to the sex- and age-specific quartiles, with high-risk values defined as those in the highest quartile of the sex- and age-specific distribution, except for HDL-c, and albumin, where the lowest quartile was the high-risk value. For LDL, SBP and DBP the associations were U-shaped, and the high-risk values for these biomarkers were therefore defined as including both the lower and the upper quartiles, see Supplementary Table 1.

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4 For all biomarkers, the highest and lowest quartile of risk scores were either lower or
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6 similar to clinical cut-points [22-26].
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9 BMI was divided into underweight (BMI less than 18.5) normal (BMI 18.5–
10 24.9), overweight (BMI 25.0–29.9), or obese (BMI 30.0 or greater); reported diseases into
11 either present or not; and smoking status into never, former, or current.
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16 Allostatic load scores

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19 The AL scores were computed using biomarkers from: the cardiovascular system (systolic
20 blood pressure (SBP), diastolic blood pressure (DBP), and pulse rate (PR)); the metabolic
21 system (LDL-c, triglycerides, HDL-c, WHR, HbA1c); and the inflammatory system (CRP,
22 serum albumin).
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29 Each system-specific AL score was then defined as the number of biomarkers
30 with a high-risk value, hence as an integer value between 0 and 3 for the cardiovascular
31 system (CVS), 0 and 5 for the metabolic system (MS), and 0 and 2 for inflammatory system
32 (IS). Each of these integer values were divided into low [CVS: 0; MS: 0; IS: 0], mid (CVS: 1;
33 MS: 1; IS: 1), and high [CVS: 2-3; MS: 2-5; IS: 2]. The AL index was defined as the sum of
34 all scores and divided into low [AL:0-1], mid [AL: 2-3], and high [AL: 4-10]. Note that, all
35 biomarkers were given equal weight in accordance with previous studies [14,16].
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45 All-cause mortality

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48 LOFUS participants were followed up for death with data obtained from the Danish Civil
49 Registration System on 26 February 2021.
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52 Data management and statistical analyses

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4 Observations with missing values in any of the variables were excluded from the analyses
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6 (1989 out of 15714, i.e. 12.6%, see Supplementary Table 2). Values below the lower limit of
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8 detection were replaced with random numbers sampled with replacement from the set $\{k$
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10 $\times 10^{-n}$, $k = 1, \dots, L\}$, where n is the variable-specific number of decimals reported in the
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12 data and $L \times 10^{-n}$ the limit of detection, see Supplementary Table 3.
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16 Participants were followed up from date of participation in the LOFUS study
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18 until date of death or end of follow-up on 26 February 2021, whichever came first. In order
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20 to define the biomarkers' high-risk values, we first studied the association between levels
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22 of each individual biomarker and mortality, allowing for possible nonlinear relations. This
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24 analysis was carried out via Cox proportional-hazard models with biomarker levels as
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26 continuous covariates, modelled with natural cubic splines with 2 degrees of freedom
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28 (except for LDL, where 3 degrees of freedom were used), and further adjusting for sex and
29
30 age. By graphical inspection, a U-shaped association was found for LDL, SBP and DBP (see
31
32 Supplementary Figure 1). Therefore, for these biomarkers the sex and age-specific (i.e.
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34 below or above age 60) lower and upper quartiles were defined as high-risk, while only one
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36 quartile for the others (upper or lower, in accordance with the existing literature); see
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38 Supplementary Table 1.
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44 Associations between all-cause mortality and dichotomized biomarkers levels
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46 (low/high risk), system-specific AL scores, and total AL index, were modelled with Cox
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48 proportional-hazard models. Here, we present two models: Model 1, where HRs are
49
50 adjusted for sex and age; Model 2, where results are further adjusted for BMI, prevalent
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52 diseases, and smoking status. HRs for the individual biomarkers (Table 2) and for system-
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54 specific AL scores (Table 3) are mutually adjusted. Proportional hazards assumptions in
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4 the above models have been tested using Schoenfeld residuals. Numbers below 5 are not
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6 reported.
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9 Data management, statistical analyses and plots were done in R ver. 4.0.3 [27], with
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11 packages splines [27], survival [28] and tidyverse [29].
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14 **Results**

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17 The LOFUS database used for this study included 13,725 persons, of whom 53% were
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19 women and 47% men. The median follow-up time was 2.6 years (IQR 1.5) and the median
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21 age was 57.6 in women and 59.9 in men. One-fourth of the participants were obese, and
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23 one-fifth were current smokers. Presence of cardiovascular disease at the time of LOFUS
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25 participation was reported by 28%, diabetes by 5%, and cancer by 4%. On the value of total
26
27 AL index, participants were divided between 14% low, 40% mid, and 46% high. During the
28
29 follow-up period, 198 participants died; of these 39% were women and 61% men (Table 1).
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34 The multivariate Cox proportional hazard regression for individual biomarker
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36 and all-cause mortality, adjusted for sex and age and additionally for BMI, reported
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38 diseases, and smoking, are listed in Table 2. For all biomarkers, apart from triglycerides, a
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40 high risk value was associated with an increased mortality level. However, only the HRs for
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42 low albumin and high CRP were statistically significantly elevated; HR 1.54 (95% CI: 1.16-
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44 2.06) and 1.41 (95% CI: 1.04-1.91), respectively.
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48 The HR for all-cause mortality increased with increasing level of the AL from
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50 low as the reference over mid to high, Table 3 and Figure 1. For the inflammatory system
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52 AL score, the HR was 1.02 (95% CI: 0.73-1.42) for mid AL, and 2.38 (95% CI: 1.67-3.39) for
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54 high AL. For the metabolic system AL score, the HRs were 1.18 (95% CI 0.75-1.85) and 1.55
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56 (95% CI: 1.00-2.38), respectively. For the cardiovascular system AL score, the HRs were
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4 1.65 (95% CI 1.02-2.65) and 1.89 (95% CI: 1.20-2.99), respectively. The steepest gradient
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6 was found for the total AL index, with HRs of 1.76 (95% CI: 0.90-3.44) for mid AL, and
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8 2.94 (95% CI: 1.53-5.66) for high AL.
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11 **Discussion**

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14 In this population-based study from a rural-provincial area of Denmark, we followed the
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16 adult population up for a mean period of 2.7 years. High levels of individual biomarkers
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18 were overall associated with increased mortality, but most of them at a modest level of 20-
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20 40%, and statistically significantly elevated for only CRP and albumin. High levels of
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22 physiologic system-specific AL scores were associated with increased mortality at the level
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24 of 50-100%; statistically significantly for the inflammatory and cardiovascular systems,
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26 and at borderline of significance for the metabolic system. The composite measure of total
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28 AL index was the strongest predictor of mortality. Persons with a high vs. low total AL
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30 index had almost 3-times the mortality. The total AL index was thus a better predictor of
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32 all-cause mortality than individual biomarkers and physiological systems AL scores, a
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34 pattern consistent with previous studies [14,16, 30].
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41 The most comprehensive studies on AL and mortality all used data from the
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43 National Health and Nutrition Examination Survey (NHANES) conducted in 1988-1994.
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45 Levine and Crimmins [31] examined ten-year all-cause and disease-specific mortality in a
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47 sample of 9942 adults, aged 30+ of whom 1076 had died. They constructed three AL
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49 scores; an AL score based on nine biomarkers in line with previous studies defined by
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51 clinical cut-off points; an expanded AL score that included five additional biomarkers
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53 defined by quintiles; and a continuous AL score constructed by using a continuous z-score
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55 measure for all fourteen biomarkers. They found that high values of all three AL scores
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57 were associated with increased mortality. Borrell et al. [32] examined twelve-year
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4 mortality by using data from 13,715 adults aged 25+ years of whom 2491 had died. Using a
5 clinical cut-off AL score, they found that, compared to persons with an AL score of 1, those
6 with AL scores of 2 and 3+ had 155% and 429% increased all-cause mortality, respectively.
7
8 Adjustment for ethnicity, age and sex reduced these excess risks to 35% and 99%,
9 respectively, while further adjustment for socioeconomic status had limited impact.
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11 Howard and Sparks [33] used the same AL construct as Borell et al. and found that a one
12 unit increase in AL represented a 7% increase in risk of death when adjusted for age, sex,
13 ethnicity, socioeconomic status, and health behaviour.
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23 For individual biomarkers in our study, HRs were highest for CRP and
24 albumin. CRP is the prototypical acute-phase response protein that increases during
25 systemic inflammation [34], while albumin is a major component of plasma protein,
26 required for transportation and to maintain oncotic pressure, acid–base function,
27 microvascular permeability, and to prevent platelet aggregation [35]. Inflammation
28 increases capillary permeability and thereby escape of serum albumin, leading to
29 expansion of interstitial space and increasing the distribution volume of albumin causing
30 lower serum albumin concentrations. High level of CRP and low level of albumin have thus
31 previously been linked with a variety of health outcomes including morbidity and mortality
32 [7,8,36].
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46 We found a U-shaped association between LDL-c and mortality. Elevated
47 LDL-c is a well-established risk factor of atherosclerosis and cardiovascular disease, and
48 the general perception is that high level of LDL-c is associated with an increased risk of
49 morbidity and mortality [37,38]. Nevertheless, studies on the association between LDL-c
50 levels and mortality have provided conflicting results. Some studies found increasing level
51 of LDL-c to be associated with lower mortality [39-40], and some studies found no
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4 association [38,41-42]. However, most studies were conducted in elderly people often with
5 an intake of lipid-lowering agents. A more recent study in young Koreans found an
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7 association between low level of LDL-c and an increased risk of cancer, cardiovascular, and
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9 all-cause mortality [43]. These findings were supported by a Chinese study of participants
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11 aged 40+ years [44]. A recent Danish study among 108,243 individuals aged 20-100 years
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13 found the lowest all-cause mortality at an LDL-c concentration of 3.6 mmol/L (140
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15 mg/dL), and higher mortality at both lower and higher levels [45]. Our findings for LDL-c
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17 were thus in accordance with these recent observations. Seplaki et al. suggested that both
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19 high and low ends of the risk continuum for the construct of AL could be more informative
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21 than simply using high-risk quartiles. They assigned a value of “1” for values above the 75th
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23 percentile and below the 25th percentile of the distribution, and a value of “0” for
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25 intermediate values [46].
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32 We found both higher and lower levels of diastolic blood pressure DBP to be
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34 associated with an increased mortality, and a similar tendency was indicated for SBP. The
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36 association between lower blood pressure and mortality is still of discussion [47-49]. Most
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38 studies have found this association among elderly people and linked it to chronic disease,
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40 e.g. cardiovascular disease (cardiac failure or ischaemic heart disease), cancer, poor
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42 functional status or frailty. Low BP has also been associated with poor function and low
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44 quality of life [50-51], but in previous studies only the highest quartile or the clinical cut-
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46 off value have been used as predictor of all-cause mortality.
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51 Several methods have been used to define an AL composite index, including
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53 the count-based, canonical correlation, z-score, and grade of membership method [52-53].
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55 The most commonly used method is the count-based method, where a summary index is
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57 calculated by summing the number of biomarkers falling within the high-risk category,
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4 either defined by the percentile (i.e., upper or lower 25th percentile of the sample's
5 distribution) or by the clinical cut-off value. In our analysis with the two-tail cut-off points,
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7 we found HRs for LDL of 1.13 (95% CI: 0.85-1.51); for SBP of 1.17 (95%CI: 0.88-1.57; and
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9 for DBP of 1.28 (95% CI: 0.95-1.72). If we have used instead the single high-risk quartile
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11 cut-off point, we would have found HRs for LDL of 0.71 (95% CI: 0.49-1.03); for SBP of
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13 0.96 (95% CI: 0.68-1.35) vs), and for DBP of 1.24 (95% CI: 0.86-1.81). The two-tail cut-off
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15 points thus provided a better identification of persons with high mortality than the one-tail
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17 cut-off points.
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23 The issue of whether a clinical or sample-based cut-off criteria should be used
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25 is still of discussion [15], however, studies comparing distinct measurement approaches
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27 have found only modest differences in their predictive utility [13, 54-55].
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30 **Strengths and limitations**

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33 The strengths of our study included the size of the cohort in terms of the large number of
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35 individuals recruited from a general adult population, and the complete follow-up for
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37 death by linkage with the Danish Civil Registration System.
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41 Our study also had some limitations. First, the choice of biomarkers used
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43 to construct the AL index. The AL theory emphasises the importance of measuring
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45 dysregulation across different physiological systems, including biomarkers from the
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47 neuroendocrine, cardiovascular, metabolic, and immune systems [13]. The neuroendocrine
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49 system (stress response) is believed to play a key role in allostasis and subsequent AL, as a
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51 series of physiological changes takes place before initial stress responses occur (such as
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53 rapid increases in blood sugar and blood pressure that supply the body with additional
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55 energy). However, biomarkers from the neuroendocrine system are difficult to measure, as
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4 repeated measurements over 1–2 days are recommended. These requirements cannot be
5 fulfilled in population studies, where participants are examined only once, and biomarkers
6 from the neuroendocrine system were therefore not available for our study.
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11 Secondly, the initial stress responses are followed by secondary outcomes
12 from the metabolic, inflammatory and cardiovascular systems, and these markers were all
13 available in our data. Nevertheless, greater sensitivity could have been achieved by
14 studying the dynamic changes over time in these markers to fully capture the flexibility of
15 stress response mechanisms across the lifespan.
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24 Finally, differences across studies in construction of AL indices could
25 influence the comparison of results. We used the shape of the association between level of
26 a given biomarker and all-cause mortality as the basis for the categorization of the
27 biomarker into low and high values. One can argue therefore that our analysis was circular
28 in the way that we used outcome on the dependent variable to categorize levels of the
29 independent variable. We believe that this was justifiable in the context here where the
30 purpose was to optimize the predictive power of the AL index. However, validation in other
31 datasets are needed before our approach can be recommended for research in general and
32 for eventual clinical use.
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45 **Conclusion**

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47 Our findings demonstrated that an optimally constructed AL index was a strong predictor
48 of all-cause mortality. This supported the conceptual validity of AL as an effective marker
49 of the cumulative physiological burden on the body. These findings can contribute to the
50 evidence for the use of an AL index as a basis for targeted efforts to bring down continued
51 stress exposures, and in this way prevent the potential detrimental effect of these
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4 exposures on health. Our findings on the U-shaped association with LDL-c, DBP and SBP
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6 and all-cause mortality suggested that AL measures incorporating risks at both the low and
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8 the high-end of biomarkers may yield the best prediction of all-cause mortality.
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Declarations

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Conflicts of interest

None.

Availability of data and materials and code availability

Data from the study can be made available via Region Sjælland following the Danish Data Protection Regulation.

Author Contributions

All authors contributed significantly to the study. Randi Jepsen provided the LOFUS data. Neda Bruun-Rasmussen, Elsebeth Lyngø and George Napolitano designed the study, interpreted the data, and drafted the manuscript. George Napolitano performed the statistical analysis. Christina Ellervik, Christian Christiansen, Randi Jepsen, Knud Rasmussen and Stig Bojesen contributed to the interpretation and writing of the manuscript. All authors critically revised and approved the final manuscript.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki.

