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Complete List of Authors:	Bruun-Rasmussen, Neda; Nykøbing Falster Hospital Napolitano, George; University of Copenhagen, Department of Public Health Christiansen, Christian; Nykøbing Falster Hospital, Department of Internal Medicine Bojesen, Stig E; Herlev and Gentofte Hospital, Department of Clinical Biochemistry Ellervik, Christina; University of Copenhagen; Havard Medical School, Department of Laboratory Medicine Jepsen, Randi; Nykøbing Falster Hospital Rasmussen, Knud; Region Zealand, Production, Research and Innovation Lynge, Elsebeth ; Nykøbing Falster Hospital
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Biomarkers and allostatic load as predictors of mortality: Lolland-Falster Health Study

Neda Esmailzadeh Bruun-Rasmussen[1] (neebruun@gmail.com), George Napolitano[2] (gena@sund.ku.dk), Christian Christiansen[3] (chchris@regionsjaelland.dk), Stig Egil Bojesen[4] (Stig.Egil.Bojesen@regionh.dk), Christina Ellervik[5,6,7] (christina@ellervik.dk), Randi Jepsen[1] (rjep@regionsjaelland.dk), Knud Rasmussen[5] (kra@regionsjaelland.dk), Elsebeth Lynge[1] (elsebeth@sund.ku.dk)

1. Center for Epidemiological Research, Nykøbing Falster Hospital, Nykøbing Falster, Denmark.
2. Department of Public Health, University of Copenhagen, Copenhagen, Denmark.
3. Department of Internal Medicine, Nykøbing Falster Hospital, Nykøbing Falster, Denmark
4. Department of Clinical Biochemistry, Herlev and Gentofte Hospital, Copenhagen, Denmark.
5. Data and Development Support, Region Zealand, Sorø, Denmark.
6. University of Copenhagen, Faculty of Health and Medical Sciences, Denmark.
7. Department of Laboratory Medicine, Boston Children's Hospital & Havard Medical School, Boston, MA. USA.

Corresponding author: MD and PhD-student, Neda Esmailzadeh Bruun-Rasmussen, Center for Epidemiological Research, Nykøbing Falster Hospital, Strandboulevarden 64, DK-4800 Nykøbing Falster, Denmark, neebruun@gmail.com, phone: +45 42423132

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.

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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

of mortality than individual biomarkers, however, there are still gaps in the understanding of the associations [15-16].

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

Methods

Study population

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

Self-reported data

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

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.

For all biomarkers, the highest and lowest quartile of risk scores were either lower or similar to clinical cut-points [22-26].

BMI was divided into underweight (BMI less than 18.5) normal (BMI 18.5–24.9), overweight (BMI 25.0–29.9), or obese (BMI 30.0 or greater); reported diseases into either present or not; and smoking status into never, former, or current.

Allostatic load scores

The AL scores were computed using biomarkers from: the cardiovascular system (systolic blood pressure (SBP), diastolic blood pressure (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). Each of these integer values were divided into low [CVS: 0; MS: 0; IS: 0], mid (CVS: 1; MS: 1; IS: 1], and high [CVS: 2-3; MS: 2-5; IS: 2]. The AL index was defined as the sum of all scores and divided into low [AL:0-1], mid [AL: 2-3], and high [AL: 4-10]. Note that, all biomarkers were given equal weight in accordance with previous studies [14,16].

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 ×10^(-n), k = 1, ..., L }, where n is the variable-specific number of decimals reported in the data and L ×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 analysis was carried out via Cox proportional-hazard models with biomarker levels as continuous covariates, modelled with natural cubic splines with 2 degrees of freedom (except for LDL, where 3 degrees of freedom were used), and further adjusting for sex and age. By graphical inspection, a U-shaped association was found for LDL, SBP and DBP (see Supplementary Figure 1). Therefore, for these biomarkers the sex and age-specific (i.e. below or above age 60) lower and upper quartiles were defined as high-risk, while only one quartile for the others (upper or lower, in accordance with the existing literature); see Supplementary Table 1.

Associations between all-cause mortality and dichotomized biomarkers levels (low/high risk), system-specific AL scores, and total AL index, were modelled with Cox proportional-hazard models. Here, we present two models: Model 1, where HRs are adjusted for sex and age; Model 2, where results are further adjusted for BMI, prevalent diseases, and smoking status. HRs for the individual biomarkers (Table 2) and for system-specific AL scores (Table 3) are mutually adjusted. Proportional hazards assumptions in

the above models have been tested using Schoenfeld residuals. Numbers below 5 are not reported.

Data management, statistical analyses and plots were done in R ver. 4.0.3 [27], with packages splines [27], survival [28] and tidyverse [29].

Results

The LOFUS database used for this study included 13,725 persons, of whom 53% were women and 47% men. The median follow-up time was 2.6 years (IQR 1.5) and the median age was 57.6 in women and 59.9 in men. One-fourth of the participants were obese, and one-fifth were current smokers. Presence of cardiovascular disease at the time of LOFUS participation was reported by 28%, diabetes by 5%, and cancer by 4%. On the value of total AL index, participants were divided between 14% low, 40% mid, and 46% high. During the follow-up period, 198 participants died; of these 39% were women and 61% men (Table 1).

The multivariate Cox proportional hazard regression for individual biomarker and all-cause mortality, adjusted for sex and age and additionally for BMI, reported diseases, and smoking, are listed in Table 2. For all biomarkers, apart from triglycerides, a high risk value was associated with an increased mortality level. However, only the HRs for 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 1.55 (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 steepest gradient was found for the total AL index, with HRs of 1.76 (95% CI: 0.90-3.44) for mid AL, and 2.94 (95% CI: 1.53-5.66) for high AL.

Discussion

In this population-based study from a rural-provincial area of Denmark, we followed the adult population up for a mean period of 2.7 years. High levels of individual biomarkers were overall associated with increased mortality, but most of them at a modest level of 20-40%, 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 50-100%; 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 the strongest predictor of mortality. Persons with a high vs. low total AL index had almost 3-times the mortality. The total AL index was thus a better predictor of all-cause mortality than individual biomarkers and physiological systems AL scores, a pattern consistent with previous studies [14,16, 30].

The most comprehensive studies on AL and mortality all used data from the National Health and Nutrition Examination Survey (NHANES) conducted in 1988-1994. Levine and Crimmins [31] examined ten-year all-cause and disease-specific mortality in a sample of 9942 adults, aged 30+ of whom 1076 had died. They constructed three AL scores; an AL score based on nine biomarkers in line with previous studies defined by clinical cut-off points; an expanded AL score that included five additional biomarkers defined by quintiles; and a continuous AL score constructed by using a continuous z-score measure for all fourteen biomarkers. They found that high values of all three AL scores were associated with increased mortality. Borrell et al. [32] examined twelve-year

mortality by using data from 13,715 adults aged 25+ years of whom 2491 had died. Using a clinical cut-off AL score, they found that, compared to persons with an AL score of 1, those with AL scores of 2 and 3+ had 155% and 429% increased all-cause mortality, respectively. Adjustment for ethnicity, age and sex reduced these excess risks to 35% and 99%, respectively, while further adjustment for socioeconomic status had limited impact. Howard and Sparks [33] used the same AL construct as Borell et al. and found that a one unit increase in AL represented a 7% increase in risk of death when adjusted for age, sex, ethnicity, socioeconomic status, and health behaviour.

For individual biomarkers in our study, HRs were highest for CRP and albumin. CRP is the prototypical acute-phase response protein that increases during systemic inflammation [34], while albumin is a major component of plasma protein, required for transportation and to maintain oncotic pressure, acid–base function, microvascular permeability, and to prevent platelet aggregation [35]. Inflammation increases capillary permeability and thereby escape of serum albumin, leading to expansion of interstitial space and increasing the distribution volume of albumin causing lower serum albumin concentrations. High level of CRP and low level of albumin have thus previously been linked with a variety of health outcomes including morbidity and mortality [7,8,36].

We found a U-shaped association between LDL-c and mortality. Elevated LDL-c is a well-established risk factor of atherosclerosis and cardiovascular disease, and the general perception is that high level of LDL-c is associated with an increased risk of morbidity and mortality [37,38]. Nevertheless, studies on the association between LDL-c levels and mortality have provided conflicting results. Some studies found increasing level of LDL-c to be associated with lower mortality [39-40], and some studies found no

association [38,41-42]. However, most studies were conducted in elderly people often with an intake of lipid-lowering agents. A more recent study in young Koreans found an association between low level of LDL-c and an increased risk of cancer, cardiovascular, and all-cause mortality [43]. These findings were supported by a Chinese study of participants aged 40+ years [44]. A recent Danish study among 108,243 individuals aged 20-100 years found the lowest all-cause mortality at an LDL-c concentration of 3.6 mmol/L (140 mg/dL), and higher mortality at both lower and higher levels [45]. Our findings for LDL-c were thus in accordance with these recent observations. Seplaki et al. suggested that both high and low ends of the risk continuum for the construct of AL could be more informative than simply using high-risk quartiles. They assigned a value of "1" for values above the 75th percentile and below the 25th percentile of the distribution, and a value of "0" for intermediate values [46].

