Sedentary behaviour and cerebrovascular disease: molecular mechanisms and the impact of bout duration.

A multicentre cohort study.

Katinka Nordheim Alme

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

This research was performed at the Department for Internal Medicine at Haraldsplass Deaconess Hospital (HDS) and the Department of Clinical Science (K1) at the University of Bergen, Norway. The research project was organised through the Norwegian Cognitive Impairment After Stroke (Nor-COAST) study. Fellow researchers at the office and the Bergen Geriatric Research Group have been valuable for exploring ideas, practising presentation skills, and problem-solving. The work with the biomarkers has been in cooperation with Professor Tom Eirik Mollnes from the Research Laboratory, Nordland Hospital and Professor Emeritus Per Magne Ueland and Arve Ulvik from Bevital A/S.

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Table of contents

Abbreviations

Acetyl CoA: acetyl coenzyme A

AhR: aryl hydrocarbon receptor

BI: Barthel Index

BMI: body mass index

CANTOS: Canakinumab Antiinflammatory Thrombosis Outcome Study

CI: confidence interval

CHD: coronary heart disease

CeVD: cerebrovascular disease

CRP: C-reactive protein

CSVD: cerebral small vessel disease

CVD: cardiovascular disease

eGFR: estimated glomerular filtration rate

FPG: fasting plasma glucose

GLUT4: glucose transporter type 4

HbA1c: glycated haemoglobin A

HOMA-IR: homeostatic model assessment of insulin resistance

HR: hazard ratio

hs-CRP: high sensitive C-reactive protein

IDO: indoleamine 2,3-dioxygenases

IFN-ɣ: interferon gamma

IL-1β: interleukin-1β

IL-10: interleukin-10

IL-6: interleukin-6

IQR: interquartile range

JUPITER: The Justification for the Use of Statins in Prevention

KA: kynurenic acid

KAT: kynurenine aminotransferase

KP: Kynurenine pathway

KTR: kynurenine/tryptophan ratio

Kyn: kynurenine

LDL: low-density lipoprotein

METs: metabolic equivalents

MRI: magnetic resonance imaging

mRS: modified Rankin Scale

MVPA: moderate-to-vigorous physical activity

NAD+: oxidized nicotinamide adenine dinucleotide

Neopt: neopterin

NIHSS: National Institutes of Health Stroke Scale

Nor-COAST: Norwegian Cognitive Impairment After Stroke Study

NSR: Norwegian Stroke Registry

OGTT: oral glucose tolerance test

PA: pyridoxic acid

PAr-index: pyridoxic acid ratio index

PGC-1α: peroxisome proliferator-activated receptor-gamma coactivator-1 alpha

PL: pyridoxal

PLP: pyridoxal-5-phosphate

PPARα: peroxisome proliferator-activated receptor alpha

REK: regional ethics committee

SBRN: Sedentary Behaviour Research Network

SD: standard deviation

TDO: tryptophan 2,3-dioxygenase

TNF-α: tumour necrosis factor-α

TOAST: Trial of Org 10172 in Acute Stroke Treatment

WHO: World Health Organization

WBC: white blood cell

List of publications

Paper I:

Alme KN, Knapskog AB, Næss H, Naik M, Beyer M, Ellekjaer H, English C, Ihle-Hansen H, Kummeneje CS, Munthe-Kaas R, Saltvedt I, Seljeseth Y, Tan X, Thingstad P, Askim T

"Is long-bout sedentary behaviour associated with long-term glucose levels three months after acute ischaemic stroke? A prospective observational cohort study." Published in *BMJ Open* 2020; **10**:e037475. Doi:10.1136/bmjopen-2020-037475

Paper II:

Alme KN, Askim T, Assmus J, Mollnes TE, Naik M, Næss H, Saltvedt I, Ueland PM, Ulvik A, Knapskog AB

"Investigating novel biomarkers of immune activation and modulation in the context of sedentary behaviour: a multicentre prospective ischemic stroke cohort study."

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Paper III

Alme KN, Ulvik A, Askim T, Assmus J, Mollnes TE, Naik M, Næss H, Saltvedt I, Ueland PM, Knapskog AB

"Neopterin and kynurenic acid as predictors of stroke recurrence and mortality. A multicentre prospective cohort study on biomarkers of inflammation measured three months after ischemic stroke."