Approval was granted by Region Zealand's Ethical Committee on Health Research (Reg:

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4 SJ-421). All data storage and management were approved by the Regional Data Protection
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6 Agency of Zealand (REG-024-2019 & REG-24-2015). LOFUS is registered in
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8 Clinicaltrials.gov (NCT02482896).
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11 Consent to participate

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14 Participants provided written informed consent.
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17 Consent for publication

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31
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34 interpretation conducted within this study.
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Table 1. Lolland-Falster Health Study (LOFUS). Baseline characteristics of study population and deaths in follow-up period, n(%). For definition of cut-off values, see Supplementary Table 1

Characteristics	Females	Males	Total	Female death	Male death	Total death
Population	7270 (53)	6455 (47)	13725 (100)	78 (39)	120 (61)	198 (100)
Follow-up time, median (IQR)	2.6 (1.6)	2.7 (1.5)	2.6 (1.5)	2.0 (1.6)	1.9 (1.8)	1.9 (1.8)
Median age (IQR)	57.6 (21.9)	59.9 (21.6)	58.7 (22.0)	70.5 (16.4)	74.0 (15.2)	72.8 (16.2)
BMI, kg/m²						
- Underweight	134 (1.8)	42 (0.7)	176 (1.3)	5 (6.4)	Not reported	6 (3.0)
- Normal weight	3038 (41.8)	1862 (28.8)	4900 (35.7)	29 (37.2)	40 (33.3)	69 (34.8)
- Overweight	2335 (32.1)	2940 (45.5)	5275 (38.4)	26 (33.3)	52 (43.3)	78 (39.4)
- Obese	1763 (24.3)	1611 (25.0)	3374 (24.6)	18 (23.1)	27 (22.5)	45 (22.7)
Smoking						
- Never	3586 (49.3)	2737 (42.4)	6323 (46.1)	21 (26.9)	24 (20.0)	45 (22.7)
- Former	2342 (32.2)	2425 (37.6)	4767 (34.7)	32 (41.0)	70 (58.3)	102 (51.5)
- Current	1342 (18.5)	1293 (20.0)	2635 (19.2)	25 (32.1)	26 (21.7)	51 (25.8)
Chronic conditions						
Cardiovascular disease reported	1828 (25.1)	1999 (31.0)	3827 (27.9)	42 (53.8)	60 (50.0)	102 (60.6)
Diabetes reported	264 (3.6)	440 (6.8)	704 (5.1)	9 (11.5)	15 (12.5)	24 (12.1)

Cancer reported	245 (3.4)	275 (4.3)	520 (3.8)	13 (16.7)	24 (20.0)	37 (18.7)
Cardiovascular system						
<i>Systolic blood pressure</i>						
- Low risk	3548 (96.6)	3165 (95.7)	6713 (96.2)	32 (41.0)	52 (43.3)	84 (42.4)
- High risk	3722 (3.4)	3290 (4.3)	7012 (3.8)	46 (59.0)	68 (56.7)	114 (57.6)
<i>Diastolic blood pressure</i>						
- Low risk	3426 (47.1)	3164 (49.0)	6590 (48.0)	26 (33.3)	52 (43.3)	78 (39.4)
- High risk	3844 (52.9)	3291 (51.0)	7135 (52.0)	52 (66.7)	68 (56.7)	120 (60.6)
<i>Pulse rate</i>						
- Low risk	5366 (73.8)	4721 (73.1)	10087 (73.5)	50 (64.1)	81 (67.5)	131 (66.2)
- High risk	1904 (26.2)	1734 (26.9)	3638 (26.5)	28 (35.9)	39 (32.5)	67 (33.8)
<i>AL cardiovascular system score</i>						
- Low	1815 (25.0)	1506 (23.3)	3321 (24.2)	7 (9.0)	16 (13.3)	23 (11.6)
- Mid	2117 (29.1)	2154 (33.4)	4271 (31.1)	27 (34.6)	43 (35.8)	70 (35.4)
- High	3338 (45.9)	2795 (43.4)	6133 (44.7)	44 (56.4)	61 (50.8)	105 (53.0)
Metabolic system						
<i>HDL-c</i>						
- Low risk	4934 (67.9)	4706 (72.9)	9640 (70.2)	46 (59.0)	85 (70.8)	131 (66.2)

- High risk	2336 (32.1)	1749 (27.1)	4085 (29.8)	32 (41.0)	35 (29.2)	67 (33.8)
<i>Triglycerides</i>						
- Low risk	5299 (72.9)	4761 (73.8)	10060 (73.3)	50 (64.1)	95 (79.2)	145 (73.2)
- High risk	1971 (27.1)	1694 (26.2)	3665 (26.7)	28 (35.9)	25 (20.8)	53 (26.8)
<i>HbA1c</i>						
- Low risk	5156 (70.9)	4438 (68.8)	9594 (69.9)	46 (59.0)	69 (57.5)	115 (58.1)
- High risk	2114 (29.1)	2017 (31.2)	4131 (30.1)	32 (41.0)	51 (42.5)	83 (41.9)
<i>Waist-hip ratio</i>						
- Low risk	5452 (75.0)	4831 (74.8)	10283 (74.9)	57 (73.1)	85 (70.8)	142 (71.7)
- High risk	1818 (25.0)	1624 (25.2)	3442 (25.1)	21 (26.9)	35 (29.2)	56 (28.3)
<i>LDL-c</i>						
- Low risk	3459 (47.6)	2989 (46.3)	6448 (47.0)	31 (39.7)	51 (42.5)	82 (41.4)
- High risk	3811 (52.4)	3466 (53.7)	7277 (53.0)	47 (60.3)	69 (57.5)	116 (58.6)
<i>AL metabolic system score</i>						
- Low	1401 (19.3)	1249 (19.3)	2650 (19.3)	11 (14.1)	18 (15.0)	29 (14.6)
- Mid	2413 (33.2)	2135 (33.1)	4548 (33.1)	18 (23.1)	37 (30.8)	55 (27.8)
- High	3456 (47.5)	3071 (47.6)	6527 (47.6)	49 (62.8)	65 (54.2)	114 (57.6)
Inflammation system						

<i>CRP</i>						
- Low risk	5451 (75.0)	4837 (74.9)	10288 (75.0)	51 (65.4)	73 (60.8)	124 (62.6)
- High risk	1819 (25.0)	1618 (25.1)	3437 (25.0)	27 (34.6)	47 (39.2)	74 (37.4)
<i>Albumin</i>						
- Low risk	4953 (68.1)	4655 (72.1)	9608 (70.0)	49 (62.8)	54 (45.0)	103 (52.0)
- High risk	2317 (31.9)	1800 (27.9)	4117 (30.0)	29 (37.2)	66 (55.0)	95 (48.0)
<i>AL inflammation system score</i>						
- Low	4027 (55.4)	3692 (57.2)	7719 (56.2)	41 (52.6)	42 (35.0)	83 (41.9)
- Mid	2350 (32.3)	2108 (32.7)	4458 (32.5)	18 (23.1)	43 (35.8)	61 (30.8)
- High	893 (12.3)	655 (10.1)	1548 (11.3)	19 (24.4)	35 (29.2)	54 (27.3)
Total AL index						
- Low	1009 (13.9)	915 (14.2)	1924 (14.0)	4 (5.1)	6 (5.0)	10 (5.1)
- Mid	2895 (39.8)	2638 (40.9)	5533 (40.3)	24 (30.8)	39 (32.5)	63 (31.8)
- High	3366 (46.3)	2902 (45.0)	6268 (45.7)	50 (64.1)	75 (62.5)	125 (63.1)

Table 2. Multivariate Cox proportional hazard regression of all-cause mortality for LOFUS participants by individual biomarkers

Variable	Non exposed	Exposed	Hazard ratio (95 % CI) Model 1*	Hazard ratio (95 % CI) Model 2**
HDL cholesterol, mg/dl	High	Low	1.22 (0.88-1.69)	1.24 (0.89-1.73)
LDL cholesterol, mg/dl	High	Low	1.22 (0.91-1.62)	1.19 (0.85-1.51)
Triglycerides, mg/dl	Low	High	0.93 (0.66-1.32)	0.94 (0.67-1.33)
Albumin, g/dl	High	Low	1.55 (1.17-2.07)	1.54 (1.16-2.06)
CRP, mg/L	Low	High	1.42 (1.05-1.92)	1.41 (1.04-1.91)
HbA1c, mmol/mol	Low	High	1.25 (0.93-1.68)	1.24 (0.89-1.73)
Systolic blood pressure, mmHg	Low	High	1.20 (0.90-1.61)	1.17 (0.88-1.57)
Diastolic blood pressure, mmHg	High	Low	1.31 (0.98-1.76)	1.29 (0.95-1.72)
Pulse rate, PM	High	Low	1.34 (0.99-1.81)	1.29 (0.91-1.66)
Waist-hip ratio	Low	High	1.02 (0.74-1.41)	1.08 (0.76-1.52)

*Adjusted for age and sex

** Additionally adjusted for BMI, reported diseases, and smoking status

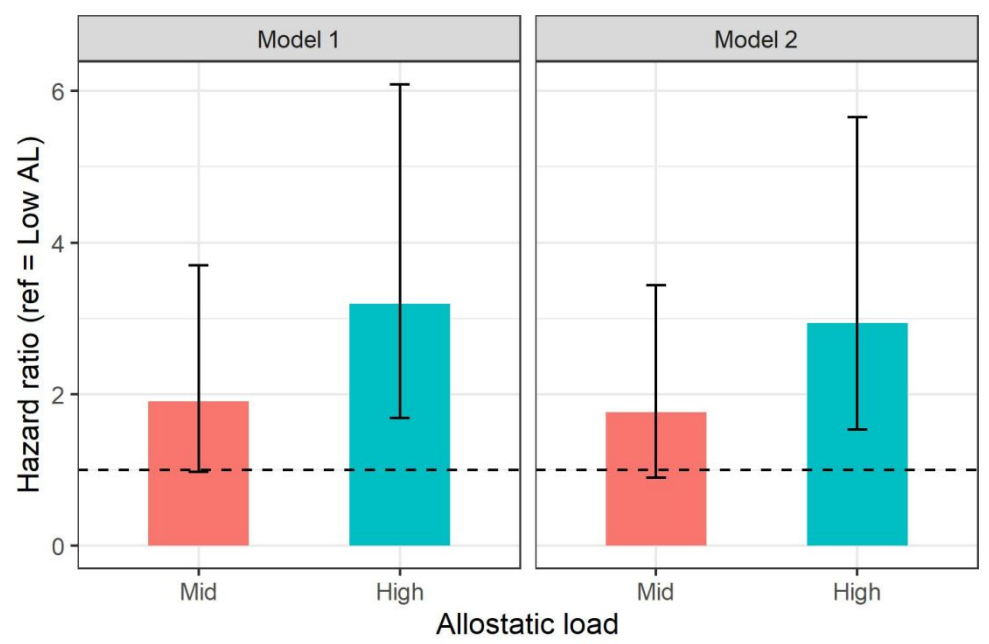
Table 3. Multivariate Cox proportional hazard regression of all-cause mortality for LOFUS participants by allostatic load index

Variable	Reference	Level	Hazard ratio (95 % CI)	
			Model 1*	Model 2**
Allostatic load index	Low [0:1]	Mid [2:3]	1.90 (0.97-3.70)	1.76 (0.90-3.44)
		High [4:10]	3.19 (1.68-6.09)	2.94 (1.53-5.66)
Inflammatory system score	Low [0]	Mid [1]	1.03 (0.74-1.44)	1.02 (0.73-1.42)
		High [2:3]	2.39 (1.69-3.38)	2.38 (1.67-3.39)
Metabolic system score	Low [0]	Mid [1]	1.19 (0.76-1.86)	1.18 (0.75-1.85)
		High [2:5]	1.54 (1.02-2.33)	1.55 (1.00-2.38)
Cardiovascular system score	Low [0]	Mid [1]	1.73 (1.08-1.78)	1.65 (1.02-2.65)
		High [2:3]	2.06 (1.31-3.24)	1.89 (1.20-2.99)

*Adjusted for age and sex

** Additionally adjusted for BMI, reported diseases, and smoking status

Figure 1. All-cause mortality by level of allostatic load index, as hazard ratio (95% confidence interval)



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Supplementary Table 1. Descriptive statistics and high-risk cut points for individual biomarkers

Characteristics	ALL	Males			Females		
	N = 13,725	N = 6455			N = 7270		
	Median (IQR)	Median (IQR)	High-risk cut point <60 years	High-risk cut point ≥60 years	Median (IQR)	High-risk cut point <60 years	High-risk cut point ≥60 years
HDL cholesterol, mg/dl	1.4 (0.5)	1.2 (0.5)	≤1.0	≤1.1	1.5 (0.5)	≤1.3	≤1.4
LDL cholesterol, mg/dl	2.8 (1.2)	2.8 (1.2)	≤2.2 or ≥3.4	≤2.4 or ≥3.5	2.8 (1.2)	≤2.2 or ≥3.4	≤2.1 or ≥3.8
Triglycerides, mg/dl	1.5 (1.1)	1.6 (1.3)	≥2.4	≥2.2	1.2 (0.9)	≥1.8	≥2.0
Albumin, g/dl	40.0 (3.0)	41.0 (4.0)	≤39.0	≤37.0	40.0 (3.0)	≤38.0	≤37.0
CRP, mg/L	1.40 (2.53)	1.06 (1.83)	≥2.37	≥3.42	1.40 (2.96)	≥3.59	≥3.53
HbA1c, mmol/mol	36.0 (5.0)	35.0 (4.0)	≥37.0	≥40.0	35.0 (5.0)	≥37.0	≥40.0
Systolic blood pressure, mmHg	130.0 (26.5)	127.5 (18.5)	≤120.0 or ≥138.5	≤128.5 or ≥153.5	119.5 (18.5)	≤112.0 or ≥130.5	≤124.5 or ≥152.5
Diastolic blood pressure, mmHg	78.5 (10.5)	79 (11.1)	≤73.5 or ≥84.6	≤75.0 or ≥85.5	77.0 (11.0)	≤72.0 or ≥83.0	≤74.5 or ≥83.5

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Pulse rate, PM	66.0 (13.0)	65.0 (14.0)	≥72.0	≥72.0	67.0 (13.0)	≥74.0	≥74.0
Waist hip ratio	0.90 (0.14)	0.94 (0.10)	≥1.0	≥1.0	0.83 (0.10)	≥0.9	≥0.9

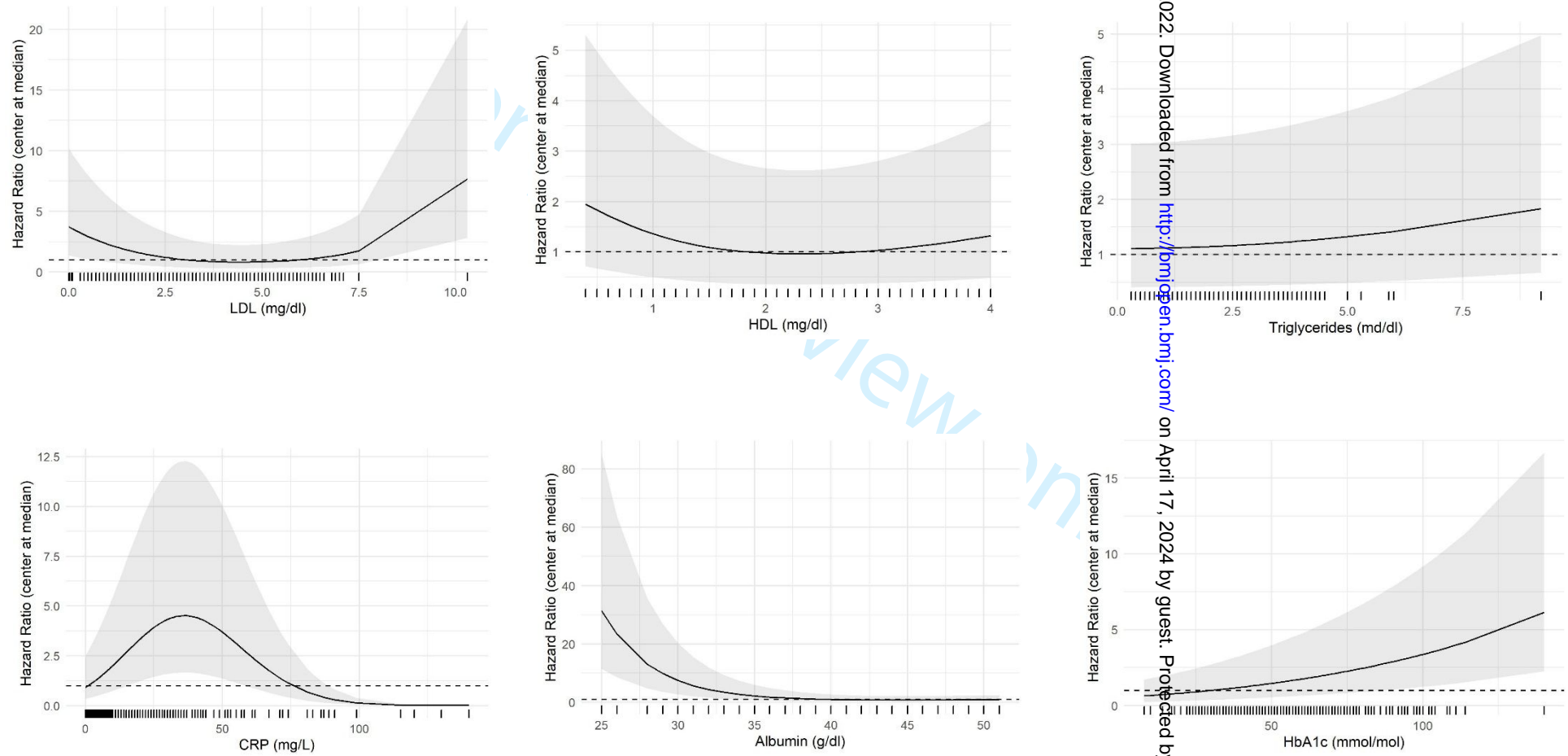
Supplementary Table 2. Number and proportion of missing values in analysis sample

Variable	Missing values	Proportion of total analysis sample (N= 15,714)
Biomarkers		
Date of biomarker sample	39	0.25%
C-Reactive Protein	171	1.08%
Albumin	168	1.07%
HDL cholesterol	168	1.07%
LDL Cholesterol	635	4.04%
Triglycerides	169	1.07%
HbA1c	207	1.32%
Waist-hip ratio	139	0.88%
Diastolic Blood Pressure	21	0.13%
Systolic Blood Pressure	21	0.13%
Pulse Rate	69	0.44%
BMI	292	1.86%
Smoking	867	5.52%
Chronic conditions	810	5.15%