We found both higher and lower levels of diastolic blood pressure DBP to be associated with an increased mortality, and a similar tendency was indicated for SBP. The association between lower blood pressure and mortality is still of discussion [47-49]. Most studies have found this association among elderly people and linked it to chronic disease, e.g. cardiovascular disease (cardiac failure or ischaemic heart disease), cancer, poor functional status or frailty. Low BP has also been associated with poor function and low quality of life [50-51], but in previous studies only the highest quartile or the clinical cut-off value have been used as predictor of all-cause mortality.

Several methods have been used to define an AL composite index, including the count-based, canonical correlation, z-score, and grade of membership method [52-53]. The most commonly used method is the count-based method, where a summary index is calculated by summing the number of biomarkers falling within the high-risk category,

either defined by the percentile (i.e., upper or lower 25th percentile of the sample's distribution) or by the clinical cut-off value. In our analysis with the two-tail cut-off points, 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 for DBP of 1.28 (95% CI: 0.95-1.72). If we have used instead the single high-risk quartile cut-off point, we would have found HRs for LDL of 0.71 (95% CI: 0.49-1.03); for SBP of 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 points thus provided a better identification of persons with high mortality than the one-tail cut-off points.

The issue of whether a clinical or sample-based cut-off criteria should be used is still of discussion [15], however, studies comparing distinct measurement approaches have found only modest differences in their predictive utility [13, 54-55].

Strengths and limitations

The strengths of our study included the size of the cohort in terms of the large number of individuals recruited from a general adult population, and the complete follow-up for death by linkage with the Danish Civil Registration System.

Our study also had some limitations. First, the choice of biomarkers used to construct the AL index. The AL theory emphasises the importance of measuring dysregulation across different physiological systems, including biomarkers from the neuroendocrine, cardiovascular, metabolic, and immune systems [13]. The neuroendocrine system (stress response) is believed to play a key role in allostasis and subsequent AL, as a series of physiological changes takes place before initial stress responses occur (such as rapid increases in blood sugar and blood pressure that supply the body with additional energy). However, biomarkers from the neuroendocrine system are difficult to measure, as

repeated measurements over 1–2 days are recommended. These requirements cannot be fulfilled in population studies, where participants are examined only once, and biomarkers from the neuroendocrine system were therefore not available for our study.

Secondly, the initial stress responses are followed by secondary outcomes from the metabolic, inflammatory and cardiovascular systems, and these markers were all available in our data. Nevertheless, greater sensitivity could have been achieved by studying the dynamic changes over time in these markers to fully capture the flexibility of stress response mechanisms across the lifespan.

Finally, differences across studies in construction of AL indices could influence the comparison of results. We used the shape of the association between level of a given biomarker and all-cause mortality as the basis for the categorization of the biomarker into low and high values. One can argue therefore that our analysis was circular in the way that we used outcome on the dependent variable to categorize levels of the independent variable. We believe that this was justifiable in the context here where the purpose was to optimize the predictive power of the AL index. However, validation in other datasets are needed before our approach can be recommended for research in general and for eventual clinical use.

Conclusion

Our findings demonstrated that an optimally constructed AL index was a strong predictor of all-cause mortality. This supported the conceptual validity of AL as an effective marker of the cumulative physiological burden on the body. These findings can contribute to the evidence for the use of an AL index as a basis for targeted efforts to bring down continued stress exposures, and in this way prevent the potential detrimental effect of these

exposures on health. Our findings on the U-shaped association with LDL-c, DBP and SBP and all-cause mortality suggested that AL measures incorporating risks at both the low and the high-end of biomarkers may yield the best prediction of all-cause mortality.

Declarations

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The funding bodies had no role in the design of the study, neither in the collection, analysis, and interpretation of data, nor in the writing of the manuscript.

Conflicts of interest

None.

Availability of data and materials and code availability

Data from the study can be made available via Region Sjaelland 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 Lynge 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:

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

Consent to participate

Participants provided written informed consent.

Consent for publication

Not applicable.

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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						
Systolic blood pressure						
- 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)
Diastolic blood pressure						
- 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)
Pulse rate						
- 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)
AL cardiovascular system score						
- 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						
HDL-c						
- 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)
Triglycerides						
- 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)
HbA1c						
- 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)
Waist-hip ratio						
- 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)
LDL-c						
- 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)
AL metabolic system score						
- 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						

CRP						
- 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)
Albumin						
- 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)
AL inflammation system score						
- 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)	1.13 (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)	1.28 (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

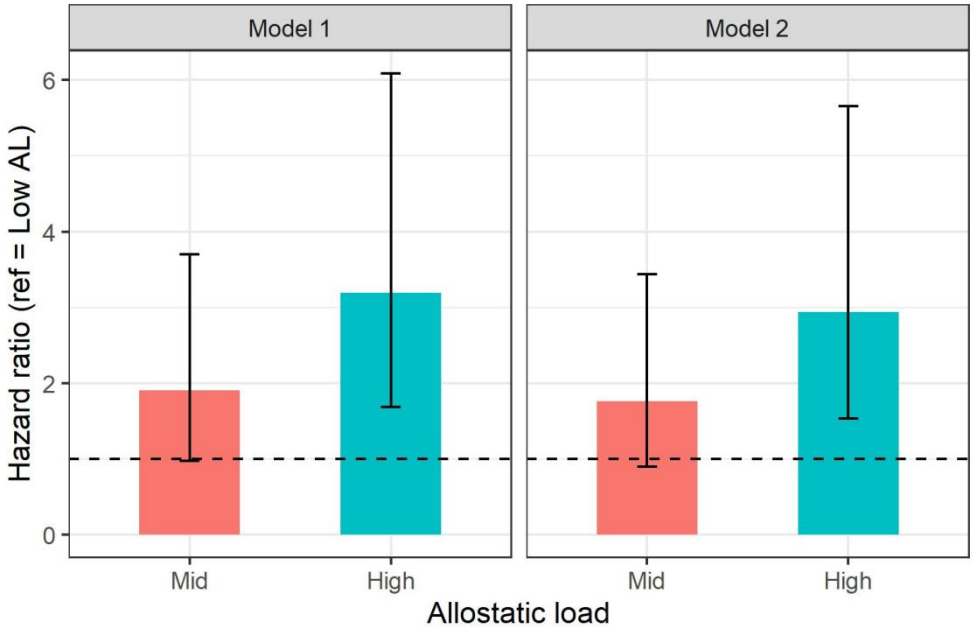
Table 3. Multivariate Cox proportional hazard regression of all-cause mortality for LOFUS participants by allostatic load index

Variable	Reference	Level	Hazard ratio (95 % CI) Model 1*	Hazard ratio (95 % CI) 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)



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

Characteristics	ALL N = 13,725	Males N = 6455			Females 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