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Abstract

Background: Sedentary behaviour is associated with vascular disease, and being sedentary for long periods at a time is believed to be associated with the highest risk. The molecular mechanisms are presumed to follow metabolic and inflammatory pathways. Details about these pathways or the length of a clinical significant sedentary bout is not known.

Aims: The primary aim of this study was to investigate the association between sedentary behaviour and novel blood biomarkers with potential predictive and explanatory properties. The secondary aim was to investigate the impact of sedentary behaviour bout length on these biomarkers.

Materials and methods: Patients admitted to hospital for acute stroke were included in the multicentre cohort study entitled the Norwegian Cognitive Impairment After Stroke (Nor-COAST) study (n=815). At the three-month assessment (n=700), sedentary behaviour was measured using the body-worn sensor ActivPAL. Blood samples were drawn for analyses at the local laboratory directly, and biobank samples were stored and later analysed for inflammatory biomarkers at two research laboratories. The long-term outcomes, ischemic stroke recurrence and mortality was identified using national registries.

Results: Glycated haemoglobin A (HbA1c) was positively associated with sedentary behaviour accumulated through bouts of 90 minutes or more. Total sedentary time was associated with higher levels of the inflammatory biomarkers C-reactive protein (CRP), interleukin-6 (IL-6), the pyridoxic acid ratio-index (PAr-index), and neopterin, and lower levels of kynurenic acid (KA). The study did not have enough power for investigating the impact of bout length on these biomarkers. There were no associations between the biomarkers and ischemic stroke recurrence. Mortality was associated with higher levels of CRP. When added to the same model, neopterin and KA showed positive and negative associations to mortality, respectively.

Conclusion and implications: The results support that the impact of sedentary behaviour on disease progression is mediated through known vascular risk factors and novel biomarkers can be useful for future intervention studies.

1. Introduction

Stroke is a leading cause of disability and mortality worldwide (1, 2). With the ageing society, this will continue to increase (3), and reducing the risk of stroke is imperative.

In 1953, a study showing increased risk of coronary heart disease in London bus drivers was published in *The Lancet* (4). Almost 70 years later, the challenges of sedentary behaviour in high-income countries are continuously increasing, and sedentary behaviour has been linked to several types of age-related diseases (5, 6), including stroke (7). Stroke patients are more sedentary than their age-matched peers, independent of functional level (8-11), and the impact of sedentary behaviour is largest in the most sedentary (12-14). Hence, targeting sedentary behaviour in a stroke population as part of the secondary preventive strategy might significantly impact future disease progression (15).

There are several unanswered questions regarding sedentary behaviour and disease development (16). How much is too much sedentary behaviour? Is long-bout sedentary behaviour worse than short? What frequency and intensity of physical activity are needed to counteract the effect of sedentary behaviour (16, 17)? Currently, the research methodology lacks coherence, using a broad range of different methods of measuring and analysing sedentary behaviour. Consequently, the results are hard to compare, and it is difficult to draw any conclusions (17).

The molecular mechanisms mediating the hazards of sedentary behaviour are not known, but they are presumed to follow metabolic and inflammatory pathways (13, 16, 18). Vascular disease progression happens over several years, often decades. During this time, several confounders occur, such as diet, smoking, alcohol consumption, concurrent disease and drug use, occur (16, 17). Biomarkers from the involved pathways with predictive properties might be useful as surrogate endpoints for future disease development (16, 19). Additionally, uncovering molecular pathways will increase our understanding of disease development in general and may serve as targets for preventive and therapeutic strategies (16).

This project investigates the association between sedentary behaviour and blood biomarkers associated with glucose regulation and inflammation in an ischemic stroke population. The predictive properties of the biomarkers, in terms of future ischemic stroke recurrence and mortality, have also been investigated. Finally, the importance of sedentary bout duration has been studied.

The literature search for this thesis was ended at 13.10.21.