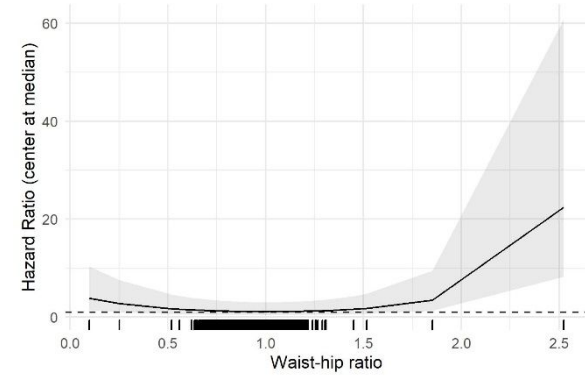
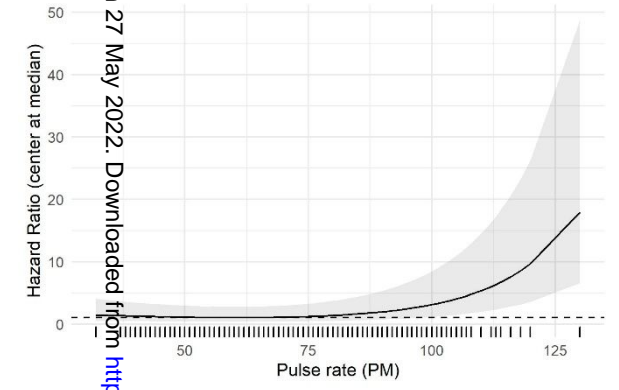
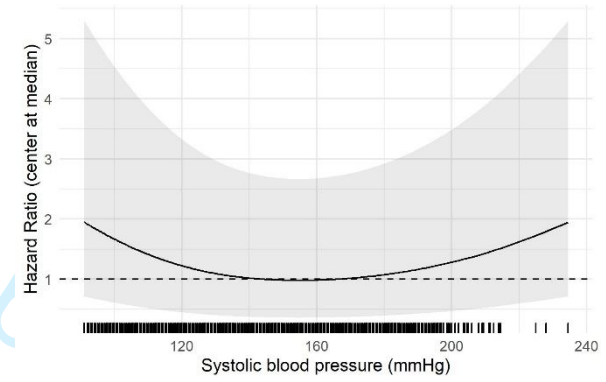
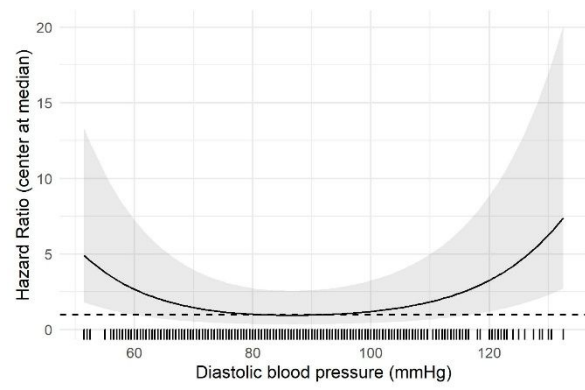
Supplementary Table 3. Number of LoD values replaced in each variable

Biomarker	# LoD replaced	LoD
Haemoglobin A1c	1	<31
C-reactive protein	242	<0.16
Alanine aminotransferase	1	<6
Bilirubin	2	<2.0
Low-density lipoprotein	3	<0.10

Supplementary Figure 1. Hazard ratio for individual biomarkers, centered at median



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Reporting checklist for cohort study.

Based on the STROBE cohort guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the STROBE cohort reporting guidelines, and cite them as:

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	Reporting Item	Page Number
Title and abstract		
Title	#1a Indicate the study's design with a commonly used term in the title or the abstract	

1	Abstract	#1b	Provide in the abstract an informative and balanced summary
2			of what was done and what was found
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6	Introduction		
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10	Background /	#2	Explain the scientific background and rationale for the
11	rationale		investigation being reported
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15	Objectives	#3	State specific objectives, including any prespecified
16			hypotheses
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20	Methods		
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24	Study design	#4	Present key elements of study design early in the paper
25			
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27	Setting	#5	Describe the setting, locations, and relevant dates, including
28			periods of recruitment, exposure, follow-up, and data
29			collection
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34	Eligibility criteria	#6a	Give the eligibility criteria, and the sources and methods of
35			selection of participants. Describe methods of follow-up.
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39	Eligibility criteria	#6b	For matched studies, give matching criteria and number of
40			exposed and unexposed
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45	Variables	#7	Clearly define all outcomes, exposures, predictors, potential
46			confounders, and effect modifiers. Give diagnostic criteria, if
47			applicable
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53	Data sources /	#8	For each variable of interest give sources of data and details
54	measurement		of methods of assessment (measurement). Describe
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one group. Give information separately for for exposed and unexposed groups if applicable.

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6 Bias [#9](#) Describe any efforts to address potential sources of bias
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9 Study size [#10](#) Explain how the study size was arrived at
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12 Quantitative [#11](#) Explain how quantitative variables were handled in the
13 variables analyses. If applicable, describe which groupings were
14 chosen, and why
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19 Statistical [#12](#) Describe all statistical methods, including those used to control for
20 methods [a](#) confounding
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28 Statistical [#12](#) Describe any methods used to examine subgroups and
29 methods [b](#) interactions
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33 Statistical [#12](#) Explain how missing data were addressed
34 methods [c](#)
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39 Statistical [#12](#) If applicable, explain how loss to follow-up was addressed
40 methods [d](#)
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44 Statistical [#12](#) Describe any sensitivity analyses
45 methods [e](#)
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52 Results

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56 Participants [#13](#) Report numbers of individuals at each stage of study—eg
57 numbers potentially eligible, examined for eligibility, confirmed
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1 eligible, included in the study, completing follow-up, and
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3 analysed. Give information separately for for exposed and
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5 unexposed groups if applicable.
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8 Participants [#13](#) Give reasons for non-participation at each stage
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13 Participants [#13](#) Consider use of a flow diagram
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15 [c](#)
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22 Descriptive data [#14](#) Give characteristics of study participants (eg demographic,
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24 [a](#) clinical, social) and information on exposures and potential
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26 confounders. Give information separately for exposed and
27
28 unexposed groups if applicable.
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31 Descriptive data [#14](#) Indicate number of participants with missing data for each variable of
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33 [b](#) interest
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40 Descriptive data [#14](#) Summarise follow-up time (eg, average and total amount)
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43 [c](#)
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49 Outcome data [#15](#) Report numbers of outcome events or summary measures over time.
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51 Give information separately for exposed and unexposed groups if
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53 applicable.
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- 1 Main results [#16](#) Give unadjusted estimates and, if applicable, confounder-
2 [a](#) adjusted estimates and their precision (eg, 95% confidence
3 interval). Make clear which confounders were adjusted for
4 and why they were included
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11 Main results [#16](#) Report category boundaries when continuous variables were
12 [b](#) categorized
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16 Main results [#16](#) If relevant, consider translating estimates of relative risk into absolute
17 [c](#) risk for a meaningful time period
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25 Other analyses [#17](#) Report other analyses done—eg analyses of subgroups and
26 interactions, and sensitivity analyses
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30 **Discussion**
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33 Key results [#18](#) Summarise key results with reference to study objectives
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36 Limitations [#19](#) Discuss limitations of the study, taking into account sources of
37 potential bias or imprecision. Discuss both direction and
38 magnitude of any potential bias.
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44 Interpretation [#20](#) Give a cautious overall interpretation considering objectives,
45 limitations, multiplicity of analyses, results from similar
46 studies, and other relevant evidence.
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51 Generalisability [#21](#) Discuss the generalisability (external validity) of the study
52 results
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57 **Other Information**
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1 Funding [#22](#) Give the source of funding and the role of the funders for the
2
3 present study and, if applicable, for the original study on
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5 which the present article is based
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Allostatic load as predictor of mortality. A cohort study from Lolland-Falster, Denmark

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Nykøbing Falster Hospital,

2nd March 2022

Allostatic load as predictor of mortality. A cohort study from Lolland-Falster, Denmark

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Abstract

Objectives: The purposes of the present study were to determine the association between 1) 10 individual biomarkers and all-cause mortality; and between 2) allostatic load (AL), across three physiological systems (cardiovascular, inflammatory, metabolic), and all-cause mortality. **Design:** Prospective cohort study. **Setting:** We used data from the Lolland-Falster Health Study undertaken in Denmark in 2016-2020 and used data on systolic (SBP) and diastolic blood pressure (DBP), pulse rate (PR), waist-hip ratio (WHR), and levels of low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), triglycerides, glycated haemoglobin (HbA1c), c-reactive protein (CRP), and serum albumin. All biomarkers were divided into quartiles with high-risk values defined as those in the highest (PR, WHR, triglycerides, HbA1c, CRP) or lowest (HDL-c, albumin) quartile, or a combination hereof (LDL-c, SBP, DBP). The ten biomarkers were combined into a summary measure of AL index. Participants were followed up for death for an average of 2.6 years. **Participants:** We examined a total of 13,725 individuals aged 18+ years. **Primary outcome measure:** Cox proportional hazard regression (HR) analysis were performed to examine the association between AL index and mortality in men and women. **Results:** All-cause mortality increased with increasing AL index. With low AL index as reference, the HR was 1.33 (95% confidence interval (CI) 0.89-1.97) for mid AL, and HR 2.37 (95% CI: 1.58-3.54) for high AL. **Conclusions:** Elevated physiological burden measured by mid and high AL index was associated with a steeper increase of mortality than individual biomarkers.

Abstract word count: 240

Keywords: Biomarker, Allostatic Load, Blood, Mortality, population-based, LOFUS.

Manuscript word count: 3870

Article Summary

Strengths and limitations

- Analysis based on a large population-based health study.
- Complete follow-up for death via linkage with Danish Civil Registration System.
- Biomarkers from only one point in time.
- No biomarker from neuroendocrine system available.

Introduction

Biological markers (biomarkers) were originally defined as “cellular, biochemical or molecular alterations that are measurable in biological media such as cells, human tissues or fluids” [1]. Later the definition was extended to include “indicators of normal biological processes, pathogenic processes and pharmacological responses to therapeutic interventions” [2]. In clinical settings, measurement of biomarkers in blood samples is used to detect and diagnose medical conditions. Biomarkers as independent predictors of all-cause mortality are therefore of considerable clinical and research interest [3]; dyslipidaemia including high levels of triglycerides and low-density lipoprotein cholesterol (LDL-c), and low levels of high-density lipoprotein cholesterol (HDL-c), have been reported to be independent risk factors for all-cause mortality [4-6]. Lower levels of albumin [7] and higher levels of C-reactive protein (CRP) [8], and haemoglobin A1c (HbA1c) [9] have likewise been linked to mortality. Also, there is some evidence that the relationship between some of these biomarkers and all-cause mortality varies across sex and age-groups [10,11].

The concept of allostatic load (AL) refers to the “wear and tear” of the body resulting from repeated stimulation of stress responses via the hypothalamic–pituitary–adrenal axis and the sympathetic-adrenal-medullary system [12]. As a latent variable, AL cannot be directly measured but it can be estimated using an AL index, which is composite of biomarkers from multiple organ systems integrated into a single score. The first AL developed by Seeman et al. in 1997 included 10 biomarkers monitoring various physiological systems [13]. However, the type and number of biomarkers used in published studies have ranged from 6 to 24 [14]. The most frequently used AL construct, originally proposed by Gruenewald et al in 2012 [15], includes 24 biomarkers. It has been suggested

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4 that in the calculation of AL, the threshold of risk for each biomarker should be obtained
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6 by the quartiles or quintiles of the values of the biomarker [16]. AL has been reported to be
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8 a better predictor of mortality than individual biomarkers, however, there are still gaps in
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10 the understanding of the associations [17-18]. AL has been suggested also as a tool for
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12 allocation of nursing resources [19].
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16 This study provides data from the Lolland-Falster Health Study (LOFUS) [20],
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18 a population-based survey undertaken in 2016-2020 in Lolland-Falster, a rural-provincial
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20 region in Denmark with a life expectancy much below the national average [21], and with
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22 health problems reported more frequently than in the rest of the country [22]. Using the
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24 LOFUS data, the purposes of the present study were 1) to determine the association
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26 between 10 individual biomarkers and all-cause mortality; and 2) to examine the
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28 association between AL, across three physiological systems (cardiovascular, inflammatory,
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30 metabolic system), and all-cause mortality. The hypothesis is that AL can be used as an
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32 informative tool in predicting future risk of death in the general adult population.
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37 **Methods**

38 **Patient and Public Involvement**

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41 We undertook a prospective cohort study of participants from LOFUS; a household-based
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43 population study with data collected between February 2016 and February 2020. Persons
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45 aged 18 years and above were randomly sampled from the Danish Civil Registration
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47 System and invited to participate together with the rest of their households. Participation
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49 required informed consent. The study was approved by Region Zealand's Ethical
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51 Committee on Health Research (Reg: SJ-421). A detailed description of the study protocol
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53 [20] and information on the socio-economic determinants of participation [23] have been
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4 published previously. Persons below 18 years, and pregnant women were excluded from
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6 the present study. Once the paper has been published in the international literature, the
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8 key results will be reported also in the local press.
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10 11 Self-reported data

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14 From questionnaires, we used data on smoking (never, former, current), and presence of
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16 chronic conditions (cardiovascular disease, diabetes, cancer) at the time of participation in
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18 LOFUS.
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20 21 Biomarkers

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24 Non-fasting blood samples were collected in vacutainer blood collection tubes (Becton,
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26 Dickinson and Company; Franklin Lakes, NJ, USA) and kept at room temperature until
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28 same day analysis at the Department of Clinical Biochemistry at Nykøbing Falster
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30 Hospital, accredited by the standard ISO 15189. We used data on HDL-c, LDL-c,
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32 triglycerides, albumin, CRP, and HbA1c. LDL-c was calculated by using Friedewald
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34 formula [24] when the plasma triglyceride concentration was below 4.5 mmol/L. Systolic
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36 and diastolic blood pressure were based on three consecutive digital measurements on the
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38 upper left arm (apparatus type Welch Allyn Connex pro BPO 3400). The mean values of
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40 the second and third measurement were used in this study (only one measurement was
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42 used if the other was missing). Waist-hip ratio (WHR) was calculated by waist-
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44 circumference divided by hip-circumference. Body mass index (BMI) was defined as
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46 weight in kilograms divided by height in meters squared (kg/m²).
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53 In the calculation of AL, biomarkers are most often dichotomized into low and
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55 high values based on either a percentile or a predetermined cut-off value [16]. However,
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57 before doing so, we mapped for each biomarker the association between level of the
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4 marker and all-cause mortality, see method below. For most biomarkers the association
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6 was monotonic, see Supplementary Figure 1. These biomarkers were then dichotomized
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8 according to the sex- and age-specific quartiles, as variations across these parameters were
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10 found in our previous study of reference intervals [25]. We dichotomized biomarkers with
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12 high-risk values defined as those in the highest quartile of the sex- and age-specific
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14 distribution, except for HDL-c, and albumin, where the lowest quartile was the high-risk
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16 value. For LDL-c, SBP and DBP the associations were U-shaped, and the high-risk values
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18 for these biomarkers were therefore defined as including both the lower and the upper
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20 quartiles, see Supplementary Table 1. For all biomarkers, the highest and lowest quartile of
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22 risk scores were either lower or similar to clinical cut-points [26-30].
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27 BMI was divided into underweight (BMI less than 18.5) normal (BMI 18.5–
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29 24.9), overweight (BMI 25.0–29.9), or obese (BMI 30.0 or greater); reported diseases into
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31 either present or not; and smoking status into never, former, or current.
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34 35 Allostatic load scores 36

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38 The AL scores were computed using biomarkers from: the cardiovascular system (systolic
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40 blood pressure (SBP), diastolic blood pressure (DBP), and pulse rate (PR)); the metabolic
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42 system (LDL-c, triglycerides, HDL-c, WHR, HbA1c); and the inflammatory system (CRP,
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44 serum albumin).
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48 Each system-specific AL score was then defined as the number of biomarkers
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50 with a high-risk value, hence as an integer value between 0 and 3 for the cardiovascular
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52 system (CVS), 0 and 5 for the metabolic system (MS), and 0 and 2 for inflammatory system
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54 (IS). The AL index was defined as the sum of all scores and divided in three groups based
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4 on tertiles contrasting individuals with [AL:0-2], mid [AL: 3-4], and high [AL: 5-10]. Note
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6 that, all biomarkers were given equal weight in accordance with previous studies [16,18].
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9 All-cause mortality

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12 LOFUS participants were followed up for death with data obtained from the Danish Civil
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14 Registration System on 26 February 2021.
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17 Data management and statistical analyses

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20 Observations with missing values in any of the variables were excluded from the analyses
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22 (1989 out of 15714, i.e. 12.6%, see Supplementary Table 2). Values below the lower limit of
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24 detection were replaced with random numbers sampled with replacement from the set $\{k$
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26 $\times 10^{(-n)}$, $k = 1, \dots, L\}$, where n is the variable-specific number of decimals reported in the
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28 data and $L \times 10^{(-n)}$ the limit of detection, see Supplementary Table 3.
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33 Participants were followed up from date of participation in the LOFUS study
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35 until date of death or end of follow-up on 26 February 2021, whichever came first. In order
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37 to define the biomarkers' high-risk values, we first studied the association between levels
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39 of each individual biomarker and mortality, allowing for possible nonlinear relations. This
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41 analysis was carried out via Cox proportional-hazard models with biomarker levels as
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43 continuous covariates, modelled with natural cubic splines with 2 degrees of freedom
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45 (except for LDL-c, where 3 degrees of freedom were used), and further adjusting for sex
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47 and age. By graphical inspection, a U-shaped association was found for LDL-c, SBP and
48
49 DBP (see Supplementary Figure 1). Therefore, for these biomarkers both the sex and age-
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51 specific (i.e. below or above age 60) lower and upper quartiles were defined as high-risk,
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53 while only one quartile for the others (upper or lower, in accordance with the existing
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55 literature); see Supplementary Table 1.
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4 Associations between all-cause mortality and dichotomized biomarkers levels
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6 (low/high risk), system-specific AL scores, and total AL index, were modelled with Cox
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8 proportional-hazard models. Here, we present two models: Model 1, where HRs are
9
10 adjusted for sex and age; Model 2, where results are further adjusted for BMI, prevalent
11
12 diseases, and smoking status. HRs for the individual biomarkers (Table 2) and for system-
13
14 specific AL scores (Table 3) are mutually adjusted. Proportional hazards assumptions in
15
16 the above models have been tested using Schoenfeld residuals. Numbers below 5 are not
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18 reported. In addition, we report HRs for a one-point increase in the AL index.
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23 Data management, statistical analyses and plots were done in R ver. 4.0.3 [31],
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25 with packages splines [31], survival [32], tidyverse [33], ggplot2 [34] and ggpubr [35].
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28 **Results**

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31 The LOFUS database used for this study included 13,725 persons, of whom 53% were
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33 women and 47% men. The median follow-up time was 2.6 years (IQR 1.5) and the median
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35 age was 57.6 in women and 59.9 in men. One-fourth of the participants were obese, and
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37 one-fifth were current smokers. Presence of cardiovascular disease at the time of LOFUS
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39 participation was reported by 28%, diabetes by 5%, and cancer by 4%. On the value of total
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41 AL index, participants were divided approximately into tertiles; 32% low, 40% mid, and
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43 38% high. During the follow-up period, 198 participants died; of these 39% were women
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45 and 61% men (Table 1).
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50 The multivariate Cox proportional hazard regression for individual biomarker
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52 and all-cause mortality, adjusted for sex and age and additionally for BMI, reported
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54 diseases, and smoking, are listed in Table 2. For all biomarkers, apart from triglycerides, a
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56 high risk value was associated with an increased mortality level. However, only the HRs for
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low albumin and high CRP were statistically significantly elevated; HR 1.54 (95% CI: 1.16-2.06) and 1.41 (95% CI: 1.04-1.91), respectively.