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

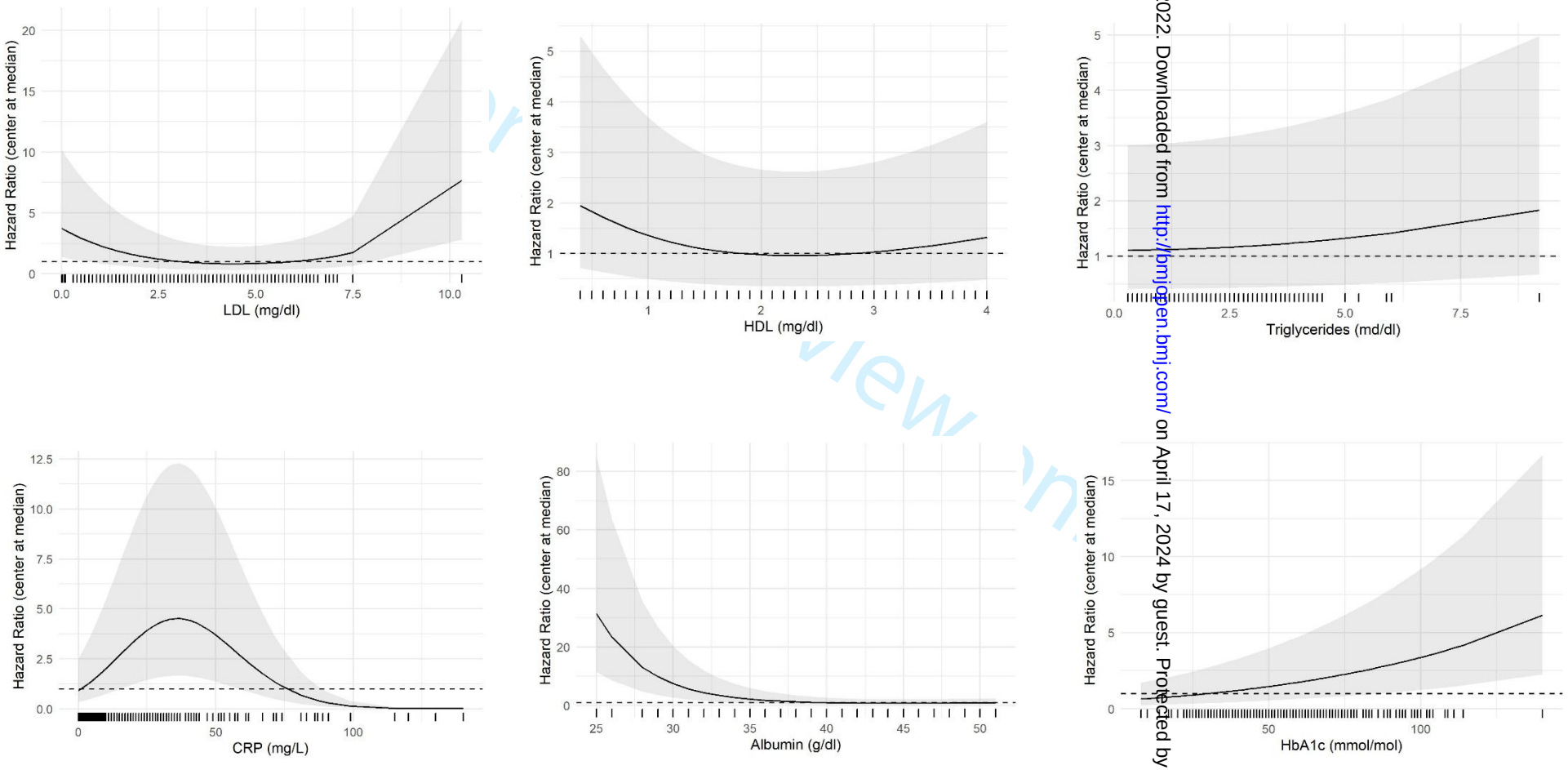
Supplementary Table 2. Number and proportion of missing values in analysis sample

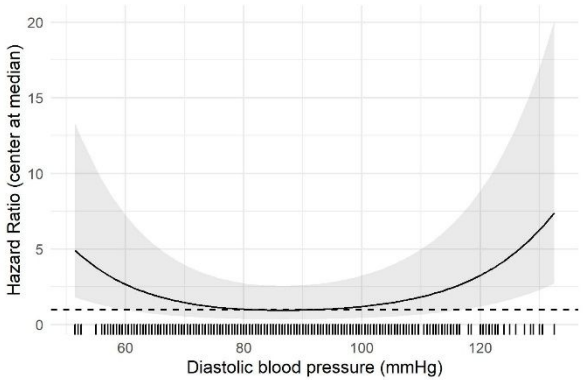
Variable	Missing values	Proportion of total analysis sample (N= 15,714)
Biomarkers		
Date of biomarker sample	39	0.25%
C-Reactive Protein	171	1.08%
Albumin	168	1.07%
HDL cholesterol	168	1.07%
LDL Cholesterol	635	4.04%
Triglycerides	169	1.07%
HbA1c	207	1.32%
Waist-hip ratio	139	0.88%
Diastolic Blood Pressure	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%

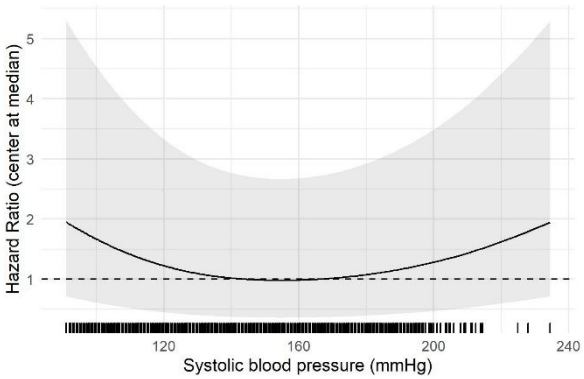
Supplementary Table 3. Number of LoD values replaced in each variable

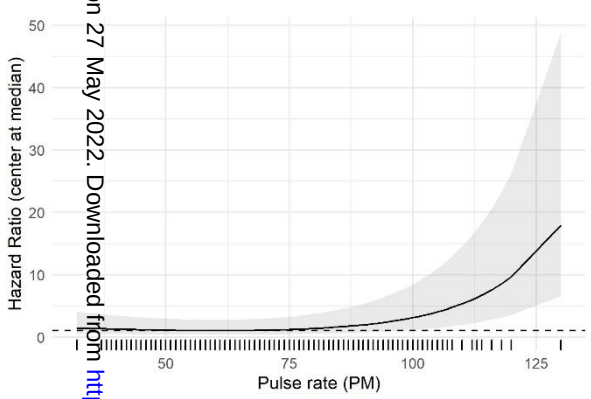
Biomarker	# LoD replaced	LoD
Haemoglobin A1c	1	<31
C-reactive protein	242	<0.16
Alanine aminotransferase	1	<6
Bilirubin	2	<2.0
Low-density lipoprotein	3	<0.10

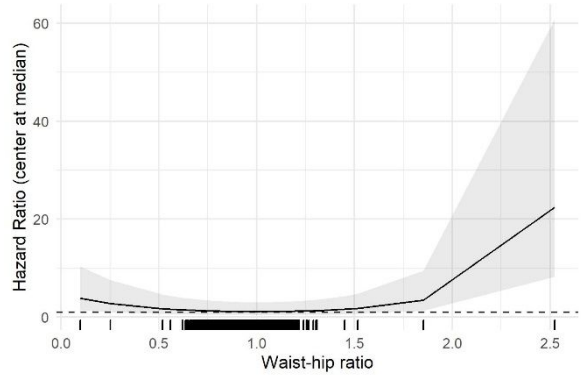
Supplementary Figure 1. Hazard ratio for individual biomarkers, centered at median











Reporting checklist for cohort study.

Based on the STROBE cohort guidelines.

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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	

Abstract	#1b	Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background / rationale	#2	Explain the scientific background and rationale for the investigation being reported
Objectives	#3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	#4	Present key elements of study design early in the paper
Setting	#5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Eligibility criteria	#6a	Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up.
Eligibility criteria	#6b	For matched studies, give matching criteria and number of exposed and unexposed
Variables	#7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources / measurement	#8	For each variable of interest give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than

		one group. Give information separately for for exposed and unexposed groups if applicable.
Bias	#9	Describe any efforts to address potential sources of bias
Study size	#10	Explain how the study size was arrived at
Quantitative variables	#11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why
Statistical methods	#12a	Describe all statistical methods, including those used to control for confounding
Statistical methods	#12b	Describe any methods used to examine subgroups and interactions
Statistical methods	#12c	Explain how missing data were addressed
Statistical methods	#12d	If applicable, explain how loss to follow-up was addressed
Statistical methods	#12e	Describe any sensitivity analyses

Results

Participants	#13a	Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed

		eligible, included in the study, completing follow-up, and analysed. Give information separately for for exposed and unexposed groups if applicable.
Participants	#13b	Give reasons for non-participation at each stage
Participants	#13c	Consider use of a flow diagram
Descriptive data	#14a	Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders. Give information separately for exposed and unexposed groups if applicable.
Descriptive data	#14b	Indicate number of participants with missing data for each variable of interest
Descriptive data	#14c	Summarise follow-up time (eg, average and total amount)
Outcome data	#15	Report numbers of outcome events or summary measures over time. Give information separately for exposed and unexposed groups if applicable.

Main results	#16a	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
Main results	#16b	Report category boundaries when continuous variables were categorized
Main results	#16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	#17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	#18	Summarise key results with reference to study objectives
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.
Interpretation	#20	Give a cautious overall interpretation considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.
Generalisability	#21	Discuss the generalisability (external validity) of the study results
Other Information		

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

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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

Neda Esmailzadeh Bruun-Rasmussen[1] (neebruun@gmail.com), George Napolitano[2] (gena@sund.ku.dk), Christian Christiansen[3] (chchris@regionsjaelland.dk), Stig Egil Bojesen[4] (Stig.Egil.Bojesen@regionh.dk), Christina Ellervik[5,6] (christina@ellervik.dk), Randi Jepsen[1] (rjep@regionsjaelland.dk), Knud Rasmussen[5] (kra@regionsjaelland.dk), Elsebeth Lynge[1] (elsebeth@sund.ku.dk)

1. Centre for Epidemiological Research, Nykøbing Falster Hospital, Nykøbing Falster, Denmark.
2. Department of Public Health, University of Copenhagen, Copenhagen, Denmark.
3. Department of Internal Medicine, Nykøbing Falster Hospital, Nykøbing Falster, Denmark
4. Department of Clinical Biochemistry, Herlev Hospital, Herlev, Denmark.
5. Department of Data and Development Support, Region Zealand, Sorø, Denmark.
6. Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark.