1.1 Stroke

1.1.1 Stroke definition

Stroke is defined by the World Health Organisation (WHO) as "rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin" (20). In societies where magnetic resonance imaging (MRI) is widely available, this definition is insufficient. MRI can identify ischemic lesions without overt symptoms, so called silent infarctions, and lesions where the clinical symptoms have disappeared (21). The more recent definition from the American Heart Association also includes findings on imaging (21). Stroke is included in the term cerebrovascular disease (CeVD), which has overlapping features with coronary heart disease (CHD). Stroke and CeVD are often used interchangeably. Both CeVD and CHD are included in the term cardiovascular disease (CVD) (2), often used as combined outcomes in studies. Stroke can be ischemic or haemorrhagic. The proportion of ischemic strokes depends on the population in question, but in high-income countries, this is reported to be 90% (22). Ischemic stroke is defined as a central nervous system infarction due to reduced blood flow (ischemia) (21). Ischemic strokes are categorized into five different subtypes: 1) large-artery atherosclerosis (embolus/thrombosis), 2) cardioembolism, 3) cerebrovascular small vessel disease (lacune), 4) stroke of other determined aetiology (dissection, vasculitis, specific genetic disorders, and others), and 5) stroke of undetermined aetiology (23). Undetermined can be "unknown" or more than one underlying aetiology (23). Ischemic stroke will be the focus for the rest of the outline, emphasising the three main

subtypes: large-artery atherosclerosis, cardioembolism, and cerebrovascular small vessel disease.

1.1.2 Stroke risk factors

Ischemic stroke risk factors can be described through three main categories: lifestyle, metabolism and inflammation (24, 25). The impacts of the risk factors are different but overlapping between the stroke subtypes, and the main differences will be outlined below. Lifestyle risk factors – in addition to physical inactivity and sedentary behaviour – are diet, smoking and alcohol. Their effect is mediated through their impact on metabolic (26) and inflammatory pathways (18, 24). There are five metabolic risk factors: hypertension, hypercholesterolemia, dyslipidaemia, diabetes mellitus and obesity (24, 26). Inflammation can be acute or chronic, and in the rest of the thesis, the focus will be on chronic low-grade inflammation (27).

1.1.3 Stroke subtypes

Large-artery atherosclerosis

Atherosclerosis of the carotids, vertebral arteries, and the major intracranial arteries are the leading causes of stroke due to large-artery atherosclerosis (19). In a population study, this accounted for 8.4% of ischemic strokes (28). The primary risk factors for atherosclerosis are dyslipidaemia, diabetes mellitus, hypertension, and smoking (29, 30). The phenomena of "residual risk of atherosclerotic disease" despite optimal risk factor control are emphasised in the current literature (30). Inflammation has risen as one of the most important explanations for this residual risk, based on studies showing an effect of targeting inflammation on the risk of atherosclerotic disease (30, 31).

Cardioembolism

Cardioembolic strokes are caused by an embolus originating from the heart, and cardioembolism has been found to account for 23.0% of ischemic strokes (28). The underlying cause is most often atrial fibrillation (19, 24). The risk of atrial fibrillation-

related thromboembolism is associated with alterations in blood flow, endothelial injury, and hypercoagulability (32). In the clinical setting, the assessment of these risk factors is done using the CHA2DS2-VASc score. This is a clinical decision-making tool for anticoagulant drug therapy use, which includes information about age, sex, and the presence of congestive heart failure, hypertension, diabetes mellitus, and prior vascular disease or thromboembolism (33, 34). Biomarkers indicating altered blood flow, endothelial injury, or hypercoagulability might be useful for additional risk assessment but are not used routinely (32, 33).

Cerebral small vessel disease

Cerebral small vessel disease (CSVD) is identified as lesions in subcortical grey and white matter (19), presenting as lacunes, small subcortical infarctions, white matter hyperintensities, enlarged perivascular spaces, microbleeds and atrophy in magnetic resonance imaging (MRI) (35). CSVD, in the form of small vessel occlusion, accounts for around 25% of all ischemic strokes (28). Often, they do not present like clinically overt strokes but as a gradual cognitive and functional decline, and findings of lacunas on MRI (35, 36). The risk factors of small vessel disease follow the traditional cardiovascular risk factors described above, but with an emphasis on hypertension and diabetes mellitus (37, 38).