The HR for all-cause mortality increased with increasing level of the AL from low as the reference over mid to high, Table 3 and Figure 1. For the inflammatory system AL score, the HR was 1.02 (95% CI: 0.73-1.42) for mid AL, and 2.38 (95% CI: 1.67-3.39) for high AL. For the metabolic system AL score, the HRs were 1.18 (95% CI 0.75-1.85) and 1.54 (95% CI: 1.00-2.38), respectively. For the cardiovascular system AL score, the HRs were 1.65 (95% CI 1.02-2.65) and 1.89 (95% CI: 1.20-2.99), respectively. The gradient for the total AL index was a HR of 1.33 (95% CI: 0.89-1.98) for mid AL, and 2.37 (95% CI: 1.58-3.54) for high AL. HRs for 1 unit increase in AL (continuous AL) was 1.23 (1.14 – 1.32) when adjusted for age and sex, and 1.22 (1.13 – 1.32), when additionally adjusted for BMI, reported diseases, and smoking status.

Discussion

In this population-based study from a rural-provincial area of Denmark, we followed the adult population up for a median period of 2.6 years. High levels of individual biomarkers were overall associated with increased mortality, but most of them at a modest level of 20-30%, and statistically significantly elevated for only CRP and albumin. High levels of physiologic system-specific AL scores were associated with increased mortality at the level of 50-140%; statistically significantly for the inflammatory and cardiovascular systems, and at borderline of significance for the metabolic system. The composite measure of total AL index was a strong predictor of all-cause mortality. Persons with a high vs. low total AL index had about 2.5 times the mortality. The total AL index was thus a better predictor of all-cause mortality than individual biomarkers and the metabolic and cardiovascular systems AL scores, a pattern consistent with previous studies [16,18, 36].

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4 The most comprehensive studies on AL and mortality all used data from the
5
6 National Health and Nutrition Examination Survey (NHANES). Borrell et al. [37]
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8 examined twelve-year mortality by using data from 13,715 adults aged 25+ years of whom
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10 2491 had died. They calculated AL based on 9 biomarkers; albumin, CRP, total cholesterol,
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12 HDL, haemoglobin A1c, waist-to-hip-ratio, SBP, DBP, and PR. Using a clinical cut-off AL
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14 score, they found that, compared to persons with an AL score of ≤ 1 , those with AL scores of
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16 2 and 3+ had adjusted HRs of 1.40 (95% CI 1.11-1.76) and 1.88 (95% CI 1.56-2.26),
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18 respectively.
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23 Levine and Crimmins [38] examined ten-year all-cause and disease-specific
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25 mortality. In total, 15,042 persons were eligible, but biomarker data were available for only
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27 9942 adults aged 30+, of whom 1076 had died. They included data on albumin, CRP,
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29 waist-to-hip ratio, total cholesterol, HDL, haemoglobin A1c, PR, SBP, and DBP. For each of
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31 the nine biomarkers, a person was classified as high or low based on clinical cut-off points,
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33 and the AL score was the number of biomarkers classified as high. In addition, an
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35 expanded AL score included five additional biomarkers defined by quintiles; and a
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37 continuous AL score used a continuous z-score measure for all fourteen biomarkers. For
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39 the first AL score, a HR of 2.75 ($p < .001$) was found for all-cause mortality when persons
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41 with the highest quintile of AL were compared with those with the lowest. Somewhat
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43 stronger gradients were found for the expanded; 3.62 ($p < .0001$) and continuous; 6.97
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45 ($p < .0001$), ALs.
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51 Howard and Sparks [39] studied 11,733 participants from NHANES.
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53 Imputation was used to estimate missing values. Their AL measure was based on DBP,
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55 SBP, PR, total cholesterol, HDL, triglycerides, haemoglobin A1c, BMI, albumin and CRP.
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4 They found that a one-unit increase in AL represented a 7% increase in risk of death when
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6 adjusted for age, sex, ethnicity, socioeconomic status, and health behaviour.
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9 The National Child Development Study was followed up for deaths from birth
10 in 1958 to 1 December 2013, i.e. to the age of 55 years [18]. AL based on 10 biomarkers was
11 calculated and divided into three levels. All-cause mortality for persons with mid or high
12 AL was compared with that of persons with low AL, and adjusted for early life, childhood,
13 young and adulthood confounders. The HR of death was 1.71 (95% CI 1.07-2.72) for
14 persons with mid AL, and 2.57 (95% CI 1.59-4.15) for those with high AL. The association
15 between AL and all-cause mortality was stronger than the associations between of the
16 individual 10 biomarkers and all-cause mortality.
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28 The NHANES studies vary in number of participants included in the studies,
29 in length of follow-up for mortality, in biomarkers included, in the definition of AL, and in
30 methods used for AL calculation. Nevertheless, all the studies indicated that all-cause
31 mortality increased with increasing AL. The study by Borell et al. [37] is the one
32 methodologically most similar to our study and the gradient of 1.88 (95% CI 1.56-2.26) is
33 compatible with the one of 2.37 (95% CI 1.58-3.54) found in our study, and so is the
34 gradient of 2.57 (95% CI 1.59-4.15) found in the National Child Development Study.
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45 For individual biomarkers in our study, HRs were highest for CRP and
46 albumin. CRP is the prototypical acute-phase response protein that increases during
47 systemic inflammation [40], while albumin is a major component of plasma protein,
48 required for transportation and to maintain oncotic pressure, acid–base function,
49 microvascular permeability, and to prevent platelet aggregation [41]. Inflammation
50 increases capillary permeability and thereby escape of serum albumin, leading to
51 expansion of interstitial space and increasing the distribution volume of albumin causing
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4 lower serum albumin concentrations. High level of CRP and low level of albumin have thus
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6 previously been linked with a variety of health outcomes including morbidity and mortality
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8 [7,8,42].
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11 We found a U-shaped association between LDL-c and mortality. Elevated
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13 LDL-c is a well-established risk factor of atherosclerosis and cardiovascular disease, and
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15 the general perception is that high level of LDL-c is associated with an increased risk of
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17 morbidity and mortality [43,44]. Nevertheless, studies on the association between LDL-c
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19 levels and mortality have provided conflicting results. Some studies found increasing level
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21 of LDL-c to be associated with lower mortality [45-46], and some studies found no
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23 association [44,47-48]. However, most studies were conducted in elderly people often with
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25 an intake of lipid-lowering agents. A more recent study in young Koreans found an
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27 association between low level of LDL-c and an increased risk of cancer, cardiovascular, and
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29 all-cause mortality [49]. These findings were supported by a Chinese study of participants
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31 aged 40+ years [50]. A recent Danish study among 108,243 individuals aged 20-100 years
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33 found the lowest all-cause mortality at an LDL-c concentration of 3.6 mmol/L (140
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35 mg/dL), and higher mortality at both lower and higher levels [51]. Our findings for LDL-c
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37 were thus in accordance with these recent observations. Seplaki et al. suggested that both
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39 high and low ends of the risk continuum for the construct of AL could be more informative
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41 than simply using high-risk quartiles. They assigned a value of “1” for values above the 75th
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43 percentile and below the 25th percentile of the distribution, and a value of “0” for
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45 intermediate values [52].
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53 We found both higher and lower levels of DBP to be associated with an
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55 increased mortality, and a similar tendency was indicated for SBP. The association
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57 between lower blood pressure and mortality is still of discussion [53-55]. Most studies have
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4 found this association among elderly people and linked it to chronic disease, e.g.
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6 cardiovascular disease (cardiac failure or ischemic heart disease), cancer, poor functional
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8 status or frailty. Low BP has also been associated with poor function and low quality of life
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10 [56-57], but in previous studies only the highest quartile or the clinical cut-off value have
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12 been used as predictor of all-cause mortality.
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17 Several methods have been used to define an AL composite index, including
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19 the count-based, canonical correlation, z-score, and grade of membership method [58-59].
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21 The most commonly used method is the count-based method, where a summary index is
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23 calculated by summing the number of biomarkers falling within the high-risk category,
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25 either defined by the percentile (i.e., upper or lower 25th percentile of the sample's
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27 distribution) or by the clinical cut-off value. In our analysis with the two-tail cut-off points,
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29 we found HRs for LDL-c of 1.13 (95% CI: 0.85-1.51); for SBP of 1.17 (95%CI: 0.88-1.57; and
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31 for DBP of 1.28 (95% CI: 0.95-1.72). If we have used instead the single high-risk quartile
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33 cut-off point, we would have found HRs for LDL-c of 0.71 (95% CI: 0.49-1.03); for SBP of
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35 0.96 (95% CI: 0.68-1.35) vs), and for DBP of 1.24 (95% CI: 0.86-1.81). The two-tail cut-off
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37 points thus provided a better identification of persons with high mortality than the one-tail
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39 cut-off points.
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45 The issue of whether a clinical or sample-based cut-off criteria should be used
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47 is still of discussion [17], however, studies comparing distinct measurement approaches
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49 have found only modest differences in their predictive utility [15, 60-61].
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51 **Strengths and limitations**

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4 The strengths of our study included the size of the cohort in terms of the large number of
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6 individuals recruited from a general adult population, and the complete follow-up for
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8 death by linkage with the Danish Civil Registration System.
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11 Our study also had some limitations. First, the choice of biomarkers used
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13 to construct the AL index. The AL theory emphasises the importance of measuring
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15 dysregulation across different physiological systems, including biomarkers from the
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17 neuroendocrine, cardiovascular, metabolic, and immune systems [13]. The neuroendocrine
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19 system (stress response) is believed to play a key role in allostasis and subsequent AL, as a
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21 series of physiological changes takes place before initial stress responses occur (such as
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23 rapid increases in blood sugar and blood pressure that supply the body with additional
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25 energy). However, biomarkers from the neuroendocrine system are difficult to measure, as
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27 repeated measurements over 1–2 days are recommended. These requirements cannot be
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29 fulfilled in population studies, where participants are examined only once, and biomarkers
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31 from the neuroendocrine system were therefore not available for our study.
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37 Secondly, the initial stress responses are followed by secondary outcomes
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39 from the metabolic, inflammatory and cardiovascular systems, and these markers were all
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41 available in our data. Nevertheless, greater sensitivity could have been achieved by
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43 studying the dynamic changes over time in these markers to fully capture the flexibility of
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45 stress response mechanisms across the lifespan.
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49 Finally, differences across studies in construction of AL indices could
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51 influence the comparison of results. We used the shape of the association between level of
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53 a given biomarker and all-cause mortality as the basis for the categorization of the
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55 biomarker into low and high values. One can argue therefore that our analysis was circular
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57 in the way that we used outcome on the dependent variable to categorize levels of the
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4 independent variable. We believe that this was justifiable in the context here where the
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6 purpose was to optimize the predictive power of the AL index. However, validation in other
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8 datasets are needed before our approach can be recommended for research in general and
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10 for eventual clinical use.
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13 14 **Conclusion**

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17 Our findings demonstrated that an optimally constructed AL index was a strong predictor
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19 of all-cause mortality. This supported the conceptual validity of AL as an effective marker
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21 of the cumulative physiological burden on the body. These findings can contribute to the
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23 evidence for the use of an AL index as a basis for targeted efforts to bring down continued
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25 stress exposures, and in this way prevent the potential detrimental effect of these
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27 exposures on health. Our findings on the U-shaped association with LDL-c, DBP and SBP
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29 and all-cause mortality suggested that AL measures incorporating risks at both the low and
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31 the high-end of biomarkers may yield the best prediction of all-cause mortality.
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Declarations

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Conflicts of interest

None.

Availability of data and materials and code availability

Data from the study can be made available via Region Sjælland following the Danish Data Protection Regulation.

Author Contributions

All authors contributed significantly to the study. Randi Jepsen provided the LOFUS data. Neda Bruun-Rasmussen, Elsebeth Lyngé and George Napolitano designed the study, interpreted the data, and drafted the manuscript. George Napolitano performed the statistical analysis. Christina Ellervik, Christian Christiansen, Randi Jepsen, Knud Rasmussen and Stig Bojesen contributed to the interpretation and writing of the manuscript. All authors critically revised and approved the final manuscript.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. All data storage and management were approved by the Regional Data Protection Agency of

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4 Zealand (REG-024-2019 & REG-24-2015). LOFUS is registered in Clinicaltrials.gov
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6 (NCT02482896).
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9 Consent to participate
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12 Participants provided written informed consent.
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15 Consent for publication
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18 Not applicable.
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30 study.
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Figure Legends

Figure 1. All-cause mortality by level of allostatic load index, as hazard ratio (95% confidence interval).

Supplementary Figure 1. Hazard ratio for individual biomarkers, centered at median.

Vertical lines denote cut-off values. Upper and lower limits are labelled by U and L, respectively.