Corresponding author: MD and PhD-student, Neda Esmailzadeh Bruun-Rasmussen, Centre for Epidemiological Research, Nykøbing Falster Hospital, Strandboulevarden 64, DK-4800 Nykøbing Falster, Denmark, neebruun@gmail.com, phone: +45 42423132

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

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 [16]. AL has been reported to be a better predictor of mortality than individual biomarkers, however, there are still gaps in the understanding of the associations [17-18]. AL has been suggested also as a tool for allocation of nursing resources [19].

This study provides data from the Lolland-Falster Health Study (LOFUS) [20], a population-based survey undertaken in 2016-2020 in Lolland-Falster, a rural-provincial region in Denmark with a life expectancy much below the national average [21], and with health problems reported more frequently than in the rest of the country [22]. Using the LOFUS data, the purposes of the present study were 1) to determine the association between 10 individual biomarkers and all-cause mortality; and 2) to examine the association between AL, across three physiological systems (cardiovascular, inflammatory, metabolic system), and all-cause mortality. The hypothesis is that AL can be used as an informative tool in predicting future risk of death in the general adult population.

Methods

Patient and Public Involvement

We undertook a prospective cohort study of participants from LOFUS; a household-based population study with data collected between February 2016 and February 2020. Persons aged 18 years and above were randomly sampled from the Danish Civil Registration System and invited to participate together with the rest of their households. Participation required informed consent. The study was approved by Region Zealand's Ethical Committee on Health Research (Reg: SJ-421). A detailed description of the study protocol [20] and information on the socio-economic determinants of participation [23] have been

published previously. Persons below 18 years, and pregnant women were excluded from the present study. Once the paper has been published in the international literature, the key results will be reported also in the local press.

Self-reported data

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

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-c was calculated by using Friedewald formula [24] 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 [16]. 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, as variations across these parameters were found in our previous study of reference intervals [25]. We dichotomized biomarkers 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-c, 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. For all biomarkers, the highest and lowest quartile of risk scores were either lower or similar to clinical cut-points [26-30].

BMI was divided into underweight (BMI less than 18.5) normal (BMI 18.5–24.9), overweight (BMI 25.0–29.9), or obese (BMI 30.0 or greater); reported diseases into either present or not; and smoking status into never, former, or current.

Allostatic load scores

The AL scores were computed using biomarkers from: the cardiovascular system (systolic blood pressure (SBP), diastolic blood pressure (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 ×10^(-n), k = 1, ..., L }, where n is the variable-specific number of decimals reported in the data and L ×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 analysis was carried out via Cox proportional-hazard models with biomarker levels as continuous covariates, modelled with natural cubic splines with 2 degrees of freedom (except for LDL-c, where 3 degrees of freedom were used), and further adjusting for sex and age. By graphical inspection, a U-shaped association was found for LDL-c, SBP and DBP (see Supplementary Figure 1). Therefore, for these biomarkers both the sex and age-specific (i.e. below or above age 60) lower and upper quartiles were defined as high-risk, while only one quartile for the others (upper or lower, in accordance with the existing literature); see Supplementary Table 1.

Associations between all-cause mortality and dichotomized biomarkers levels (low/high risk), system-specific AL scores, and total AL index, were modelled with Cox proportional-hazard models. Here, we present two models: Model 1, where HRs are adjusted for sex and age; Model 2, where results are further adjusted for BMI, prevalent diseases, and smoking status. HRs for the individual biomarkers (Table 2) and for system-specific AL scores (Table 3) are mutually adjusted. Proportional hazards assumptions in the above models have been tested using Schoenfeld residuals. Numbers below 5 are not reported. In addition, we report HRs for a one-point increase in the AL index.

Data management, statistical analyses and plots were done in R ver. 4.0.3 [31], with packages splines [31], survival [32],tidyverse [33], ggrepel [34] and ggpubr [35].

Results

The LOFUS database used for this study included 13,725 persons, of whom 53% were women and 47% men. The median follow-up time was 2.6 years (IQR 1.5) and the median age was 57.6 in women and 59.9 in men. One-fourth of the participants were obese, and one-fifth were current smokers. Presence of cardiovascular disease at the time of LOFUS participation was reported by 28%, diabetes by 5%, and cancer by 4%. On the value of total AL index, participants were divided approximately into tertiles; 32% low, 40% mid, and 38% high. During the follow-up period, 198 participants died; of these 39% were women and 61% men (Table 1).

The multivariate Cox proportional hazard regression for individual biomarker and all-cause mortality, adjusted for sex and age and additionally for BMI, reported diseases, and smoking, are listed in Table 2. For all biomarkers, apart from triglycerides, a high risk value was associated with an increased mortality level. However, only the HRs for

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].

The most comprehensive studies on AL and mortality all used data from the National Health and Nutrition Examination Survey (NHANES). Borrell et al. [37] examined twelve-year mortality by using data from 13,715 adults aged 25+ years of whom 2491 had died. They calculated AL based on 9 biomarkers; albumin, CRP, total cholesterol, HDL, haemoglobin A1c, waist-to-hip-ratio, SBP, DBP, and PR. Using a clinical cut-off AL score, they found that, compared to persons with an AL score of ≤1, those with AL scores of 2 and 3+ had adjusted HRs of 1.40 (95% CI 1.11-1.76) and 1.88 (95% CI 1.56-2.26), respectively.

Levine and Crimmins [38] examined ten-year all-cause and disease-specific mortality. In total, 15,042 persons were eligible, but biomarker data were available for only 9942 adults aged 30+, of whom 1076 had died. They included data on albumin, CRP, waist-to-hip ratio, total cholesterol, HDL, haemoglobin A1c, PR, SBP, and DBP. For each of the nine biomarkers, a person was classified as high or low based on clinical cut-off points, and the AL score was the number of biomarkers classified as high. In addition, an expanded AL score included five additional biomarkers defined by quintiles; and a continuous AL score used a continuous z-score measure for all fourteen biomarkers. For the first AL score, a HR of 2.75 (p<.001) was found for all-cause mortality when persons with the highest quintile of AL were compared with those with the lowest. Somewhat stronger gradients were found for the expanded; 3.62 (p<.0001) and continuous; 6.97 (p<.0001), ALs.

Howard and Sparks [39] studied 11,733 participants from NHANES. Imputation was used to estimate missing values. Their AL measure was based on DBP, SBP, PR, total cholesterol, HDL, triglycerides, haemoglobin A1c, BMI, albumin and CRP.

They found that a one-unit increase in AL represented a 7% increase in risk of death when adjusted for age, sex, ethnicity, socioeconomic status, and health behaviour.

The National Child Development Study was followed up for deaths from birth in 1958 to 1 December 2013, i.e. to the age of 55 years [18]. AL based on 10 biomarkers was calculated and divided into three levels. All-cause mortality for persons with mid or high AL was compared with that of persons with low AL, and adjusted for early life, childhood, young and adulthood confounders. The HR of death was 1.71 (95% CI 1.07-2.72) for persons with mid AL, and 2.57 (95% CI 1.59-4.15) for those with high AL. The association between AL and all-cause mortality was stronger than the associations between of the individual 10 biomarkers and all-cause mortality.

The NHANES studies vary in number of participants included in the studies, in length of follow-up for mortality, in biomarkers included, in the definition of AL, and in methods used for AL calculation. Nevertheless, all the studies indicated that all-cause mortality increased with increasing AL. The study by Borell et al. [37] is the one methodologically most similar to our study and the gradient of 1.88 (95% CI 1.56-2.26) is compatible with the one of 2.37 (95% CI 1.58-3.54) found in our study, and so is the gradient of 2.57 (95% CI 1.59-4.15) found in the National Child Development Study.

For individual biomarkers in our study, HRs were highest for CRP and albumin. CRP is the prototypical acute-phase response protein that increases during systemic inflammation [40], while albumin is a major component of plasma protein, required for transportation and to maintain oncotic pressure, acid–base function, microvascular permeability, and to prevent platelet aggregation [41]. Inflammation increases capillary permeability and thereby escape of serum albumin, leading to expansion of interstitial space and increasing the distribution volume of albumin causing

lower serum albumin concentrations. High level of CRP and low level of albumin have thus previously been linked with a variety of health outcomes including morbidity and mortality [7,8,42].