1.1.4 Stroke prevention

The preventive strategies in stroke are organised as primary or secondary prevention and differ between the subtypes. The common principle for secondary prevention is "risk factor control", targeting lipids, glucose regulation, blood pressure, smoking, diet, physical inactivity and adiposity. Also, blood thinners are essential (24).

1.1.5 Measuring functional stroke outcomes

Advances in acute stroke care and primary- and secondary preventive treatment have led to a need for more refined outcome measures beyond mortality and recurrence rate,

such as functional abilities (39). Functional abilities can be described in terms of impairment, activity, and participation (40), and these domains can be assessed using a range of tools. The tools need to be valid; measure what they are supposed to be measuring, and reliable; be consistent between measures (39).

With the introduction of acute stroke treatment, the 15-item stroke scale, the National Institutes of Health Stroke Scale (NIHSS) was developed to measure the immediate effects of treatment (41). This is now the most frequently used tool to assess stroke severity as an expression of impairment (39). The NIHSS is valid compared to stroke volume and for prediction of future care needs when used as categories but not for functional impact when used as a continuous variable. It has high reliability between several measures made by the same rater (intra-rater reliability), but it is less reliable between raters (inter-rater reliability) (39).

Barthel Index (BI) of function in activities of daily living and the modified Rankin Scale (mRS) of global disability are measures of activity and participation, respectively (39). The BI was first described in 1965 (42) and is a 10-item scale depicting different basic activities of everyday life which are crucial for independence (43). The validity is moderate, showing less correlation to infarct size and more to other measures of function, future recovery and care needs. The reliability is moderate (39). The mRS scale is a five-point scale measuring global disability, where 0–2 is defined as 'good outcomes', 3–5 indicates increasingly disability, and 6 is death (44). The validity is moderate and best compared with other measures of function and less compared to stroke volume. The inter-rater reliability has been found to be low (39).

For both the BI and mRS, the validity is reduced if the assessment is made too close to ictus (39). The NIHSS has been found to be more sensitive to meaningful change over time compared to the mRS and the BI. In this study, functional outcomes were used to describe the population.

1.2 Sedentary behaviour

Sedentary behaviour is a risk factor for stroke and is associated with vascular risk factors such as diabetes mellitus, dyslipidaemia, adiposity and inflammation. The dangers of sedentary behaviour are probably mediated through these risk factors (16). Sedentary behaviour has been defined as "any waking behavior characterized by an energy expenditure ≤1.5 metabolic equivalents (METs), while in a sitting, reclining or lying posture" (17). On the other hand, the term "physically inactive" refers to a person who is not reaching the physical activity guideline recommendations (45). Hence, a person can be highly sedentary but physically active. Physical exercise is a subset of physical activity but with the additional objective of improving or maintaining physical fitness. Exercise might have a higher intensity than everyday activities (46). Therefore, when sleep is excluded, time in sedentary behaviour and physically activity are inverse sizes (Figure 1) (17). This dichotomous definition does not capture energy expenditure. The issue of measuring energy expenditure will be addressed below.

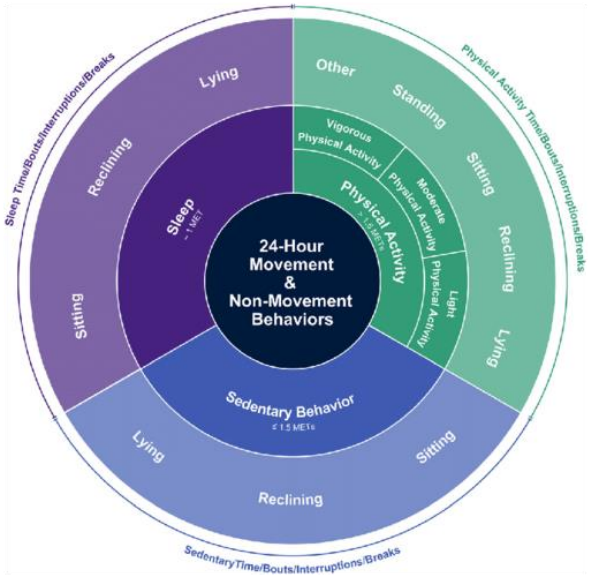

Figure 1: 24-hour movement and non-movement behaviours as defined by the Sedentary Behavior Research Network (SBRN). From Tremblay et al. 2017 (17). Reproduced in line with the terms of Creative Commons Attribution 4.0 International Licence https://creativecommons.org/publicdomain/zero/1.0/.