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Table 1. Lolland-Falster Health Study (LOFUS). Baseline characteristics of study population and deaths in follow-up period, n(%). For definition of cut-off values, see Supplementary Table 1

Characteristics	Females	Males	Total	Female death	Male death	Total death
Population	7270 (53)	6455 (47)	13725 (100)	78 (39)	120 (61)	198 (100)
Follow-up time, median (IQR)	2.6 (1.6)	2.7 (1.5)	2.6 (1.5)	2.0 (1.6)	1.9 (1.8)	1.9 (1.8)
Median age (IQR)	57.6 (21.9)	59.9 (21.6)	58.7 (22.0)	70.5 (16.4)	74.0 (15.2)	72.8 (16.2)
BMI, kg/m²						
- Underweight	134 (1.8)	42 (0.7)	176 (1.3)	5 (6.4)	Not reported	6 (3.0)
- Normal weight	3038 (41.8)	1862 (28.8)	4900 (35.7)	29 (37.2)	40 (33.3)	69 (34.8)
- Overweight	2335 (32.1)	2940 (45.5)	5275 (38.4)	26 (33.3)	52 (43.3)	78 (39.4)
- Obese	1763 (24.3)	1611 (25.0)	3374 (24.6)	18 (23.1)	27 (22.5)	45 (22.7)
Smoking						
- Never	3586 (49.3)	2737 (42.4)	6323 (46.1)	21 (26.9)	24 (20.0)	45 (22.7)
- Former	2342 (32.2)	2425 (37.6)	4767 (34.7)	32 (41.0)	70 (58.3)	102 (51.5)
- Current	1342 (18.5)	1293 (20.0)	2635 (19.2)	25 (32.1)	26 (21.7)	51 (25.8)
Chronic conditions						
Cardiovascular disease reported	1828 (25.1)	1999 (31.0)	3827 (27.9)	42 (53.8)	60 (50.0)	102 (51.5)
Diabetes reported	264 (3.6)	440 (6.8)	704 (5.1)	9 (11.5)	15 (12.5)	24 (12.1)

Cancer reported	245 (3.4)	275 (4.3)	520 (3.8)	13 (16.7)	24 (20.0)	37 (18.7)
Cardiovascular system						
<i>Systolic blood pressure</i>						
- Low risk	3548 (48.8)	3165 (49.0)	6713 (48.9)	32 (41.0)	52 (43.3)	84 (42.4)
- High risk	3722 (51.2)	3290 (51.0)	7012 (51.1)	46 (59.0)	68 (56.7)	114 (57.6)
<i>Diastolic blood pressure</i>						
- Low risk	3426 (47.1)	3164 (49.0)	6590 (48.0)	26 (33.3)	52 (43.3)	78 (39.4)
- High risk	3844 (52.9)	3291 (51.0)	7135 (52.0)	52 (66.7)	68 (56.7)	120 (60.6)
<i>Pulse rate</i>						
- Low risk	5366 (73.8)	4721 (73.1)	10087 (73.5)	50 (64.1)	81 (67.5)	131 (66.2)
- High risk	1904 (26.2)	1734 (26.9)	3638 (26.5)	28 (35.9)	39 (32.5)	67 (33.8)
<i>AL cardiovascular system score</i>						
- Low	1815 (25.0)	1506 (23.3)	3321 (24.2)	7 (9.0)	16 (13.3)	23 (11.6)
- Mid	2117 (29.1)	2154 (33.4)	4271 (31.1)	27 (34.6)	43 (35.8)	70 (35.4)
- High	3338 (45.9)	2795 (43.3)	6133 (44.7)	44 (56.4)	61 (50.8)	105 (53.0)
Metabolic system						
<i>HDL-c</i>						
- Low risk	4934 (67.9)	4706 (72.9)	9640 (70.2)	46 (59.0)	85 (70.8)	131 (66.2)

- High risk	2336 (32.1)	1749 (27.1)	4085 (29.8)	32 (41.0)	35 (29.2)	67 (33.8)
<i>Triglycerides</i>						
- Low risk	5299 (72.9)	4761 (73.8)	10060 (73.3)	50 (64.1)	95 (79.2)	145 (73.2)
- High risk	1971 (27.1)	1694 (26.2)	3665 (26.7)	28 (35.9)	25 (20.8)	53 (26.8)
<i>HbA1c</i>						
- Low risk	5156 (70.9)	4438 (68.8)	9594 (69.9)	46 (59.0)	69 (57.5)	115 (58.1)
- High risk	2114 (29.1)	2017 (31.2)	4131 (30.1)	32 (41.0)	51 (42.5)	83 (41.9)
<i>Waist-hip ratio</i>						
- Low risk	5452 (75.0)	4831 (74.8)	10283 (74.9)	57 (73.1)	85 (70.8)	142 (71.7)
- High risk	1818 (25.0)	1624 (25.2)	3442 (25.1)	21 (26.9)	35 (29.2)	56 (28.3)
<i>LDL-c</i>						
- Low risk	3459 (47.6)	2989 (46.3)	6448 (47.0)	31 (39.7)	51 (42.5)	82 (41.4)
- High risk	3811 (52.4)	3466 (53.7)	7277 (53.0)	47 (60.3)	69 (57.5)	116 (58.6)
<i>AL metabolic system score</i>						
- Low	1401 (19.3)	1249 (19.3)	2650 (19.3)	11 (14.1)	18 (15.0)	29 (14.6)
- Mid	2413 (33.2)	2135 (33.1)	4548 (33.1)	18 (23.1)	37 (30.8)	55 (27.8)
- High	3456 (47.5)	3071 (47.6)	6527 (47.6)	49 (62.8)	65 (54.2)	114 (57.6)
Inflammation system						

<i>CRP</i>						
- Low risk	5451 (75.0)	4837 (74.9)	10288 (75.0)	51 (65.4)	73 (60.8)	124 (62.6)
- High risk	1819 (25.0)	1618 (25.1)	3437 (25.0)	27 (34.6)	47 (39.2)	74 (37.4)
<i>Albumin</i>						
- Low risk	4953 (68.1)	4655 (72.1)	9608 (70.0)	49 (62.8)	54 (45.0)	103 (52.0)
- High risk	2317 (31.9)	1800 (27.9)	4117 (30.0)	29 (37.2)	66 (55.0)	95 (48.0)
<i>AL inflammation system score</i>						
- Low	4027 (55.4)	3692 (57.2)	7719 (56.2)	41 (52.6)	42 (35.0)	83 (41.9)
- Mid	2350 (32.3)	2108 (32.7)	4458 (32.5)	18 (23.1)	43 (35.8)	61 (30.8)
- High	893 (12.3)	655 (10.1)	1548 (11.3)	19 (24.4)	35 (29.2)	54 (27.3)
Total AL index						
- Low	2306 (31.7)	2112 (32.7)	4418 (32.2)	14 (17.9)	24 (20.0)	38 (19.2)
- Mid	2882 (39.6)	2599 (40.3)	5481 (39.9)	26 (33.3)	45 (37.5)	71 (35.9)
- High	2082 (28.6)	1744 (27.0)	3826 (27.9)	38 (48.7)	51 (42.5)	89 (44.9)

Table 2. Multivariate Cox proportional hazard regression of all-cause mortality for LOFUS participants by individual biomarkers

Variable	Non exposed	Exposed	Hazard ratio (95 % CI) Model 1*	Hazard ratio (95 % CI) Model 2**
HDL cholesterol, mg/dl	High	Low	1.22 (0.88-1.69)	1.24 (0.89-1.73)
LDL cholesterol, mg/dl	Mid	High and low	1.22 (0.91-1.62)	1.19 (0.85-1.51)
Triglycerides, mg/dl	Low	High	0.93 (0.66-1.32)	0.94 (0.67-1.33)
Albumin, g/dl	High	Low	1.55 (1.17-2.07)	1.54 (1.16-2.06)
CRP, mg/L	Low	High	1.42 (1.05-1.92)	1.41 (1.04-1.91)
HbA1c, mmol/mol	Low	High	1.25 (0.93-1.68)	1.24 (0.90-1.71)
Systolic blood pressure, mmHg	Mid	High and low	1.20 (0.90-1.61)	1.17 (0.88-1.57)
Diastolic blood pressure, mmHg	Mid	High and low	1.31 (0.98-1.76)	1.29 (0.95-1.72)
Pulse rate, PM	High	Low	1.34 (0.99-1.81)	1.29 (0.91-1.66)
Waist-hip ratio	Low	High	1.02 (0.74-1.41)	1.08 (0.76-1.52)

*Adjusted for age and sex

** Additionally adjusted for BMI, reported diseases, and smoking status

Table 3. Multivariate Cox proportional hazard regression of all-cause mortality for 607136 participants by allostatic load index

Variable	Reference	Level	Hazard ratio (95 % CI)	
			Model 1*	Model 2**
Allostatic load index	Low	Mid	1.39 (0.94 – 2.06)	1.33 (0.89 – 1.98)
		High	2.45 (1.68 – 3.59)	2.37 (1.58 – 3.54)
Continuous allostatic load measure			1.23 (1.14 – 1.32)	1.22 (1.13 – 1.32)
Inflammatory system score	Low	Mid	1.03 (0.74-1.44)	1.02 (0.73-1.42)
		High	2.39 (1.69-3.38)	2.38 (1.67-3.39)
Metabolic system score	Low	Mid	1.19 (0.76-1.86)	1.18 (0.75-1.85)
		High	1.54 (1.02-2.33)	1.54 (1.00-2.38)
Cardiovascular system score	Low	Mid	1.73 (1.08-1.78)	1.65 (1.02-2.65)
		High	2.06 (1.31-3.24)	1.89 (1.20-2.99)

*Adjusted for age and sex

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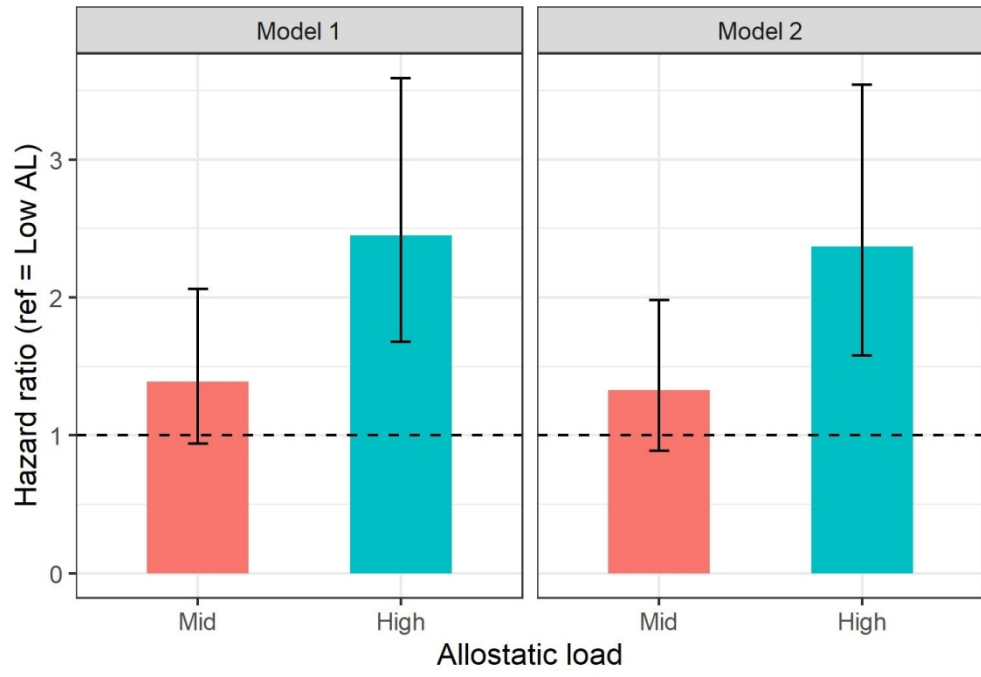
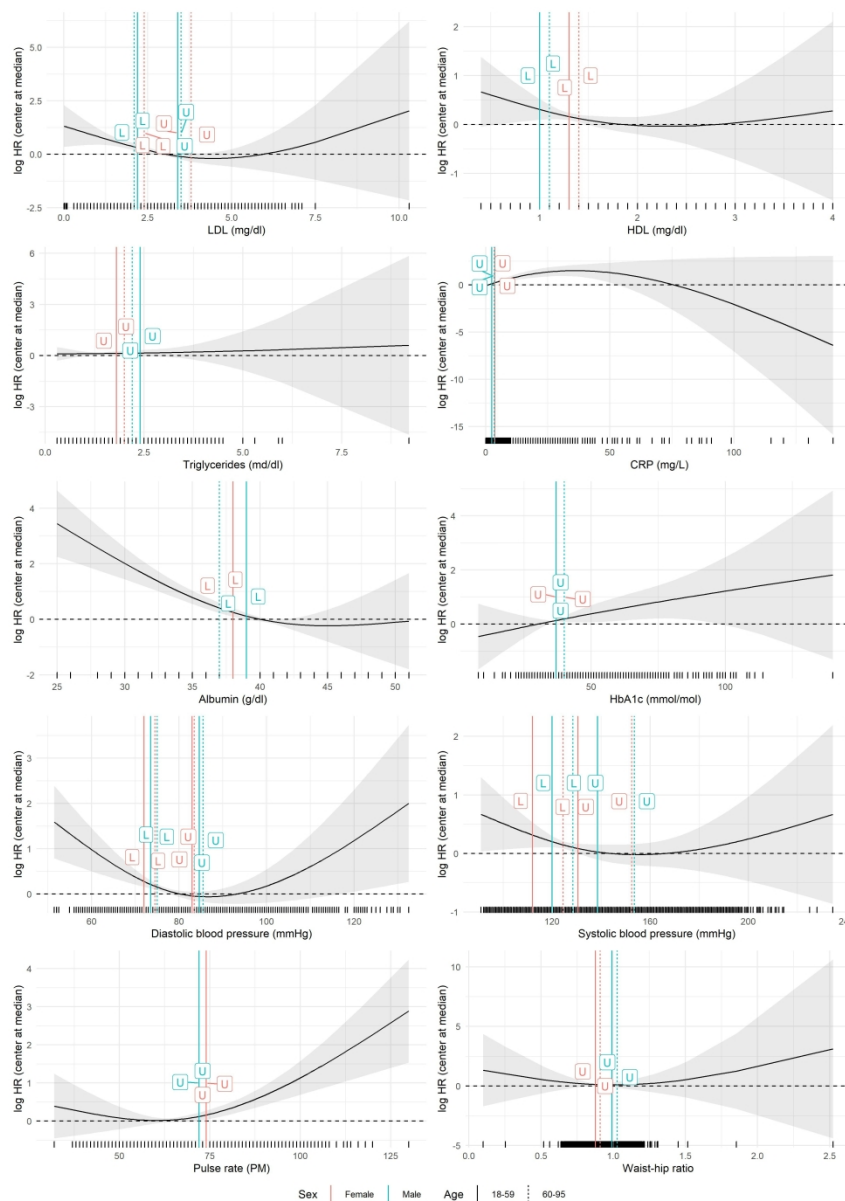


Figure 1. All-cause mortality by level of allostatic load index, as hazard ratio (95% confidence interval).

129x89mm (300 x 300 DPI)

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Supplementary Figure 1. Hazard ratio for individual biomarkers, centered at median. Vertical lines denote cut-off values. Upper and lower limits are labelled by U and L, respectively.

299x424mm (300 x 300 DPI)

Supplementary Table 1. Descriptive statistics and high-risk cut points for individual biomarkers

Characteristics	ALL	Males			Females		
	N = 13,725	N = 6455			N = 7270		
	Median (IQR)	Median (IQR)	High-risk cut point <60 years	High-risk cut point ≥60 years	Median (IQR)	High-risk cut point <60 years	High-risk cut point ≥60 years
HDL cholesterol, mg/dl	1.4 (0.5)	1.2 (0.5)	≤1.0	≤1.1	1.5 (0.5)	≤1.3	≤1.4
LDL cholesterol, mg/dl	2.8 (1.2)	2.8 (1.2)	≤2.2 or ≥3.4	≤2.1 or ≥3.5	2.8 (1.2)	≤2.2 or ≥3.4	≤2.4 or ≥3.8
Triglycerides, mg/dl	1.5 (1.1)	1.6 (1.3)	≥2.4	≥2.2	1.2 (0.9)	≥1.8	≥2.0
Albumin, g/dl	40.0 (3.0)	41.0 (4.0)	≤39.0	≤37.0	40.0 (3.0)	≤38.0	≤37.0
CRP, mg/L	1.40 (2.53)	1.06 (1.83)	≥2.37	≥3.42	1.40 (2.96)	≥3.59	≥3.53
HbA1c, mmol/mol	36.0 (5.0)	35.0 (4.0)	≥37.0	≥40.0	35.0 (5.0)	≥37.0	≥40.0
Systolic blood pressure, mmHg	130.0 (26.5)	127.5 (18.5)	≤120.0 or ≥138.5	≤128.5 or ≥153.5	119.5 (18.5)	≤112.0 or ≥130.5	≤124.5 or ≥152.5

Diastolic blood pressure, mmHg	78.5 (10.5)	79 (11.1)	≤73.5 or ≥84.6	≤75.0 or ≥85.5	77.0 (11.0)	≤72.0 or ≥83.0	≤74.5 or ≥83.5
Pulse rate, PM	66.0 (13.0)	65.0 (14.0)	≥72.0	≥72.0	67.0 (13.0)	≥74.0	≥74.0
Waist hip ratio	0.90 (0.14)	0.94 (0.10)	≥1.0	≥1.0	0.83 (0.10)	≥0.9	≥0.9

For peer review only

Supplementary Table 2. Number and proportion of missing values in analysis sample

Variable	Missing values	Proportion of total analysis sample (N= 15,714)
Biomarkers		
Date of biomarker sample	39	0.25%
C-Reactive Protein	171	1.08%
Albumin	168	1.07%
HDL cholesterol	168	1.07%
LDL Cholesterol	635	4.04%
Triglycerides	169	1.07%
HbA1c	207	1.32%
Waist-hip ratio	139	0.88%
Diastolic Blood Pressure	21	0.13%
Systolic Blood Pressure	21	0.13%
Pulse Rate	69	0.44%
BMI	292	1.86%
Smoking	867	5.52%
Chronic conditions	810	5.15%

Supplementary Table 3. Number of LoD values replaced in each variable

Biomarker	# LoD replaced	LoD
Haemoglobin A1c	1	<31
C-reactive protein	242	<0.16
Alanine aminotransferase	1	<6
Bilirubin	2	<2.0
Low-density lipoprotein	3	<0.10

Reporting checklist for cohort study.