We found a U-shaped association between LDL-c and mortality. Elevated LDL-c is a well-established risk factor of atherosclerosis and cardiovascular disease, and the general perception is that high level of LDL-c is associated with an increased risk of morbidity and mortality [43,44]. Nevertheless, studies on the association between LDL-c levels and mortality have provided conflicting results. Some studies found increasing level of LDL-c to be associated with lower mortality [45-46], and some studies found no association [44,47-48]. However, most studies were conducted in elderly people often with an intake of lipid-lowering agents. A more recent study in young Koreans found an association between low level of LDL-c and an increased risk of cancer, cardiovascular, and all-cause mortality [49]. These findings were supported by a Chinese study of participants aged 40+ years [50]. A recent Danish study among 108,243 individuals aged 20-100 years found the lowest all-cause mortality at an LDL-c concentration of 3.6 mmol/L (140 mg/dL), and higher mortality at both lower and higher levels [51]. Our findings for LDL-c were thus in accordance with these recent observations. Seplaki et al. suggested that both high and low ends of the risk continuum for the construct of AL could be more informative than simply using high-risk quartiles. They assigned a value of "1" for values above the 75th percentile and below the 25th percentile of the distribution, and a value of "0" for intermediate values [52].

We found both higher and lower levels of DBP to be associated with an increased mortality, and a similar tendency was indicated for SBP. The association between lower blood pressure and mortality is still of discussion [53-55]. Most studies have

found this association among elderly people and linked it to chronic disease, e.g. cardiovascular disease (cardiac failure or ischemic heart disease), cancer, poor functional status or frailty. Low BP has also been associated with poor function and low quality of life [56-57], but in previous studies only the highest quartile or the clinical cut-off value have been used as predictor of all-cause mortality.

Several methods have been used to define an AL composite index, including the count-based, canonical correlation, z-score, and grade of membership method [58-59]. The most commonly used method is the count-based method, where a summary index is calculated by summing the number of biomarkers falling within the high-risk category, either defined by the percentile (i.e., upper or lower 25th percentile of the sample's distribution) or by the clinical cut-off value. In our analysis with the two-tail cut-off points, 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 for DBP of 1.28 (95% CI: 0.95-1.72). If we have used instead the single high-risk quartile cut-off point, we would have found HRs for LDL-c of 0.71 (95% CI: 0.49-1.03); for SBP of 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 points thus provided a better identification of persons with high mortality than the one-tail cut-off points.

The issue of whether a clinical or sample-based cut-off criteria should be used is still of discussion [17], however, studies comparing distinct measurement approaches have found only modest differences in their predictive utility [15, 60-61].

Strengths and limitations

The strengths of our study included the size of the cohort in terms of the large number of individuals recruited from a general adult population, and the complete follow-up for death by linkage with the Danish Civil Registration System.

Our study also had some limitations. First, the choice of biomarkers used to construct the AL index. The AL theory emphasises the importance of measuring dysregulation across different physiological systems, including biomarkers from the neuroendocrine, cardiovascular, metabolic, and immune systems [13]. The neuroendocrine system (stress response) is believed to play a key role in allostasis and subsequent AL, as a series of physiological changes takes place before initial stress responses occur (such as rapid increases in blood sugar and blood pressure that supply the body with additional energy). However, biomarkers from the neuroendocrine system are difficult to measure, as repeated measurements over 1–2 days are recommended. These requirements cannot be fulfilled in population studies, where participants are examined only once, and biomarkers from the neuroendocrine system were therefore not available for our study.

Secondly, the initial stress responses are followed by secondary outcomes from the metabolic, inflammatory and cardiovascular systems, and these markers were all available in our data. Nevertheless, greater sensitivity could have been achieved by studying the dynamic changes over time in these markers to fully capture the flexibility of stress response mechanisms across the lifespan.

Finally, differences across studies in construction of AL indices could influence the comparison of results. We used the shape of the association between level of a given biomarker and all-cause mortality as the basis for the categorization of the biomarker into low and high values. One can argue therefore that our analysis was circular in the way that we used outcome on the dependent variable to categorize levels of the

independent variable. We believe that this was justifiable in the context here where the purpose was to optimize the predictive power of the AL index. However, validation in other datasets are needed before our approach can be recommended for research in general and for eventual clinical use.

Conclusion

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

Declarations

Funding

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The funding bodies had no role in the design of the study, neither in the collection, analysis, and interpretation of data, nor in the writing of the manuscript.

Conflicts of interest

None.

Availability of data and materials and code availability

Data from the study can be made available via Region Sjaelland 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 Lynge 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

Zealand (REG-024-2019 & REG-24-2015). LOFUS is registered in Clinicaltrials.gov (NCT02482896).

Consent to participate

Participants provided written informed consent.

Consent for publication

Not applicable.

Acknowledgement

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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.

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						
Systolic blood pressure						
- 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)
Diastolic blood pressure						
- 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)
Pulse rate						
- 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)
AL cardiovascular system score						
- 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						
HDL-c						
- 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)
Triglycerides						
- 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)
HbA1c						
- 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)
Waist-hip ratio						
- 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)
LDL-c						
- 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)
AL metabolic system score						
- 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						

CRP						
- 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)
Albumin						
- 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)
AL inflammation system score						
- 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)	1.13 (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)	1.28 (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*	Hazard ratio (95 % CI) 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

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

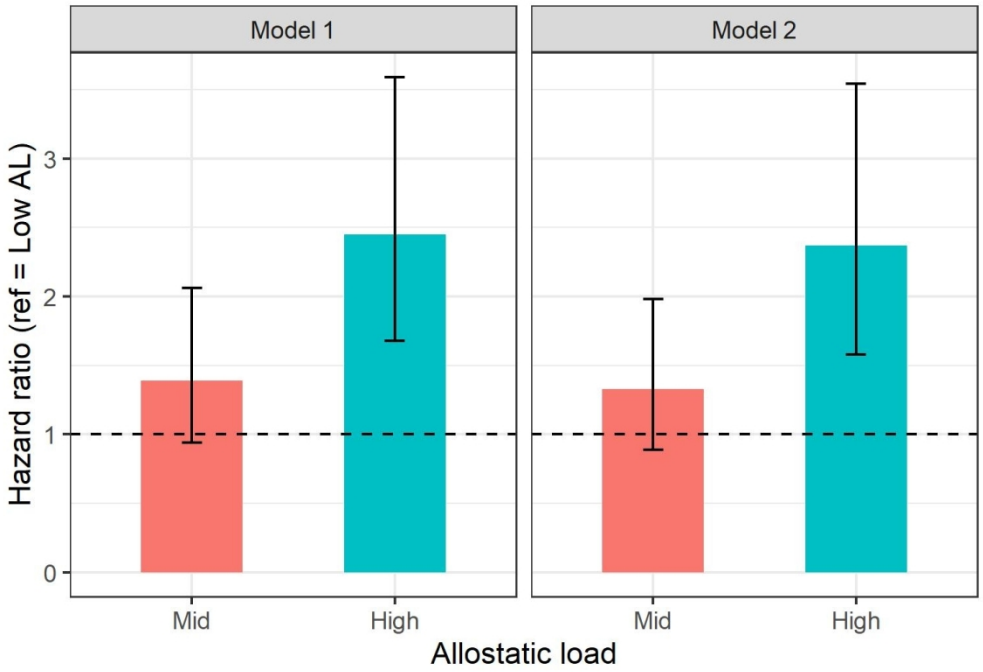
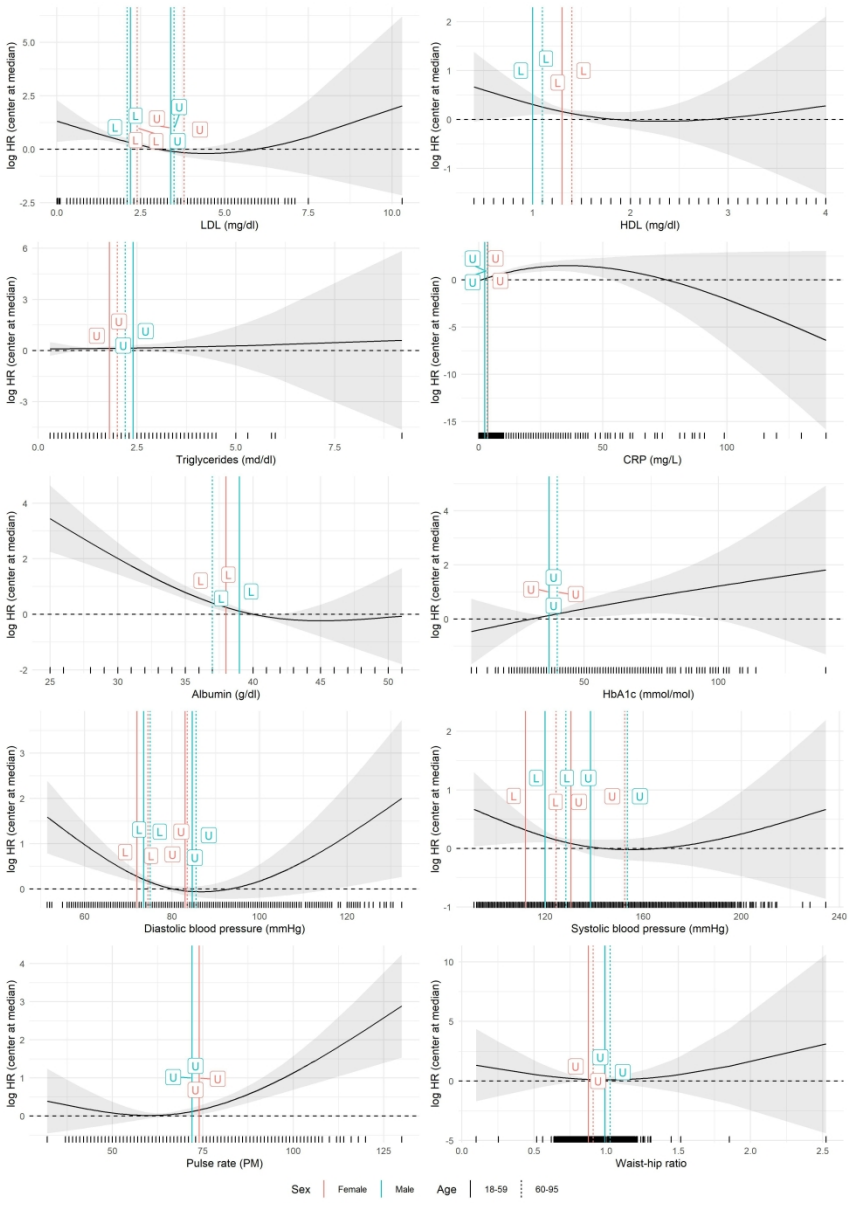