1.2.1 Measuring and analysing sedentary behaviour

Measuring sedentary behaviour

The standard method for measuring sedentary behaviour has not been defined. However, the Sedentary Behavior Research Network (SBRN) does emphasise the use of objective measures, such as body-worn accelerometers or inclinometers (17). The accelerometer counts oscillation frequency and converts this to METs (47). The reliability between different devices is low, in particular for persons with low gait speed. The discrepancy in the frequency count might be caused by different placement protocols between the devices, type of task, intensity of the activity, population and the technical properties of the device (47). Discrepancies in energy estimation can also be caused by differences in the device-specific algorithms for energy expenditure calculation (47). Hence, the validity of the conversion norm may vary between the devices. Also, the standard conversion norm for energy expenditure is based on a healthy population and is not validated for a stroke population (48). In one study, four different devices, with a total of 14 placements, were validated up against the gold standard, indirect calorimetry, in stroke patients. Only one device which was worn on the arm, the SenseWear Armband ®, showed acceptable results when estimating energy expenditure in stroke patients doing everyday tasks (49). This device also measured temperature and galvanic skin response and included these in the algorithm for energy expenditure (50).

Thus, differing results between the devices are probably due to the low reliability of the frequency measure and validity of the conversion algorithm.

Inclinometers placed on the thigh measure position. It can identify lying/sitting, standing or stepping and has been validated in a stroke population (51). In healthy persons, the energy expenditure used when standing is found to be above the threshold of sedentary behaviour (52, 53), and stroke patients are found to have an even higher energy expenditure (48). Hence, in compliance with the SBRN definition illustrated in Figure 1, when excluding sleep, time in standing or stepping is non-sedentary, and the remaining time can be used as a measure of sedentary behaviour in patients that are able to be mobilised to a standing position (54). This method does not capture non-

sedentary behaviour in a sitting/reclining/lying position, such as active sitting (55). Also, it does not differentiate between levels of energy expenditure during non-sedentary behaviour (48).

Other methods for quantifying sedentary behaviour are self- or proxy-completed questionnaires about sedentary behaviour in general or screen-time in particular. The test properties for many of the questionnaires have never been investigated. For the tools that are validated, the reliability has been acceptable, but the validity is low (17).

In this project, sedentary behaviour has been measured by identifying position using a single thigh-worn sensor and defined as the time in a sitting or lying position. This will be the focus for the further discussion.

Analysing sedentary behaviour data

Sedentary behaviour can be accumulated in different ways, giving rise to a person's sedentary behaviour pattern which is "the manner in which sedentary behaviour is accumulated" (17). When analysing the data gathered using body-worn sensors, sedentary behaviour patterns such as habit and bout duration are important, in addition to identifying sleep and non-wear time (16, 17).

Habitual sedentary behaviour

Habitual activity patterns are also called "chronic" behaviour (13). In contrast, an acute change is typically a one-day intervention. Habit is important when reading literature and designing studies because a short-term change elicits a different response in the body compared to a change of habit over several months (13). The definition of "chronic" is not uniform, but a duration of more than three months is often used in intervention studies (13, 56).

Sedentary behaviour bout duration

Breaking up sedentary behaviour into shorter periods, or bouts has been found to be important to reduce the harmful effect (13). There is no consensus of what the duration of a clinically significant sedentary bout is. In intervention studies, 30 minutes has often been used as a limit for breaking up sedentary time. The actual threshold is unknown and might vary depending on the physiological process in question (8, 17, 57). Different measuring methods have been used, such as the numbers of sedentary bouts, mean bout duration, or numbers of breaks from sedentary behaviour (17). Measuring time in sedentary behaviour accumulated through different bout-length categories is recommended because it is the only method that includes both time and bout duration in the same measure (17).