Based on the STROBE cohort guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

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		Reporting Item	Page Number
Title and abstract			
Title	#1a	Indicate the study's design with a commonly used term in the title or the abstract	1
Abstract	#1b	Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background / rationale	#2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	#3	State specific objectives, including any prespecified hypotheses	5
Methods			

1	Study design	#4	Present key elements of study design early in the paper	5
2				
3	Setting	#5	Describe the setting, locations, and relevant dates,	5
4			including periods of recruitment, exposure, follow-up, and	
5			data collection	
6				
7				
8	Eligibility criteria	#6a	Give the eligibility criteria, and the sources and methods	5
9			of selection of participants. Describe methods of follow-up.	
10				
11	Eligibility criteria	#6b	For matched studies, give matching criteria and number of	N/A
12			exposed and unexposed	
13				
14				
15				
16	Variables	#7	Clearly define all outcomes, exposures, predictors,	6,7,8
17			potential confounders, and effect modifiers. Give	
18			diagnostic criteria, if applicable	
19				
20				
21	Data sources /	#8	For each variable of interest give sources of data and	6,7,8
22	measurement		details of methods of assessment (measurement).	
23			Describe comparability of assessment methods if there is	
24			more than one group. Give information separately for for	
25			exposed and unexposed groups if applicable.	
26				
27				
28				
29				
30	Bias	#9	Describe any efforts to address potential sources of bias	15,16
31				
32	Study size	#10	Explain how the study size was arrived at	5
33				
34	Quantitative	#11	Explain how quantitative variables were handled in the	N/A
35	variables		analyses. If applicable, describe which groupings were	
36			chosen, and why	
37				
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39				
40	Statistical	#12a	Describe all statistical methods, including those used to	
41	methods		control for confounding	
42				
43				
44	8,9			
45				
46	Statistical	#12b	Describe any methods used to examine subgroups and	8
47	methods		interactions	
48				
49				
50	Statistical	#12c	Explain how missing data were addressed	8
51	methods			
52				
53				
54	Statistical	#12d	If applicable, explain how loss to follow-up was addressed	N/A
55	methods			
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1	Statistical	#12e	Describe any sensitivity analyses	
2	methods			
3				
4				
5	N/A			
6				
7	Results			
8				
9	Participants	#13a	Report numbers of individuals at each stage of study—eg	9
10			numbers potentially eligible, examined for eligibility,	
11			confirmed eligible, included in the study, completing	
12			follow-up, and analysed. Give information separately for	
13			for exposed and unexposed groups if applicable.	
14				
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16				
17	Participants	#13b	Give reasons for non-participation at each stage	N/A
18				
19	Participants	#13c	Consider use of a flow diagram	
20				
21				
22	N/A			
23				
24	Descriptive data	#14a	Give characteristics of study participants (eg	9,28
25			demographic, clinical, social) and information on	
26			exposures and potential confounders. Give information	
27			separately for exposed and unexposed groups if	
28			applicable.	
29				
30				
31				
32	Descriptive data	#14b	Indicate number of participants with missing data for each	
33			variable of interest	
34				
35	Supplementary			
36	table 2			
37				
38	Descriptive data	#14c	Summarise follow-up time (eg, average and total amount)	
39				
40				
41				
42	9			
43				
44	Outcome data	#15	Report numbers of outcome events or summary measures	
45			over time. Give information separately for exposed and	
46			unexposed groups if applicable.	
47				
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49				
50	NA			
51				
52				
53	Main results	#16a	Give unadjusted estimates and, if applicable, confounder-	10,12
54			adjusted estimates and their precision (eg, 95%	
55			confidence interval). Make clear which confounders were	
56			adjusted for and why they were included	
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1	Main results	#16b	Report category boundaries when continuous variables were categorized	NA
2				
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4	Main results	#16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
5				
6				
7				
8	NA			
9				
10				
11	Other analyses	#17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	9
12				
13				
14				
15	Discussion			
16				
17	Key results	#18	Summarise key results with reference to study objectives	9,10
18				
19	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.	14
20				
21				
22				
23				
24	Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	11,12,13,14
25				
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29				
30	Generalisability	#21	Discuss the generalisability (external validity) of the study results	15
31				
32				
33				
34	Other			
35	Information			
36				
37				
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	17
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43				

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BMJ Open

Allostatic load as predictor of mortality. A cohort study from Lolland-Falster, Denmark

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Nykøbing Falster Hospital,

5th May 2022

Allostatic load as predictor of mortality. A cohort study from Lolland-Falster, Denmark

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Abstract

Objectives: The purposes of the present study were to determine the association between 1) 10 individual biomarkers and all-cause mortality; and between 2) allostatic load (AL), across three physiological systems (cardiovascular, inflammatory, metabolic), and all-cause mortality. **Design:** Prospective cohort study. **Setting:** We used data from the Lolland-Falster Health Study undertaken in Denmark in 2016-2020 and used data on systolic (SBP) and diastolic blood pressure (DBP), pulse rate (PR), waist-hip ratio (WHR), and levels of low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), triglycerides, glycated haemoglobin (HbA1c), c-reactive protein (CRP), and serum albumin. All biomarkers were divided into quartiles with high-risk values defined as those in the highest (PR, WHR, triglycerides, HbA1c, CRP) or lowest (HDL-c, albumin) quartile, or a combination hereof (LDL-c, SBP, DBP). The ten biomarkers were combined into a summary measure of AL index. Participants were followed up for death for an average of 2.6 years. **Participants:** We examined a total of 13,725 individuals aged 18+ years. **Primary outcome measure:** Cox proportional hazard regression (HR) analysis were performed to examine the association between AL index and mortality in men and women. **Results:** All-cause mortality increased with increasing AL index. With low AL index as reference, the HR was 1.33 (95% confidence interval (CI) 0.89-1.97) for mid AL, and HR 2.37 (95% CI: 1.58-3.54) for high AL. **Conclusions:** Elevated physiological burden measured by mid and high AL index was associated with a steeper increase of mortality than individual biomarkers.

Abstract word count: 240

Keywords: Biomarker, Allostatic Load, Blood, Mortality, population-based, LOFUS.

Manuscript word count: 3982

Article Summary

Strengths and limitations

- Analysis based on a large population-based health study.
- Complete follow-up for death via linkage with Danish Civil Registration System.
- Biomarkers from only one point in time.
- No biomarker from neuroendocrine system available.

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Introduction

Biological markers (biomarkers) were originally defined as “cellular, biochemical or molecular alterations that are measurable in biological media such as cells, human tissues or fluids” [1]. Later the definition was extended to include “indicators of normal biological processes, pathogenic processes and pharmacological responses to therapeutic interventions” [2]. In clinical settings, measurement of biomarkers in blood samples is used to detect and diagnose medical conditions. Biomarkers as independent predictors of all-cause mortality are therefore of considerable clinical and research interest [3]; dyslipidaemia including high levels of triglycerides and low-density lipoprotein cholesterol (LDL-c), and low levels of high-density lipoprotein cholesterol (HDL-c), have been reported to be independent risk factors for all-cause mortality [4-6]. Lower levels of albumin [7] and higher levels of C-reactive protein (CRP) [8], and haemoglobin A1c (HbA1c) [9] have likewise been linked to mortality. Also, there is some evidence that the relationship between some of these biomarkers and all-cause mortality varies across sex and age-groups [10,11].

The concept of allostatic load (AL) refers to the “wear and tear” of the body resulting from repeated stimulation of stress responses via the hypothalamic–pituitary–adrenal axis and the sympathetic-adrenal-medullary system [12]. As a latent variable, AL cannot be directly measured but it can be estimated using an AL index, which is composite of biomarkers from multiple organ systems integrated into a single score. The first AL developed by Seeman et al. in 1997 included 10 biomarkers monitoring various physiological systems [13]. However, the type and number of biomarkers used in published studies have ranged from 6 to 24 [14]. The most frequently used Al construct, originally proposed by Gruenewald et al in 2012 [15], includes 24 biomarkers. It has been suggested

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4 that in the calculation of AL, the threshold of risk for each biomarker should be obtained
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6 by the quartiles or quintiles of the values of the biomarker [16]. AL has been reported to be
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8 a better predictor of mortality than individual biomarkers, however, there are still gaps in
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10 the understanding of the associations [17,18]. AL has been suggested also as a tool for
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12 allocation of nursing resources [19].
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16 This study provides data from the Lolland-Falster Health Study (LOFUS) [20],
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18 a population-based survey undertaken in 2016-2020 in Lolland-Falster, a rural-provincial
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20 region in Denmark with a life expectancy much below the national average [21], and with
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22 health problems reported more frequently than in the rest of the country [22]. Using the
23
24 LOFUS data, the purposes of the present study were 1) to determine the association
25
26 between 10 individual biomarkers and all-cause mortality; and 2) to examine the
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28 association between AL, across three physiological systems (cardiovascular, inflammatory,
29
30 metabolic system), and all-cause mortality. The hypothesis is that AL can be used as an
31
32 informative tool in predicting future risk of death in the general adult population.
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37 **Methods**

38 Study population

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41 We undertook a prospective cohort study of participants from LOFUS; a household-based
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43 population study with data collected between February 2016 and February 2020. Persons
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45 aged 18 years and above were randomly sampled from the Danish Civil Registration
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47 System and invited to participate together with the rest of their households. Participation
48
49 required informed consent. The study was approved by Region Zealand's Ethical
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51 Committee on Health Research (Reg: SJ-421). A detailed description of the study protocol
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53 [20] and information on the socio-economic determinants of participation [23] have been
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4 published previously. Persons below 18 years, and pregnant women were excluded from
5
6 the present study.
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8 9 Patient and Public Involvement

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12 Patients were not actively involved in any stage of the present study. Once the paper has
13
14 been published in the international literature, the key results will be reported also in the
15
16 local press.
17

18 19 Self-reported data

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22 From questionnaires, we used data on smoking (never, former, current), and presence of
23
24 chronic conditions (cardiovascular disease, diabetes, cancer) at the time of participation in
25
26 LOFUS.
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28 29 Biomarkers

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31
32 Non-fasting blood samples were collected in vacutainer blood collection tubes (Becton,
33
34 Dickinson and Company; Franklin Lakes, NJ, USA) and kept at room temperature until
35
36 same day analysis at the Department of Clinical Biochemistry at Nykøbing Falster
37
38 Hospital, accredited by the standard ISO 15189. We used data on HDL-c, LDL-c,
39
40 triglycerides, albumin, CRP, and HbA1c. LDL-c was calculated by using Friedewald
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42 formula [24] when the plasma triglyceride concentration was below 4.5 mmol/L. Systolic
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44 (SBP) and diastolic (DBP) blood pressure were based on three consecutive digital
45
46 measurements on the upper left arm (apparatus type Welch Allyn Connex pro BPO 3400).
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48 The mean values of the second and third measurement were used in this study (only one
49
50 measurement was used if the other was missing). Waist-hip ratio (WHR) was calculated by
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52 waist-circumference divided by hip-circumference. Body mass index (BMI) was defined as
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54 weight in kilograms divided by height in meters squared (kg/m²).
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4 In the calculation of AL, biomarkers are most often dichotomized into low and
5
6 high values based on either a percentile or a predetermined cut-off value [16]. However,
7
8 before doing so, we mapped for each biomarker the association between level of the
9
10 marker and all-cause mortality, see method below. For most biomarkers the association
11
12 was monotonic, see Supplementary Figure 1. These biomarkers were then dichotomized
13
14 according to the sex- and age-specific quartiles. For age, we dichotomized at age 60. Some
15
16 previous studies focused on AL in people aged 60 and above [25,26] and we intuitively
17
18 found it reasonable to distinguish in the same way between “young” and “old” people in
19
20 our data; age 60 was furthermore the median age of our study population; and with this
21
22 age-dichotomization we avoided violations of the model assumption in the statistical
23
24 analysis. We dichotomized biomarkers with high-risk values defined as those in the highest
25
26 quartile of the sex- and age-specific distribution, except for HDL-c, and albumin, where the
27
28 lowest quartile was the high-risk value. For LDL-c, SBP and DBP the associations were U-
29
30 shaped, and the high-risk values for these biomarkers were therefore defined as including
31
32 both the lower and the upper quartiles, see Supplementary Table 1. For biomarkers with U-
33
34 shaped associations, we tested out also using octiles as cut-off points. However, this
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36 resulted in some violations of the model assumptions in the statistical analysis, and for
37
38 SBP the upper octile cut-off was from a clinical point of view very high. On this basis we
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40 used the quartile cut-offs also for the biomarkers with the U-shaped association. For all
41
42 biomarkers, the highest and lowest quartile of risk scores were either lower or similar to
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44 clinical cut-points [27-31].
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52 BMI was divided into underweight (BMI less than 18.5) normal (BMI 18.5–
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54 24.9), overweight (BMI 25.0–29.9), or obese (BMI 30.0 or greater); reported diseases into
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56 either present or not; and smoking status into never, former, or current.
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Allostatic load scores

The AL scores were computed using biomarkers from: the cardiovascular system (SBP, DBP, and pulse rate (PR)); the metabolic system (LDL-c, triglycerides, HDL-c, WHR, HbA1c); and the inflammatory system (CRP, serum albumin).

Each system-specific AL score was then defined as the number of biomarkers with a high-risk value, hence as an integer value between 0 and 3 for the cardiovascular system (CVS), 0 and 5 for the metabolic system (MS), and 0 and 2 for inflammatory system (IS). The AL index was defined as the sum of all scores and divided in three groups based on tertiles contrasting individuals with [AL:0-2], mid [AL: 3-4], and high [AL: 5-10]. Note that, all biomarkers were given equal weight in accordance with previous studies [16,18].

All-cause mortality

LOFUS participants were followed up for death with data obtained from the Danish Civil Registration System on 26 February 2021.

Data management and statistical analyses

Observations with missing values in any of the variables were excluded from the analyses (1989 out of 15714, i.e. 12.6%, see Supplementary Table 2). Values below the lower limit of detection were replaced with random numbers sampled with replacement from the set $\{k \times 10^{(-n)}, k = 1, \dots, L\}$, where n is the variable-specific number of decimals reported in the data and $L \times 10^{(-n)}$ the limit of detection, see Supplementary Table 3.

Participants were followed up from date of participation in the LOFUS study until date of death or end of follow-up on 26 February 2021, whichever came first. In order to define the biomarkers' high-risk values, we first studied the association between levels of each individual biomarker and mortality, allowing for possible nonlinear relations. This

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4 analysis was carried out via Cox proportional-hazard models with biomarker levels as
5
6 continuous covariates, modelled with natural cubic splines with 2 degrees of freedom
7
8 (except for LDL-c, where 3 degrees of freedom were used), and further adjusting for sex
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10 and age. By graphical inspection, a U-shaped association was found for LDL-c, SBP and
11
12 DBP (see Supplementary Figure 1). Therefore, for these biomarkers both the sex and age-
13
14 specific (i.e. below or above age 60) lower and upper quartiles were defined as high-risk,
15
16 while only one quartile for the others (upper or lower, in accordance with the existing
17
18 literature); see Supplementary Table 1.
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23 Associations between all-cause mortality and dichotomized biomarkers levels
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25 (low/high risk), system-specific AL scores, and total AL index, were modelled with Cox
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27 proportional-hazard models. Here, we present two models: Model 1, where HRs are
28
29 adjusted for sex and age; Model 2, where results are further adjusted for BMI, prevalent
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31 diseases, and smoking status. HRs for the individual biomarkers (Table 2) and for system-
32
33 specific AL scores (Table 3) are mutually adjusted. Proportional hazards assumptions in
34
35 the above models have been tested using Schoenfeld residuals. Numbers below 5 are not
36
37 reported. In addition, we report HRs for a one-point increase in the AL index.
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42 Data management, statistical analyses and plots were done in R ver. 4.0.3
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44 [32], with packages splines [32], survival [33], tidyverse [34], ggrepel [35] and ggpubr
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46 [36].
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49 Results

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52 The LOFUS database used for this study included 13,725 persons, of whom 53% were
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54 women and 47% men. The median follow-up time was 2.6 years (IQR 1.5) and the median
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56 age was 57.6 in women and 59.9 in men. One-fourth of the participants were obese, and
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4 one-fifth were current smokers. Presence of cardiovascular disease at the time of LOFUS
5 participation was reported by 28%, diabetes by 5%, and cancer by 4%. On the value of total
6 AL index, participants were divided approximately into tertiles; 32% low, 40% mid, and
7 38% high. During the follow-up period, 198 participants died; of these 39% were women
8 and 61% men (Table 1).
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16 The multivariate Cox proportional hazard regression for individual biomarker
17 and all-cause mortality, adjusted for sex and age and additionally for BMI, reported
18 diseases, and smoking, are listed in Table 2. For all biomarkers, apart from triglycerides, a
19 high risk value was associated with an increased mortality level. However, only the HRs for
20 low albumin and high CRP were statistically significantly elevated; HR 1.54 (95% CI: 1.16-
21 2.06) and 1.41 (95% CI: 1.04-1.91), respectively.
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30 The HR for all-cause mortality increased with increasing level of the AL from
31 low as the reference over mid to high, Table 3 and Figure 1. For the inflammatory system
32 AL score, the HR was 1.02 (95% CI: 0.73-1.42) for mid AL, and 2.38 (95% CI: 1.67-3.39) for
33 high AL. For the metabolic system AL score, the HRs were 1.18 (95% CI 0.75-1.85) and 1.54
34 (95% CI: 1.00-2.38), respectively. For the cardiovascular system AL score, the HRs were
35 1.65 (95% CI 1.02-2.65) and 1.89 (95% CI: 1.20-2.99), respectively. The gradient for the
36 total AL index was a HR of 1.33 (95% CI: 0.89-1.98) for mid AL, and 2.37 (95% CI: 1.58-
37 3.54) for high AL. HRs for 1 unit increase in AL (continuous AL) was 1.23 (1.14 – 1.32)
38 when adjusted for age and sex, and 1.22 (1.13 – 1.32), when additionally adjusted for BMI,
39 reported diseases, and smoking status.
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54 Discussion