Figure 1. All-cause mortality by level of allostatic load index, as hazard ratio (95% confidence interval).

129x89mm (300 x 300 DPI)



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 N = 13,725	Males N = 6455			Females 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

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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In your methods section, say that you used the STROBE cohortreporting 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 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			

Study design	#4	Present key elements of study design early in the paper	5
Setting	#5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Eligibility criteria	#6a	Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up.	5
Eligibility criteria	#6b	For matched studies, give matching criteria and number of exposed and unexposed	N/A
Variables	#7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6,7,8
Data sources / measurement	#8	For each variable of interest give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group. Give information separately for for exposed and unexposed groups if applicable.	6,7,8
Bias	#9	Describe any efforts to address potential sources of bias	15,16
Study size	#10	Explain how the study size was arrived at	5
Quantitative variables	#11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why	N/A
Statistical methods	#12a	Describe all statistical methods, including those used to control for confounding	8,9
Statistical methods	#12b	Describe any methods used to examine subgroups and interactions	8
Statistical methods	#12c	Explain how missing data were addressed	8
Statistical methods	#12d	If applicable, explain how loss to follow-up was addressed	N/A

Statistical methods	#12e	Describe any sensitivity analyses	
N/A			
Results			
Participants	#13a	Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed. Give information separately for for exposed and unexposed groups if applicable.	9
Participants	#13b	Give reasons for non-participation at each stage	N/A
Participants	#13c	Consider use of a flow diagram	
N/A			
Descriptive data	#14a	Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders. Give information separately for exposed and unexposed groups if applicable.	9,28
Descriptive data	#14b	Indicate number of participants with missing data for each variable of interest	
Supplementary table 2			
Descriptive data	#14c	Summarise follow-up time (eg, average and total amount)	
9			
Outcome data	#15	Report numbers of outcome events or summary measures over time. Give information separately for exposed and unexposed groups if applicable.	
NA			
Main results	#16a	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	10,12

Main results	#16b	Report category boundaries when continuous variables were categorized	NA
Main results	#16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
NA			
Other analyses	#17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	9
Discussion			
Key results	#18	Summarise key results with reference to study objectives	9,10
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
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
Generalisability	#21	Discuss the generalisability (external validity) of the study results	15
Other Information			
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

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

Neda Esmailzadeh Bruun-Rasmussen[1] (neebruun@gmail.com), George Napolitano[2] (gena@sund.ku.dk), Christian Christiansen[3] (chchris@regionsjaelland.dk), Stig Egil Bojesen[4] (Stig.Egil.Bojesen@regionh.dk), Christina Ellervik[5,6] (christina@ellervik.dk), Randi Jepsen[1] (rjep@regionsjaelland.dk), Knud Rasmussen[5] (kra@regionsjaelland.dk), Elsebeth Lynge[1] (elsebeth@sund.ku.dk)

1. Centre for Epidemiological Research, Nykøbing Falster Hospital, Nykøbing Falster, Denmark.
2. Department of Public Health, University of Copenhagen, Copenhagen, Denmark.
3. Department of Internal Medicine, Nykøbing Falster Hospital, Nykøbing Falster, Denmark
4. Department of Clinical Biochemistry, Herlev Hospital, Herlev, Denmark.
5. Department of Data and Development Support, Region Zealand, Sorø, Denmark.
6. Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark.

Corresponding author: MD and PhD-student, Neda Esmailzadeh Bruun-Rasmussen, Centre for Epidemiological Research, Nykøbing Falster Hospital, Strandboulevarden 64, DK-4800 Nykøbing Falster, Denmark, neebruun@gmail.com, phone: +45 42423132

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.

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

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 [16]. AL has been reported to be a better predictor of mortality than individual biomarkers, however, there are still gaps in the understanding of the associations [17,18]. AL has been suggested also as a tool for allocation of nursing resources [19].

This study provides data from the Lolland-Falster Health Study (LOFUS) [20], a population-based survey undertaken in 2016-2020 in Lolland-Falster, a rural-provincial region in Denmark with a life expectancy much below the national average [21], and with health problems reported more frequently than in the rest of the country [22]. Using the LOFUS data, the purposes of the present study were 1) to determine the association between 10 individual biomarkers and all-cause mortality; and 2) to examine the association between AL, across three physiological systems (cardiovascular, inflammatory, metabolic system), and all-cause mortality. The hypothesis is that AL can be used as an informative tool in predicting future risk of death in the general adult population.

Methods

Study population

We undertook a prospective cohort study of participants from LOFUS; a household-based population study with data collected between February 2016 and February 2020. Persons aged 18 years and above were randomly sampled from the Danish Civil Registration System and invited to participate together with the rest of their households. Participation required informed consent. The study was approved by Region Zealand's Ethical Committee on Health Research (Reg: SJ-421). A detailed description of the study protocol [20] and information on the socio-economic determinants of participation [23] have been

published previously. Persons below 18 years, and pregnant women were excluded from the present study.

Patient and Public Involvement

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

Self-reported data

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

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-c was calculated by using Friedewald formula [24] when the plasma triglyceride concentration was below 4.5 mmol/L. Systolic (SBP) and diastolic (DBP) 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 [16]. 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. For age, we dichotomized at age 60. Some previous studies focused on AL in people aged 60 and above [25,26] and we intuitively found it reasonable to distinguish in the same way between "young" and "old" people in our data; age 60 was furthermore the median age of our study population; and with this age-dichotomization we avoided violations of the model assumption in the statistical analysis. We dichotomized biomarkers 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-c, 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. For biomarkers with U-shaped associations, we tested out also using octiles as cut-off points. However, this resulted in some violations of the model assumptions in the statistical analysis, and for SBP the upper octile cut-off was from a clinical point of view very high. On this basis we used the quartile cut-offs also for the biomarkers with the U-shaped association. For all biomarkers, the highest and lowest quartile of risk scores were either lower or similar to clinical cut-points [27-31].

BMI was divided into underweight (BMI less than 18.5) normal (BMI 18.5–24.9), overweight (BMI 25.0–29.9), or obese (BMI 30.0 or greater); reported diseases into either present or not; and smoking status into never, former, or current.

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 ×10^(-n), k = 1, ..., L }, where n is the variable-specific number of decimals reported in the data and L ×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

analysis was carried out via Cox proportional-hazard models with biomarker levels as continuous covariates, modelled with natural cubic splines with 2 degrees of freedom (except for LDL-c, where 3 degrees of freedom were used), and further adjusting for sex and age. By graphical inspection, a U-shaped association was found for LDL-c, SBP and DBP (see Supplementary Figure 1). Therefore, for these biomarkers both the sex and age-specific (i.e. below or above age 60) lower and upper quartiles were defined as high-risk, while only one quartile for the others (upper or lower, in accordance with the existing literature); see Supplementary Table 1.

Associations between all-cause mortality and dichotomized biomarkers levels (low/high risk), system-specific AL scores, and total AL index, were modelled with Cox proportional-hazard models. Here, we present two models: Model 1, where HRs are adjusted for sex and age; Model 2, where results are further adjusted for BMI, prevalent diseases, and smoking status. HRs for the individual biomarkers (Table 2) and for system-specific AL scores (Table 3) are mutually adjusted. Proportional hazards assumptions in the above models have been tested using Schoenfeld residuals. Numbers below 5 are not reported. In addition, we report HRs for a one-point increase in the AL index.