Sleep

Sleep is distinct from sedentary behaviour (17), but the inclinometers cannot discern between these two non-movement behaviours (53). Sleeping patterns change with age (58) and disease (59, 60), and finding a valid method for assessing sleep time is challenging (53). One commonly used method is to predefine a time period of assumed wake-time based on commonly accepted diurnal rhythms (53). Some studies also include the use of a diary. This method is time-consuming for the participant and the researcher. The information is often registered in retrospect, which reduces the precision of the registration and increases the risk of missing data (53). It is also possible to use the visual output of the individual recording to identify periods with no or minimal position transition, illustrated with a colour change (Figure 2) (53). Different processing algorithms have also been developed. They have not been validated in a stroke population and are often less precise in patients with deviating sleeping patterns (11, 61).

Non-wear time

If the monitor is not attached to the patient, the device will still be recording but in a constant position. It has been suggested that excluding periods longer than 8 hours of sitting/lying as non-wear in daytime recordings could be a valid method. Visually evaluating the data is also possible, but this can be time-consuming in large populations (53).

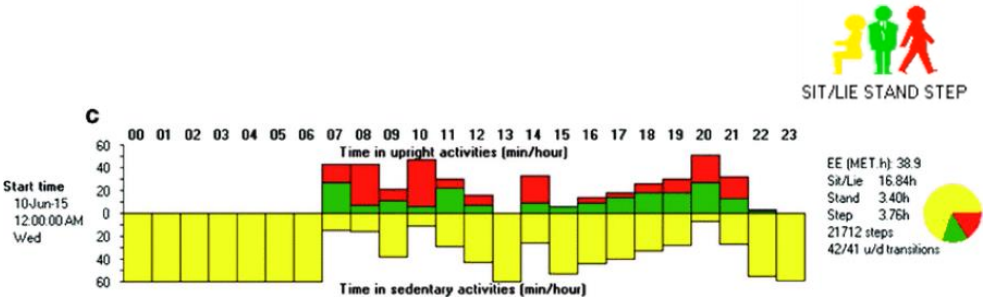

Figure 2: Graphical output of activity monitoring data. Yellow colour is sitting or lying, green is standing, and red is stepping. Credits: PAL-Technology ®. The illustration is used with permission.

1.3 Biomarkers

A biomarker is "a defined characteristic that is measured as an indicator of a normal biological process, pathogenic process or responses to an exposure or intervention" (62). From the perspective of sedentary behaviour and vascular disease, this can be measures of hyperglycaemia, hypercholesterolemia, dyslipidaemia, hypertension, obesity (31, 63), and inflammation (25).

In the rest of the thesis, the focus will be on the blood biomarkers. The blood samples in this study were taken non-fasting, and triglycerides were therefore not available. The study and the outline below are therefore limited to blood biomarkers of glucose regulation and inflammation.

1.3.1 Glucose regulation, sedentary behaviour and vascular disease

Measuring glucose regulation

Assessment of glucose regulation has changed from estimating urine glucose levels either by taste or more sophisticated methods (64) to measuring plasma glucose values in a fasting state, fasting plasma glucose (FPG), or after oral loading with a standardised amount of glucose in the oral glucose tolerance test (OGTT) (65, 66). In recent years, measuring "long-term glucose", representing the mean value of blood glucose levels in the past three months using glycated haemoglobin (HbA1c), has become the standard. The Hba1c is found to be less sensitive for the diagnosis of diabetes mellitus compared to the FPG and OGTT. Neither of these measurements can discern between insulin deficiency and insulin resistance. Measurements such as C-peptide and autoantibodies are available for this purpose, but the analyses are not used routinely in the adult population (64, 67). The homeostatic model assessment of insulin resistance (HOMA-IR) measures insulin resistance using fasting plasma glucose and insulin values (65). This method is not used routinely in the clinical setting but is frequently used in research (68).

Glucose regulation and sedentary behaviour

Glucose levels are associated with sedentary behaviour (69), with the highest impact of sedentary behaviour on the most sedentary (14) and those with impaired glucose regulation (14). The effect of increasing physical activity is presumably mediated by the recruitment of the glucose transporter type 4 (GLUT4) in the muscle cell wall by muscle contraction, which otherwise is recruited by insulin (70). Hence, this can explain the greater importance of physical activity in patients with insulin deficiency and why sedentary behaviour seems to contribute less in individuals with preserved glucose regulation (14, 70, 71).