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4 In this population-based study from a rural-provincial area of Denmark, we followed the
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6 adult population up for a median period of 2.6 years. High levels of individual biomarkers
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8 were overall associated with increased mortality, but most of them at a modest level of 20-
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10 30%, and statistically significantly elevated for only CRP and albumin. High levels of
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12 physiologic system-specific AL scores were associated with increased mortality at the level
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14 of 50-140%; statistically significantly for the inflammatory and cardiovascular systems,
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16 and at borderline of significance for the metabolic system. The composite measure of total
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18 AL index was a strong predictor of all-cause mortality. Persons with a high vs. low total AL
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20 index had about 2.5 times the mortality. The total AL index was thus a better predictor of
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22 all-cause mortality than individual biomarkers and the metabolic and cardiovascular
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24 systems AL scores, a pattern consistent with previous studies [16,18,37].
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30 The most comprehensive studies on AL and mortality all used data from the
31
32 National Health and Nutrition Examination Survey (NHANES). Borrell et al. [38]
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34 examined twelve-year mortality by using data from 13,715 adults aged 25+ years of whom
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36 2491 had died. They calculated AL based on 9 biomarkers; albumin, CRP, total cholesterol,
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38 HDL-c, haemoglobin A1c, waist-to-hip-ratio, SBP, DBP, and PR. Using a clinical cut-off AL
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40 score, they found that, compared to persons with an AL score of ≤ 1 , those with AL scores of
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42 2 and 3+ had adjusted HRs of 1.40 (95% CI 1.11-1.76) and 1.88 (95% CI 1.56-2.26),
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44 respectively.
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49 Levine and Crimmins [39] examined ten-year all-cause and disease-specific
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51 mortality. In total, 15,042 persons were eligible, but biomarker data were available for only
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53 9942 adults aged 30+, of whom 1076 had died. They included data on albumin, CRP,
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55 waist-to-hip ratio, total cholesterol, HDL-c, haemoglobin A1c, PR, SBP, and DBP. For each
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57 of the nine biomarkers, a person was classified as high or low based on clinical cut-off
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4 points, and the AL score was the number of biomarkers classified as high. In addition, an
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6 expanded AL score included five additional biomarkers defined by quintiles; and a
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8 continuous AL score used a continuous z-score measure for all fourteen biomarkers. For
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10 the first AL score, a HR of 2.75 ($p < .001$) was found for all-cause mortality when persons
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12 with the highest quintile of AL were compared with those with the lowest. Somewhat
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14 stronger gradients were found for the expanded; 3.62 ($p < .0001$) and continuous; 6.97
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16 ($p < .0001$), ALs.
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21 Howard and Sparks [40] studied 11,733 participants from NHANES.
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23 Imputation was used to estimate missing values. Their AL measure was based on DBP,
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25 SBP, PR, total cholesterol, HDL-c, triglycerides, haemoglobin A1c, BMI, albumin and CRP.
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27 They found that a one-unit increase in AL represented a 7% increase in risk of death when
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29 adjusted for age, sex, ethnicity, socioeconomic status, and health behaviour.
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34 The National Child Development Study was followed up for deaths from birth
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36 in 1958 to 1 December 2013, i.e. to the age of 55 years [18]. AL based on 10 biomarkers was
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38 calculated and divided into three levels. All-cause mortality for persons with mid or high
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40 AL was compared with that of persons with low AL, and adjusted for early life, childhood,
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42 young and adulthood confounders. The HR of death was 1.71 (95% CI 1.07-2.72) for
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44 persons with mid AL, and 2.57 (95% CI 1.59-4.15) for those with high AL. The association
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46 between AL and all-cause mortality was stronger than the associations between of the
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48 individual 10 biomarkers and all-cause mortality.
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52 The NHANES studies vary in number of participants included in the studies,
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54 in length of follow-up for mortality, in biomarkers included, in the definition of AL, and in
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56 methods used for AL calculation. Nevertheless, all the studies indicated that all-cause
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58 mortality increased with increasing AL. The study by Borell et al. [38] is the one
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4 methodologically most similar to our study and the gradient of 1.88 (95% CI 1.56-2.26) is
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6 compatible with the one of 2.37 (95% CI 1.58-3.54) found in our study, and so is the
7
8 gradient of 2.57 (95% CI 1.59-4.15) found in the National Child Development Study.
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12 For individual biomarkers in our study, HRs were highest for CRP and
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14 albumin. CRP is the prototypical acute-phase response protein that increases during
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16 systemic inflammation [41], while albumin is a major component of plasma protein,
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18 required for transportation and to maintain oncotic pressure, acid–base function,
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20 microvascular permeability, and to prevent platelet aggregation [42]. Inflammation
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22 increases capillary permeability and thereby escape of serum albumin, leading to
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24 expansion of interstitial space and increasing the distribution volume of albumin causing
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26 lower serum albumin concentrations. High level of CRP and low level of albumin have thus
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28 previously been linked with a variety of health outcomes including morbidity and mortality
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30 [7,8,43].
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36 We found a U-shaped association between LDL-c and mortality. Elevated
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38 LDL-c is a well-established risk factor of atherosclerosis and cardiovascular disease, and
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40 the general perception is that high level of LDL-c is associated with an increased risk of
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42 morbidity and mortality [44,45]. Nevertheless, studies on the association between LDL-c
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44 levels and mortality have provided conflicting results. Some studies found increasing level
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46 of LDL-c to be associated with lower mortality [46,47], and some studies found no
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48 association [45,48-49]. However, most studies were conducted in elderly people often with
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50 an intake of lipid-lowering agents. A more recent study in young Koreans found an
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52 association between low level of LDL-c and an increased risk of cancer, cardiovascular, and
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54 all-cause mortality [50]. These findings were supported by a Chinese study of participants
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56 aged 40+ years [51]. A recent Danish study among 108,243 individuals aged 20-100 years
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4 found the lowest all-cause mortality at an LDL-c concentration of 3.6 mmol/L (140
5 mg/dL), and higher mortality at both lower and higher levels [52]. Our findings for LDL-c
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7 were thus in accordance with these recent observations. Seplaki et al. suggested that both
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9 high and low ends of the risk continuum for the construct of AL could be more informative
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11 than simply using high-risk quartiles. They assigned a value of “1” for values above the 75th
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13 percentile and below the 25th percentile of the distribution, and a value of “0” for
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15 intermediate values [53].
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21 We found both higher and lower levels of DBP to be associated with an
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23 increased mortality, and a similar tendency was indicated for SBP. The association
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25 between lower blood pressure and mortality is still of discussion [54-56]. Most studies
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27 have found this association among elderly people and linked it to chronic disease, e.g.
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29 cardiovascular disease (cardiac failure or ischemic heart disease), cancer, poor functional
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31 status or frailty. Low BP has also been associated with poor function and low quality of life
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33 [57,58], but in previous studies only the highest quartile or the clinical cut-off value have
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35 been used as predictor of all-cause mortality.
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41 Several methods have been used to define an AL composite index, including
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43 the count-based, canonical correlation, z-score, and grade of membership method [59,60].
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45 The most commonly used method is the count-based method, where a summary index is
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47 calculated by summing the number of biomarkers falling within the high-risk category,
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49 either defined by the percentile (i.e., upper or lower 25th percentile of the sample's
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51 distribution) or by the clinical cut-off value. In our analysis with the two-tail cut-off points,
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53 we found HRs for LDL-c of 1.13 (95% CI: 0.85-1.51); for SBP of 1.17 (95%CI: 0.88-1.57; and
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55 for DBP of 1.28 (95% CI: 0.95-1.72). If we have used instead the single high-risk quartile
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57 cut-off point, we would have found HRs for LDL-c of 0.71 (95% CI: 0.49-1.03); for SBP of
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4 0.96 (95% CI: 0.68-1.35) vs), and for DBP of 1.24 (95% CI: 0.86-1.81). The two-tail cut-off
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6 points thus provided a better identification of persons with high mortality than the one-tail
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8 cut-off points.
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11 The issue of whether a clinical or sample-based cut-off criteria should be used
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13 is still of discussion [17], however, studies comparing distinct measurement approaches
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15 have found only modest differences in their predictive utility [15, 61-62].
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18 19 **Strengths and limitations**

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22 The strengths of our study included the size of the cohort in terms of the large number of
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24 individuals recruited from a general adult population, and the complete follow-up for
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26 death by linkage with the Danish Civil Registration System.
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30 Our study also had some limitations. First, the choice of biomarkers used
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32 to construct the AL index. The AL theory emphasises the importance of measuring
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34 dysregulation across different physiological systems, including biomarkers from the
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36 neuroendocrine, cardiovascular, metabolic, and immune systems [13]. The neuroendocrine
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38 system (stress response) is believed to play a key role in allostasis and subsequent AL, as a
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40 series of physiological changes takes place before initial stress responses occur (such as
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42 rapid increases in blood sugar and blood pressure that supply the body with additional
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44 energy). However, biomarkers from the neuroendocrine system are difficult to measure, as
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46 repeated measurements over 1–2 days are recommended. These requirements cannot be
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48 fulfilled in population studies, where participants are examined only once, and biomarkers
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50 from the neuroendocrine system were therefore not available for our study.
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55 Secondly, the initial stress responses are followed by secondary outcomes
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57 from the metabolic, inflammatory and cardiovascular systems, and these markers were all
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4 available in our data. Nevertheless, greater sensitivity could have been achieved by
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6 studying the dynamic changes over time in these markers to fully capture the flexibility of
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8 stress response mechanisms across the lifespan.
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11 Finally, differences across studies in construction of AL indices could
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13 influence the comparison of results. We used the shape of the association between level of
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15 a given biomarker and all-cause mortality as the basis for the categorization of the
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17 biomarker into low and high values. One can argue therefore that our analysis was circular
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19 in the way that we used outcome on the dependent variable to categorize levels of the
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21 independent variable. We believe that this was justifiable in the context here where the
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23 purpose was to optimize the predictive power of the AL index. However, validation in other
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25 datasets are needed before our approach can be recommended for research in general and
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27 for eventual clinical use.
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31 32 **Conclusion**

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34 Our findings demonstrated that an optimally constructed AL index was a strong predictor
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36 of all-cause mortality. This supported the conceptual validity of AL as an effective marker
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38 of the cumulative physiological burden on the body. These findings can contribute to the
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40 evidence for the use of an AL index as a basis for targeted efforts to bring down continued
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42 stress exposures, and in this way prevent the potential detrimental effect of these
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44 exposures on health. Our findings on the U-shaped association with LDL-c, DBP and SBP
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46 and all-cause mortality suggested that AL measures incorporating risks at both the low and
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48 the high-end of biomarkers may yield the best prediction of all-cause mortality.
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Declarations

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Conflicts of interest

None.

Availability of data and materials and code availability

Data from the study can be made available via Region Sjælland following the Danish Data Protection Regulation.

Author Contributions

All authors contributed significantly to the study. Randi Jepsen provided the LOFUS data. Neda Bruun-Rasmussen, Elsebeth Lyngé and George Napolitano designed the study, interpreted the data, and drafted the manuscript. George Napolitano performed the statistical analysis. Christina Ellervik, Christian Christiansen, Randi Jepsen, Knud Rasmussen and Stig Bojesen contributed to the interpretation and writing of the manuscript. All authors critically revised and approved the final manuscript.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. All data storage and management were approved by the Regional Data Protection Agency of

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4 Zealand (REG-024-2019 & REG-24-2015). LOFUS is registered in Clinicaltrials.gov
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6 (NCT02482896).
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9 Consent to participate
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12 Participants provided written informed consent.
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15 Consent for publication
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18 Not applicable.
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31
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33
34 LOFUS bears no responsibility for the analysis or the interpretation conducted within this
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36 study.
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Figure Legends

Figure 1. All-cause mortality by level of allostatic load index, as hazard ratio (95% confidence interval).

Supplementary Figure 1. Hazard ratio for individual biomarkers, centered at median.

Vertical lines denote cut-off values. Upper and lower limits are labelled by U and L, respectively.

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Table 1. Lolland-Falster Health Study (LOFUS). Baseline characteristics of study population and deaths in follow-up period, n(%). For definition of cut-off values, see Supplementary Table 1

Characteristics	Females	Males	Total	Female death	Male death	Total death
Population	7270 (53)	6455 (47)	13725 (100)	78 (39)	120 (61)	198 (100)
Follow-up time, median (IQR)	2.6 (1.6)	2.7 (1.5)	2.6 (1.5)	2.0 (1.6)	1.9 (1.8)	1.9 (1.8)
Median age (IQR)	57.6 (21.9)	59.9 (21.6)	58.7 (22.0)	70.5 (16.4)	74.0 (15.2)	72.8 (16.2)
BMI, kg/m²						
- Underweight	134 (1.8)	42 (0.7)	176 (1.3)	5 (6.4)	Not reported	6 (3.0)
- Normal weight	3038 (41.8)	1862 (28.8)	4900 (35.7)	29 (37.2)	40 (33.3)	69 (34.8)
- Overweight	2335 (32.1)	2940 (45.5)	5275 (38.4)	26 (33.3)	52 (43.3)	78 (39.4)
- Obese	1763 (24.3)	1611 (25.0)	3374 (24.6)	18 (23.1)	27 (22.5)	45 (22.7)
Smoking						
- Never	3586 (49.3)	2737 (42.4)	6323 (46.1)	21 (26.9)	24 (20.0)	45 (22.7)
- Former	2342 (32.2)	2425 (37.6)	4767 (34.7)	32 (41.0)	70 (58.3)	102 (51.5)
- Current	1342 (18.5)	1293 (20.0)	2635 (19.2)	25 (32.1)	26 (21.7)	51 (25.8)
Chronic conditions						
Cardiovascular disease reported	1828 (25.1)	1999 (31.0)	3827 (27.9)	42 (53.8)	60 (50.0)	102 (51.5)
Diabetes reported	264 (3.6)	440 (6.8)	704 (5.1)	9 (11.5)	15 (12.5)	24 (12.1)

Cancer reported	245 (3.4)	275 (4.3)	520 (3.8)	13 (16.7)	24 (20.0)	37 (18.7)
Cardiovascular system						
<i>Systolic blood pressure</i>						
- Low risk	3548 (48.8)	3165 (49.0)	6713 (48.9)	32 (41.0)	52 (43.3)	84 (42.4)
- High risk	3722 (51.2)	3290 (51.0)	7012 (51.1)	46 (59.0)	68 (56.7)	114 (57.6)
<i>Diastolic blood pressure</i>						
- Low risk	3426 (47.1)	3164 (49.0)	6590 (48.0)	26 (33.3)	52 (43.3)	78 (39.4)
- High risk	3844 (52.9)	3291 (51.0)	7135 (52.0)	52 (66.7)	68 (56.7)	120 (60.6)
<i>Pulse rate</i>						
- Low risk	5366 (73.8)	4721 (73.1)	10087 (73.5)	50 (64.1)	81 (67.5)	131 (66.2)
- High risk	1904 (26.2)	1734 (26.9)	3638 (26.5)	28 (35.9)	39 (32.5)	67 (33.8)
<i>AL cardiovascular system score</i>						
- Low	1815 (25.0)	1506 (23.3)	3321 (24.2)	7 (9.0)	16 (13.3)	23 (11.6)
- Mid	2117 (29.1)	2154 (33.4)	4271 (31.1)	27 (34.6)	43 (35.8)	70 (35.4)
- High	3338 (45.9)	2795 (43.3)	6133 (44.7)	44 (56.4)	61 (50.8)	105 (53.0)
Metabolic system						
<i>HDL-c</i>						
- Low risk	4934 (67.9)	4706 (72.9)	9640 (70.2)	46 (59.0)	85 (70.8)	131 (66.2)

- High risk	2336 (32.1)	1749 (27.1)	4085 (29.8)	32 (41.0)	35 (29.2)	67 (33.8)
<i>Triglycerides</i>						
- Low risk	5299 (72.9)	4761 (73.8)	10060 (73.3)	50 (64.1)	95 (79.2)	145 (73.2)
- High risk	1971 (27.1)	1694 (26.2)	3665 (26.7)	28 (35.9)	25 (20.8)	53 (26.8)
<i>HbA1c</i>						
- Low risk	5156 (70.9)	4438 (68.8)	9594 (69.9)	46 (59.0)	69 (57.5)	115 (58.1)
- High risk	2114 (29.1)	2017 (31.2)	4131 (30.1)	32 (41.0)	51 (42.5)	83 (41.9)
<i>Waist-hip ratio</i>						
- Low risk	5452 (75.0)	4831 (74.8)	10283 (74.9)	57 (73.1)	85 (70.8)	142 (71.7)
- High risk	1818 (25.0)	1624 (25.2)	3442 (25.1)	21 (26.9)	35 (29.2)	56 (28.3)
<i>LDL-c</i>						
- Low risk	3459 (47.6)	2989 (46.3)	6448 (47.0)	31 (39.7)	51 (42.5)	82 (41.4)
- High risk	3811 (52.4)	3466 (53.7)	7277 (53.0)	47 (60.3)	69 (57.5)	116 (58.6)
<i>AL metabolic system score</i>						
- Low	1401 (19.3)	1249 (19.3)	2650 (19.3)	11 (14.1)	18 (15.0)	29 (14.6)
- Mid	2413 (33.2)	2135 (33.1)	4548 (33.1)	18 (23.1)	37 (30.8)	55 (27.8)
- High	3456 (47.5)	3071 (47.6)	6527 (47.6)	49 (62.8)	65 (54.2)	114 (57.6)
Inflammation system						