Data management, statistical analyses and plots were done in R ver. 4.0.3 [32], with packages splines [32], survival [33], tidyverse [34], ggrepel [35] and ggpubr [36].

Results

The LOFUS database used for this study included 13,725 persons, of whom 53% were women and 47% men. The median follow-up time was 2.6 years (IQR 1.5) and the median age was 57.6 in women and 59.9 in men. One-fourth of the participants were obese, and

one-fifth were current smokers. Presence of cardiovascular disease at the time of LOFUS participation was reported by 28%, diabetes by 5%, and cancer by 4%. On the value of total AL index, participants were divided approximately into tertiles; 32% low, 40% mid, and 38% high. During the follow-up period, 198 participants died; of these 39% were women and 61% men (Table 1).

The multivariate Cox proportional hazard regression for individual biomarker and all-cause mortality, adjusted for sex and age and additionally for BMI, reported diseases, and smoking, are listed in Table 2. For all biomarkers, apart from triglycerides, a high risk value was associated with an increased mortality level. However, only the HRs for 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 [16,18,37].

The most comprehensive studies on AL and mortality all used data from the National Health and Nutrition Examination Survey (NHANES). Borrell et al. [38] examined twelve-year mortality by using data from 13,715 adults aged 25+ years of whom 2491 had died. They calculated AL based on 9 biomarkers; albumin, CRP, total cholesterol, HDL-c, haemoglobin A1c, waist-to-hip-ratio, SBP, DBP, and PR. Using a clinical cut-off AL score, they found that, compared to persons with an AL score of $\leq$1, those with AL scores of 2 and 3+ had adjusted HRs of 1.40 (95% CI 1.11-1.76) and 1.88 (95% CI 1.56-2.26), respectively.

Levine and Crimmins [39] examined ten-year all-cause and disease-specific mortality. In total, 15,042 persons were eligible, but biomarker data were available for only 9942 adults aged 30+, of whom 1076 had died. They included data on albumin, CRP, waist-to-hip ratio, total cholesterol, HDL-c, haemoglobin A1c, PR, SBP, and DBP. For each of the nine biomarkers, a person was classified as high or low based on clinical cut-off

points, and the AL score was the number of biomarkers classified as high. In addition, an expanded AL score included five additional biomarkers defined by quintiles; and a continuous AL score used a continuous z-score measure for all fourteen biomarkers. For the first AL score, a HR of 2.75 (p<.001) was found for all-cause mortality when persons with the highest quintile of AL were compared with those with the lowest. Somewhat stronger gradients were found for the expanded; 3.62 (p<.0001) and continuous; 6.97 (p<.0001), ALs.

Howard and Sparks [40] studied 11,733 participants from NHANES. Imputation was used to estimate missing values. Their AL measure was based on DBP, SBP, PR, total cholesterol, HDL-c, triglycerides, haemoglobin A1c, BMI, albumin and CRP. They found that a one-unit increase in AL represented a 7% increase in risk of death when adjusted for age, sex, ethnicity, socioeconomic status, and health behaviour.

The National Child Development Study was followed up for deaths from birth in 1958 to 1 December 2013, i.e. to the age of 55 years [18]. AL based on 10 biomarkers was calculated and divided into three levels. All-cause mortality for persons with mid or high AL was compared with that of persons with low AL, and adjusted for early life, childhood, young and adulthood confounders. The HR of death was 1.71 (95% CI 1.07-2.72) for persons with mid AL, and 2.57 (95% CI 1.59-4.15) for those with high AL. The association between AL and all-cause mortality was stronger than the associations between of the individual 10 biomarkers and all-cause mortality.

The NHANES studies vary in number of participants included in the studies, in length of follow-up for mortality, in biomarkers included, in the definition of AL, and in methods used for AL calculation. Nevertheless, all the studies indicated that all-cause mortality increased with increasing AL. The study by Borell et al. [38] is the one

methodologically most similar to our study and the gradient of 1.88 (95% CI 1.56-2.26) is compatible with the one of 2.37 (95% CI 1.58-3.54) found in our study, and so is the gradient of 2.57 (95% CI 1.59-4.15) found in the National Child Development Study.

For individual biomarkers in our study, HRs were highest for CRP and albumin. CRP is the prototypical acute-phase response protein that increases during systemic inflammation [41], while albumin is a major component of plasma protein, required for transportation and to maintain oncotic pressure, acid–base function, microvascular permeability, and to prevent platelet aggregation [42]. Inflammation increases capillary permeability and thereby escape of serum albumin, leading to expansion of interstitial space and increasing the distribution volume of albumin causing lower serum albumin concentrations. High level of CRP and low level of albumin have thus previously been linked with a variety of health outcomes including morbidity and mortality [7,8,43].

We found a U-shaped association between LDL-c and mortality. Elevated LDL-c is a well-established risk factor of atherosclerosis and cardiovascular disease, and the general perception is that high level of LDL-c is associated with an increased risk of morbidity and mortality [44,45]. Nevertheless, studies on the association between LDL-c levels and mortality have provided conflicting results. Some studies found increasing level of LDL-c to be associated with lower mortality [46,47], and some studies found no association [45,48-49]. However, most studies were conducted in elderly people often with an intake of lipid-lowering agents. A more recent study in young Koreans found an association between low level of LDL-c and an increased risk of cancer, cardiovascular, and all-cause mortality [50]. These findings were supported by a Chinese study of participants aged 40+ years [51]. A recent Danish study among 108,243 individuals aged 20-100 years

found the lowest all-cause mortality at an LDL-c concentration of 3.6 mmol/L (140 mg/dL), and higher mortality at both lower and higher levels [52]. Our findings for LDL-c were thus in accordance with these recent observations. Seplaki et al. suggested that both high and low ends of the risk continuum for the construct of AL could be more informative than simply using high-risk quartiles. They assigned a value of "1" for values above the 75th percentile and below the 25th percentile of the distribution, and a value of "0" for intermediate values [53].

We found both higher and lower levels of DBP to be associated with an increased mortality, and a similar tendency was indicated for SBP. The association between lower blood pressure and mortality is still of discussion [54-56]. Most studies have found this association among elderly people and linked it to chronic disease, e.g. cardiovascular disease (cardiac failure or ischemic heart disease), cancer, poor functional status or frailty. Low BP has also been associated with poor function and low quality of life [57,58], but in previous studies only the highest quartile or the clinical cut-off value have been used as predictor of all-cause mortality.

Several methods have been used to define an AL composite index, including the count-based, canonical correlation, z-score, and grade of membership method [59,60]. The most commonly used method is the count-based method, where a summary index is calculated by summing the number of biomarkers falling within the high-risk category, either defined by the percentile (i.e., upper or lower 25th percentile of the sample's distribution) or by the clinical cut-off value. In our analysis with the two-tail cut-off points, 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 for DBP of 1.28 (95% CI: 0.95-1.72). If we have used instead the single high-risk quartile cut-off point, we would have found HRs for LDL-c of 0.71 (95% CI: 0.49-1.03); for SBP of

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 points thus provided a better identification of persons with high mortality than the one-tail cut-off points.

The issue of whether a clinical or sample-based cut-off criteria should be used is still of discussion [17], however, studies comparing distinct measurement approaches have found only modest differences in their predictive utility [15, 61-62].

Strengths and limitations

The strengths of our study included the size of the cohort in terms of the large number of individuals recruited from a general adult population, and the complete follow-up for death by linkage with the Danish Civil Registration System.

Our study also had some limitations. First, the choice of biomarkers used to construct the AL index. The AL theory emphasises the importance of measuring dysregulation across different physiological systems, including biomarkers from the neuroendocrine, cardiovascular, metabolic, and immune systems [13]. The neuroendocrine system (stress response) is believed to play a key role in allostasis and subsequent AL, as a series of physiological changes takes place before initial stress responses occur (such as rapid increases in blood sugar and blood pressure that supply the body with additional energy). However, biomarkers from the neuroendocrine system are difficult to measure, as repeated measurements over 1–2 days are recommended. These requirements cannot be fulfilled in population studies, where participants are examined only once, and biomarkers from the neuroendocrine system were therefore not available for our study.

Secondly, the initial stress responses are followed by secondary outcomes from the metabolic, inflammatory and cardiovascular systems, and these markers were all

available in our data. Nevertheless, greater sensitivity could have been achieved by studying the dynamic changes over time in these markers to fully capture the flexibility of stress response mechanisms across the lifespan.

Finally, differences across studies in construction of AL indices could influence the comparison of results. We used the shape of the association between level of a given biomarker and all-cause mortality as the basis for the categorization of the biomarker into low and high values. One can argue therefore that our analysis was circular in the way that we used outcome on the dependent variable to categorize levels of the independent variable. We believe that this was justifiable in the context here where the purpose was to optimize the predictive power of the AL index. However, validation in other datasets are needed before our approach can be recommended for research in general and for eventual clinical use.