Glucose regulation and vascular disease

Diabetes mellitus has been associated with an increased risk of CHD and stroke after adjusting for other risk factors, including inflammation (72). In patients with diabetes mellitus, FPG (72) and HbA1c (73) are associated with CVD in general and ischemic stroke in particular. In contrast, a recent meta-analysis of studies investigating the association between insulin resistance, measured by the HOMA-IR, and the risk of stroke, did not find any association (74). The authors emphasised, however, that the results should be interpreted with caution because the meta-analysis was based on a small number of studies (74).

1.3.2 Inflammation, sedentary behaviour and vascular disease

Inflammation protects us from exogenous pathogens and is crucial for tissue damage repair. If inflammation does not resolve as it is supposed to but sustains as chronic low-grade inflammation, this is associated with disease development (27, 75, 76). Inflammageing, the tendency towards chronic low-grade inflammation associated with age, is believed to be an important contributor to biological ageing (75, 76).

The immune system

The immune system has two main divisions: the innate and the adaptive. Innate immunity is present from birth, while adaptive immunity evolves in response to experience by gene recombination in lymphoid T cells and B cells (77) (Figure 3). B cells are responsible for producing soluble antibodies, while T cells execute the inflammatory response by effector T cells known as T helper cells, T killer cells and T regulatory cells (77). T regulatory cells are essential for immune regulation, and the balance between T helper cells and T regulatory cells has been used as a measure of immune tolerance (78).

The innate immune system can react de novo in an unspecific manner. However, studies show that the responsiveness is dependent on prior triggers and the differentiation of monocyte-derived macrophages into a phenotypic spectrum ranging between two inflammatory activation patterns (79). The classic (M1) or alternative

(M2) activation pattern gives rise to a pro-inflammatory or anti-inflammatory phenotype, respectively (77, 80). In contrast to the gene recombination seen in the adaptive immune system, these changes are mediated through epigenetic reprogramming, which by nature is dynamic according to exposure (79).

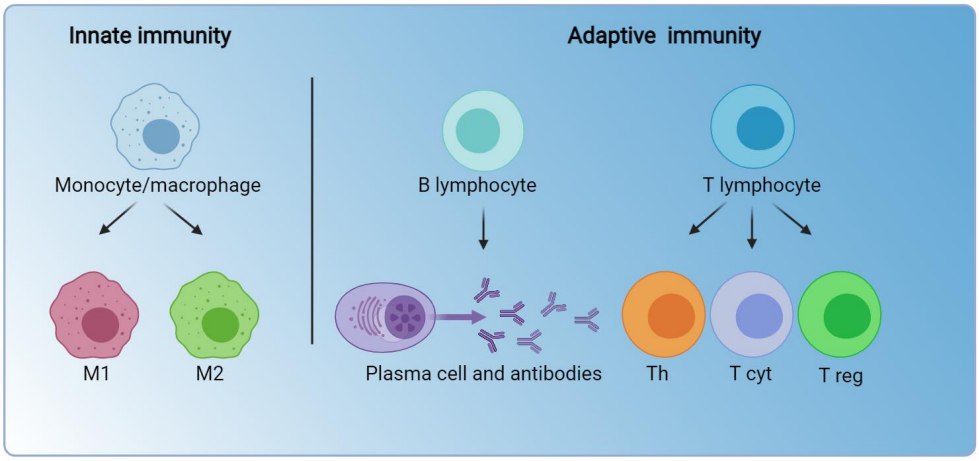

Figure 3: Innate and adaptive immunity. Simplified schematic presentation of the relevant parts of the innate and the adaptive immune system. M1= M1 macrophage. M2= M2 macrophage. Th= T helper cell. Tcyt= T cytotoxic cell. Treg= T regulatory cell. Adapted from Abbas et al., 2018 (77), made with BioRender.com.

Measuring immune system activation and functioning

In this section, there will be a short outline of a small selection of cytokines and other inflammatory biomarkers relevant for this thesis. A description of the biomarkers through their role in one of two pro-inflammatory pathways or anti-inflammation and immune tolerance will follow.