<i>CRP</i>						
- Low risk	5451 (75.0)	4837 (74.9)	10288 (75.0)	51 (65.4)	73 (60.8)	124 (62.6)
- High risk	1819 (25.0)	1618 (25.1)	3437 (25.0)	27 (34.6)	47 (39.2)	74 (37.4)
<i>Albumin</i>						
- Low risk	4953 (68.1)	4655 (72.1)	9608 (70.0)	49 (62.8)	54 (45.0)	103 (52.0)
- High risk	2317 (31.9)	1800 (27.9)	4117 (30.0)	29 (37.2)	66 (55.0)	95 (48.0)
<i>AL inflammation system score</i>						
- Low	4027 (55.4)	3692 (57.2)	7719 (56.2)	41 (52.6)	42 (35.0)	83 (41.9)
- Mid	2350 (32.3)	2108 (32.7)	4458 (32.5)	18 (23.1)	43 (35.8)	61 (30.8)
- High	893 (12.3)	655 (10.1)	1548 (11.3)	19 (24.4)	35 (29.2)	54 (27.3)
Total AL index						
- Low	2306 (31.7)	2112 (32.7)	4418 (32.2)	14 (17.9)	24 (20.0)	38 (19.2)
- Mid	2882 (39.6)	2599 (40.3)	5481 (39.9)	26 (33.3)	45 (37.5)	71 (35.9)
- High	2082 (28.6)	1744 (27.0)	3826 (27.9)	38 (48.7)	51 (42.5)	89 (44.9)

Table 2. Multivariate Cox proportional hazard regression of all-cause mortality for LOFUS participants by individual biomarkers

Variable	Non exposed	Exposed	Hazard ratio (95 % CI) Model 1*	Hazard ratio (95 % CI) Model 2**
HDL cholesterol, mmol/L	High	Low	1.22 (0.88-1.69)	1.24 (0.89-1.73)
LDL cholesterol, mmol/L	Mid	High and low	1.22 (0.91-1.62)	1.19 (0.85-1.51)
Triglycerides, mmol/L	Low	High	0.93 (0.66-1.32)	0.94 (0.67-1.33)
Albumin, g/L	High	Low	1.55 (1.17-2.07)	1.54 (1.16-2.06)
CRP, mg/L	Low	High	1.42 (1.05-1.92)	1.41 (1.04-1.91)
HbA1c, mmol/mol	Low	High	1.25 (0.93-1.68)	1.24 (0.90-1.71)
Systolic blood pressure, mmHg	Mid	High and low	1.20 (0.90-1.61)	1.17 (0.88-1.57)
Diastolic blood pressure, mmHg	Mid	High and low	1.31 (0.98-1.76)	1.29 (0.95-1.72)
Pulse rate, PM	High	Low	1.34 (0.99-1.81)	1.29 (0.91-1.66)
Waist-hip ratio	Low	High	1.02 (0.74-1.41)	1.08 (0.76-1.52)

*Adjusted for age and sex

** Additionally adjusted for BMI, reported diseases, and smoking status

Table 3. Multivariate Cox proportional hazard regression of all-cause mortality for 607136 participants by allostatic load index

Variable	Reference	Level	Hazard ratio (95 % CI)	
			Model 1*	Model 2**
Allostatic load index	Low	Mid	1.39 (0.94 – 2.06)	1.33 (0.89 – 1.98)
		High	2.45 (1.68 – 3.59)	2.37 (1.58 – 3.54)
Continuous allostatic load measure			1.23 (1.14 – 1.32)	1.22 (1.13 – 1.32)
Inflammatory system score	Low	Mid	1.03 (0.74-1.44)	1.02 (0.73-1.42)
		High	2.39 (1.69-3.38)	2.38 (1.67-3.39)
Metabolic system score	Low	Mid	1.19 (0.76-1.86)	1.18 (0.75-1.85)
		High	1.54 (1.02-2.33)	1.54 (1.00-2.38)
Cardiovascular system score	Low	Mid	1.73 (1.08-1.78)	1.65 (1.02-2.65)
		High	2.06 (1.31-3.24)	1.89 (1.20-2.99)

*Adjusted for age and sex

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3 ** Additionally adjusted for BMI, reported diseases, and smoking status
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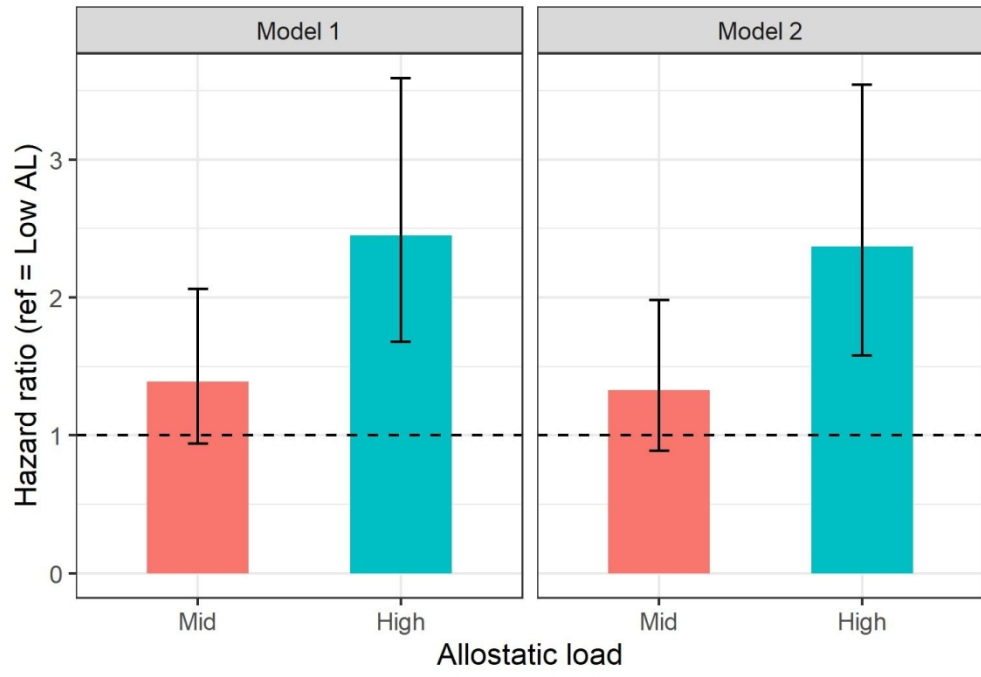
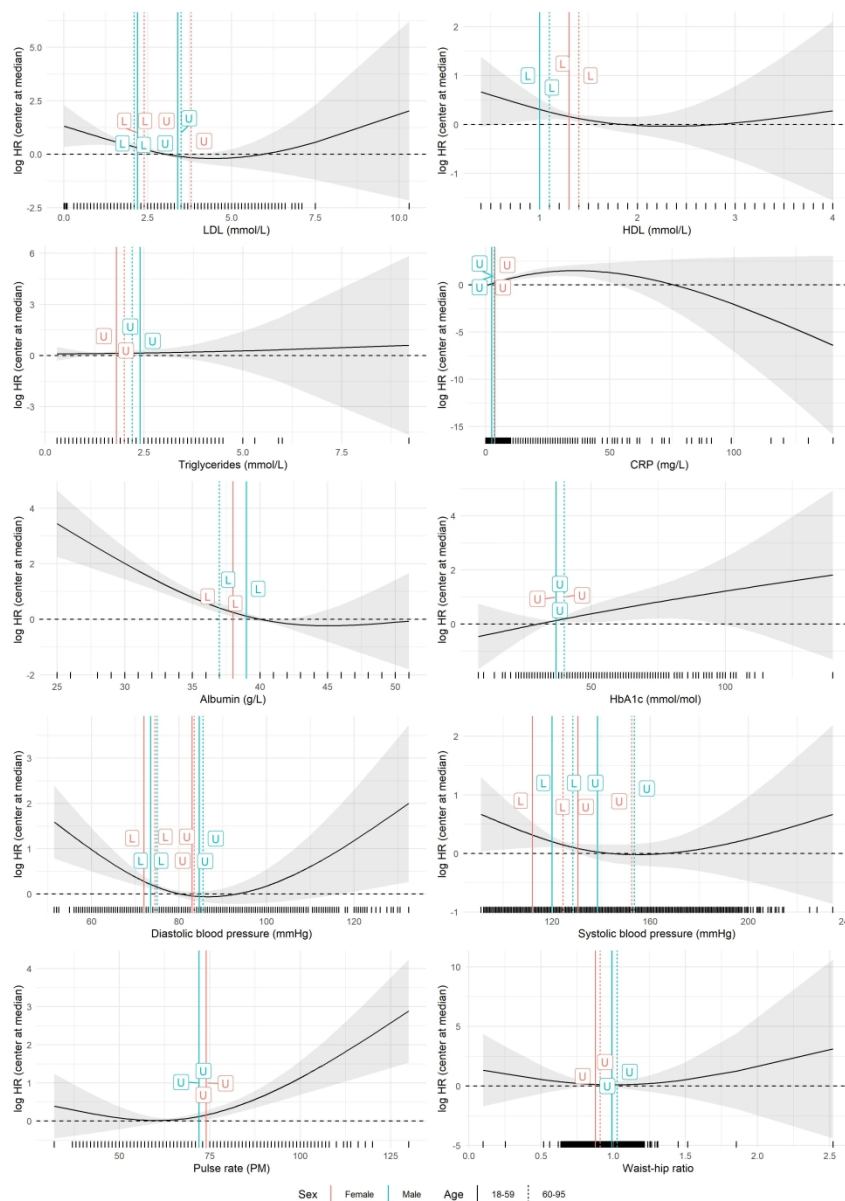


Figure 1. All-cause mortality by level of allostatic load index, as hazard ratio (95% confidence interval).

129x89mm (300 x 300 DPI)

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Supplementary Figure 1. Hazard ratio for individual biomarkers, centered at median. Vertical lines denote cut-off values. Upper and lower limits are labelled by U and L, respectively.

299x424mm (300 x 300 DPI)

Supplementary Table 1. Descriptive statistics and high-risk cut points for individual biomarkers

Characteristics	ALL	Males			Females		
	N = 13,725	N = 6455			N = 7270		
	Median (IQR)	Median (IQR)	High-risk cut point <60 years	High-risk cut point ≥60 years	Median (IQR)	High-risk cut point <60 years	High-risk cut point ≥60 years
HDL cholesterol, mmol/L	1.4 (0.5)	1.2 (0.5)	≤1.0	≤1.1	1.5 (0.5)	≤1.3	≤1.4
LDL cholesterol, mmol/L	2.8 (1.2)	2.8 (1.2)	≤2.2 or ≥3.4	≤2.1 or ≥3.5	2.8 (1.2)	≤2.2 or ≥3.4	≤2.4 or ≥3.8
Triglycerides, mmol/L	1.5 (1.1)	1.6 (1.3)	≥2.4	≥2.2	1.2 (0.9)	≥1.8	≥2.0
Albumin, g/L	40.0 (3.0)	41.0 (4.0)	≤39.0	≤37.0	40.0 (3.0)	≤38.0	≤37.0
CRP, mg/L	1.40 (2.53)	1.06 (1.83)	≥2.37	≥3.42	1.40 (2.96)	≥3.59	≥3.53
HbA1c, mmol/mol	36.0 (5.0)	35.0 (4.0)	≥37.0	≥40.0	35.0 (5.0)	≥37.0	≥40.0
Systolic blood pressure, mmHg	130.0 (26.5)	127.5 (18.5)	≤120.0 or ≥138.5	≤128.5 or ≥153.5	119.5 (18.5)	≤112.0 or ≥130.5	≤124.5 or ≥152.5

Diastolic blood pressure, mmHg	78.5 (10.5)	79 (11.1)	≤73.5 or ≥84.6	≤75.0 or ≥85.5	77.0 (11.0)	≤72.0 or ≥83.0	≤74.5 or ≥83.5
Pulse rate, PM	66.0 (13.0)	65.0 (14.0)	≥72.0	≥72.0	67.0 (13.0)	≥74.0	≥74.0
Waist hip ratio	0.90 (0.14)	0.94 (0.10)	≥1.0	≥1.0	0.83 (0.10)	≥0.9	≥0.9

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Supplementary Table 2. Number and proportion of missing values in analysis sample

Variable	Missing values	Proportion of total analysis sample (N= 15,714)
Biomarkers		
Date of biomarker sample	39	0.25%
C-Reactive Protein	171	1.08%
Albumin	168	1.07%
HDL cholesterol	168	1.07%
LDL Cholesterol	635	4.04%
Triglycerides	169	1.07%
HbA1c	207	1.32%
Waist-hip ratio	139	0.88%
Diastolic Blood Pressure	21	0.13%
Systolic Blood Pressure	21	0.13%
Pulse Rate	69	0.44%
BMI	292	1.86%
Smoking	867	5.52%
Chronic conditions	810	5.15%

Supplementary Table 3. Number of LoD values replaced in each variable

Biomarker	# LoD replaced	LoD
Haemoglobin A1c	1	<31
C-reactive protein	242	<0.16
Alanine aminotransferase	1	<6
Bilirubin	2	<2.0
Low-density lipoprotein	3	<0.10

Reporting checklist for cohort study.

Based on the STROBE cohort guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the STROBE cohort reporting guidelines, and cite them as:

von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies.

		Reporting Item	Page Number
Title and abstract			
Title	#1a	Indicate the study's design with a commonly used term in the title or the abstract	1
Abstract	#1b	Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background / rationale	#2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	#3	State specific objectives, including any prespecified hypotheses	5
Methods			

1	Study design	#4	Present key elements of study design early in the paper	5
2				
3	Setting	#5	Describe the setting, locations, and relevant dates,	5
4			including periods of recruitment, exposure, follow-up, and	
5			data collection	
6				
7				
8	Eligibility criteria	#6a	Give the eligibility criteria, and the sources and methods	5
9			of selection of participants. Describe methods of follow-up.	
10				
11	Eligibility criteria	#6b	For matched studies, give matching criteria and number of	N/A
12			exposed and unexposed	
13				
14				
15				
16	Variables	#7	Clearly define all outcomes, exposures, predictors,	6,7,8
17			potential confounders, and effect modifiers. Give	
18			diagnostic criteria, if applicable	
19				
20				
21	Data sources /	#8	For each variable of interest give sources of data and	6,7,8
22	measurement		details of methods of assessment (measurement).	
23			Describe comparability of assessment methods if there is	
24			more than one group. Give information separately for for	
25			exposed and unexposed groups if applicable.	
26				
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30	Bias	#9	Describe any efforts to address potential sources of bias	15,16
31				
32	Study size	#10	Explain how the study size was arrived at	5
33				
34	Quantitative	#11	Explain how quantitative variables were handled in the	N/A
35	variables		analyses. If applicable, describe which groupings were	
36			chosen, and why	
37				
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40	Statistical	#12a	Describe all statistical methods, including those used to	
41	methods		control for confounding	
42				
43				
44	8,9			
45				
46	Statistical	#12b	Describe any methods used to examine subgroups and	8
47	methods		interactions	
48				
49				
50	Statistical	#12c	Explain how missing data were addressed	8
51	methods			
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54	Statistical	#12d	If applicable, explain how loss to follow-up was addressed	N/A
55	methods			
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1	Statistical	#12e	Describe any sensitivity analyses	
2	methods			
3				
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5	N/A			
6				
7	Results			
8				
9	Participants	#13a	Report numbers of individuals at each stage of study—eg	9
10			numbers potentially eligible, examined for eligibility,	
11			confirmed eligible, included in the study, completing	
12			follow-up, and analysed. Give information separately for	
13			for exposed and unexposed groups if applicable.	
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17	Participants	#13b	Give reasons for non-participation at each stage	N/A
18				
19	Participants	#13c	Consider use of a flow diagram	
20				
21				
22	N/A			
23				
24	Descriptive data	#14a	Give characteristics of study participants (eg	9,28
25			demographic, clinical, social) and information on	
26			exposures and potential confounders. Give information	
27			separately for exposed and unexposed groups if	
28			applicable.	
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32	Descriptive data	#14b	Indicate number of participants with missing data for each	
33			variable of interest	
34				
35	Supplementary			
36	table 2			
37				
38	Descriptive data	#14c	Summarise follow-up time (eg, average and total amount)	
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42	9			
43				
44	Outcome data	#15	Report numbers of outcome events or summary measures	
45			over time. Give information separately for exposed and	
46			unexposed groups if applicable.	
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50	NA			
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53	Main results	#16a	Give unadjusted estimates and, if applicable, confounder-	10,12
54			adjusted estimates and their precision (eg, 95%	
55			confidence interval). Make clear which confounders were	
56			adjusted for and why they were included	
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1	Main results	#16b	Report category boundaries when continuous variables were categorized	NA
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4	Main results	#16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
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8	NA			
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11	Other analyses	#17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	9
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15	Discussion			
16				
17	Key results	#18	Summarise key results with reference to study objectives	9,10
18				
19	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.	14
20				
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24	Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.	11,12,13,14
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30	Generalisability	#21	Discuss the generalisability (external validity) of the study results	15
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34	Other			
35	Information			
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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	17
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