Conclusion

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

Declarations

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The funding bodies had no role in the design of the study, neither in the collection, analysis, and interpretation of data, nor in the writing of the manuscript.

Conflicts of interest

None.

Availability of data and materials and code availability

Data from the study can be made available via Region Sjaelland 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 Lynge 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

Zealand (REG-024-2019 & REG-24-2015). LOFUS is registered in Clinicaltrials.gov (NCT02482896).

Consent to participate

Participants provided written informed consent.

Consent for publication

Not applicable.

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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.

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						
Systolic blood pressure						
- 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)
Diastolic blood pressure						
- 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)
Pulse rate						
- 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)
AL cardiovascular system score						
- 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						
HDL-c						
- 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)
Triglycerides						
- 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)
HbA1c						
- 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)
Waist-hip ratio						
- 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)
LDL-c						
- 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)
AL metabolic system score						
- 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						

CRP						
- 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)
Albumin						
- 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)
AL inflammation system score						
- 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)	1.13 (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)	1.28 (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*	Hazard ratio (95 % CI) 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

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

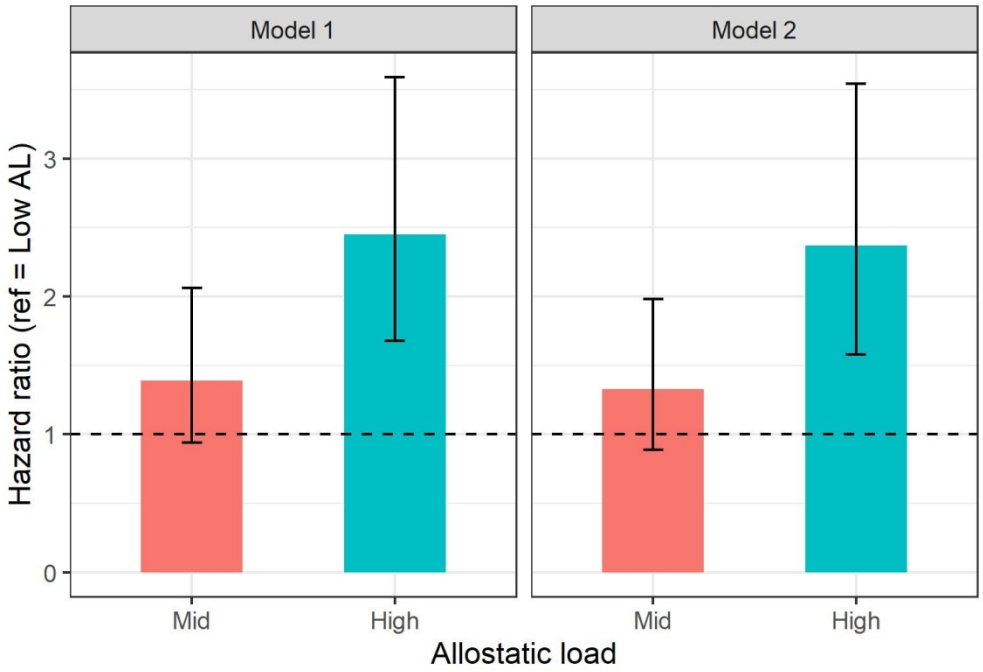
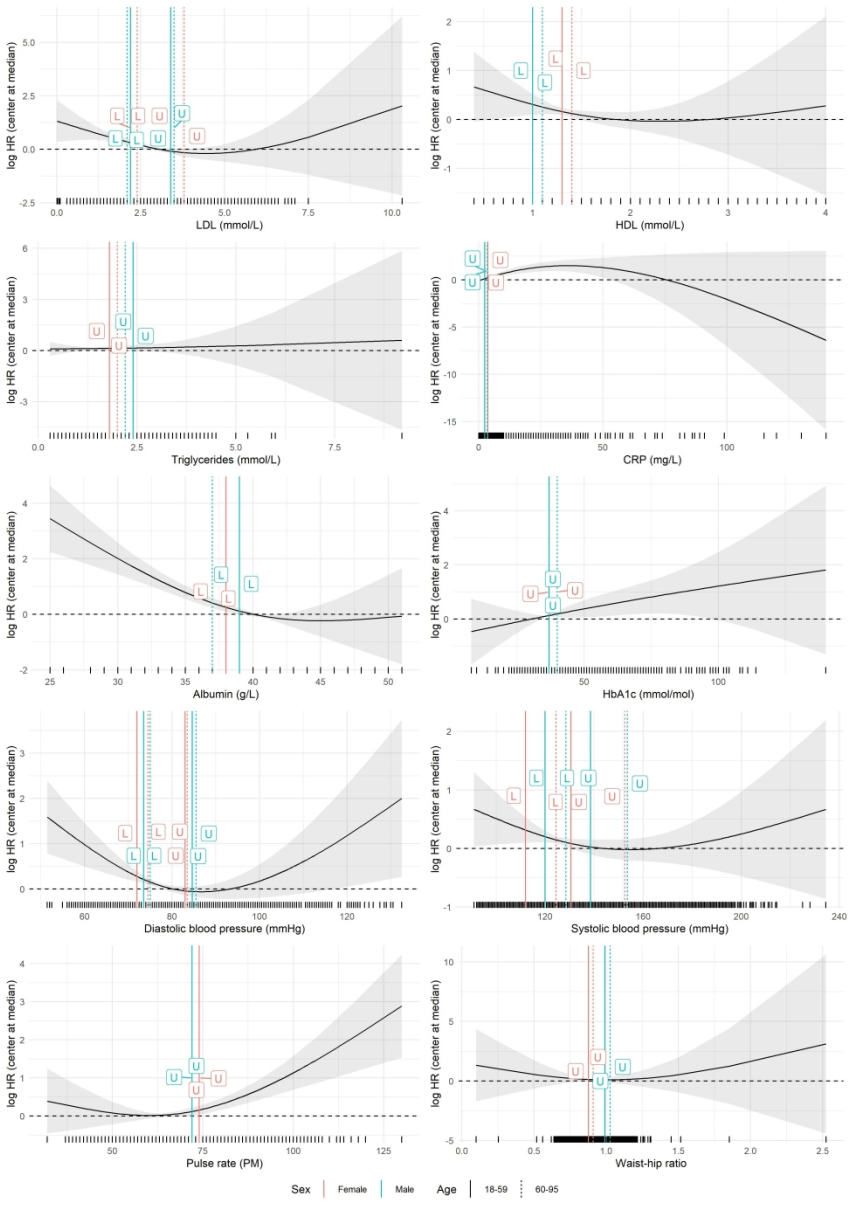


Figure 1. All-cause mortality by level of allostatic load index, as hazard ratio (95% confidence interval).

129x89mm (300 x 300 DPI)



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 N = 13,725	Males N = 6455			Females 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

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 cohortreporting guidelines, and cite them as:

von Elm E, Altman DG, Egger M, Pocock SJ, Gotzsche 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			

Study design	#4	Present key elements of study design early in the paper	5
Setting	#5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Eligibility criteria	#6a	Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up.	5
Eligibility criteria	#6b	For matched studies, give matching criteria and number of exposed and unexposed	N/A
Variables	#7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6,7,8
Data sources / measurement	#8	For each variable of interest give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group. Give information separately for for exposed and unexposed groups if applicable.	6,7,8
Bias	#9	Describe any efforts to address potential sources of bias	15,16
Study size	#10	Explain how the study size was arrived at	5
Quantitative variables	#11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why	N/A
Statistical methods	#12a	Describe all statistical methods, including those used to control for confounding	8,9
Statistical methods	#12b	Describe any methods used to examine subgroups and interactions	8
Statistical methods	#12c	Explain how missing data were addressed	8
Statistical methods	#12d	If applicable, explain how loss to follow-up was addressed	N/A

Statistical methods	#12e	Describe any sensitivity analyses	
N/A			
Results			
Participants	#13a	Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed. Give information separately for for exposed and unexposed groups if applicable.	9
Participants	#13b	Give reasons for non-participation at each stage	N/A
Participants	#13c	Consider use of a flow diagram	
N/A			
Descriptive data	#14a	Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders. Give information separately for exposed and unexposed groups if applicable.	9,28
Descriptive data	#14b	Indicate number of participants with missing data for each variable of interest	
Supplementary table 2			
Descriptive data	#14c	Summarise follow-up time (eg, average and total amount)	
9			
Outcome data	#15	Report numbers of outcome events or summary measures over time. Give information separately for exposed and unexposed groups if applicable.	
NA			
Main results	#16a	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	10,12

Main results	#16b	Report category boundaries when continuous variables were categorized	NA
Main results	#16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
NA			
Other analyses	#17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	9
Discussion			
Key results	#18	Summarise key results with reference to study objectives	9,10
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
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
Generalisability	#21	Discuss the generalisability (external validity) of the study results	15
Other Information			
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