Cytokines – general description

Cytokines are the principal mediators of communication between cells in the immune system and consist of interleukins, chemokines, interferons and growth factors (81, 82). Immune cells can secrete a range of cytokines, and different cells have different but overlapping cytokine profiles. The cytokines are often described as having a predominantly pro-inflammatory or anti-inflammatory effect (83). They are also often

described as part of the innate or the adaptive immune system, based on the principal expressing cell, even though their effect is not restricted to these borders (Figure 4) (77). For example, the defining cytokine of T helper 1 cells in the adaptive immune system, interferon-ɣ (IFN-ɣ), is the main driver of the classical activation of macrophages (M1) in the innate immune system (77). Cytokines are pleiotropic, and they also work in concert with their environment: when a cytokine is expressed in the tissue, the response depends on other cytokines nearby. They can act synergistically or antagonistically, and the response also depends on the duration and strength of the expression (81, 84). Hence, a short description will always be a simplification.

The cytokines vary in their biological and analytical properties, such as diurnal and seasonal variations and in vivo and in vitro stability (84). Consequently, some cytokines are more often measured indirectly by downstream molecules more reliably measured in blood (85-87). These downstream biomarkers might be specific for a particular inflammatory pathway or capture several modes of inflammation (85). They can be biologically active molecules or merely indicators without biological effect (85).

The cytokines used in this study are the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), and IFN-ɣ, and the anti-inflammatory cytokine interleukin-10 (IL-10). They will be more thoroughly described below.

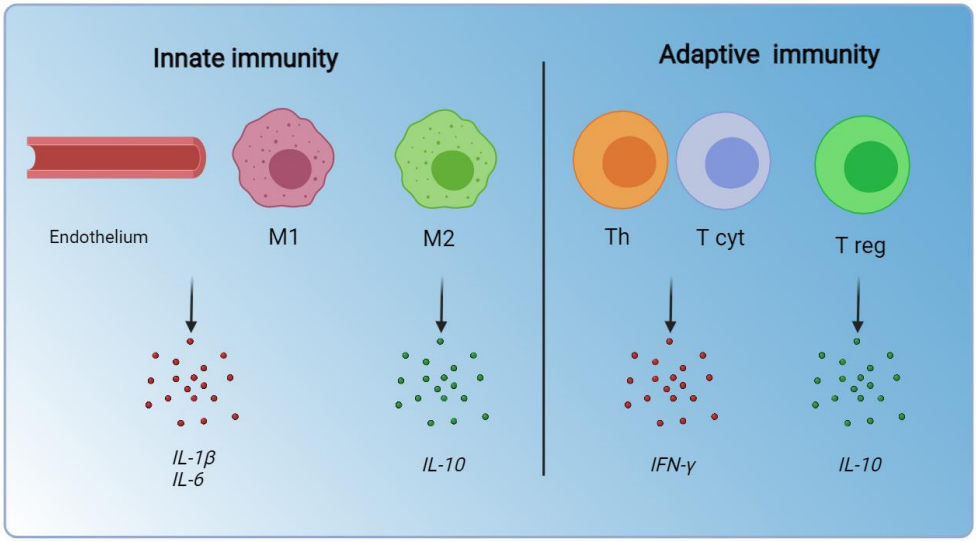

Figure 4: Principal cell source for the described cytokines. M1=M1 macrophage. M2= M2 macrophage. Th= T helper cell. T cyt= T Cytotoxic cell. T reg=T regulatory cell. IL-1β=interleukin-1β. IL-6=interleukin-6. IL-10=interleukin-10. IFN-γ=interferon- γ. Adapted from Abbas et al., 2018 (77). Graphics made with BioRender.com

Indirect biomarkers of cytokine activity

The most frequently used indirect biomarker of inflammation is the acute phase protein, C-reactive protein (CRP), produced in the liver in response to IL1-β and IL-6 during a pro-inflammatory state (85). Another non-protein cellular product, the pteridine neopterin, is produced by macrophages in response to IFN-ɣ from activated T helper 1 cells (88, 89). Metabolic pathways, such as the metabolism of tryptophan and vitamin B6, can be affected by cytokines, and the metabolites can serve as inflammatory biomarkers. These two metabolic pathways will be described before the inflammatory pathways are presented.

The metabolism of tryptophan – the kynurenine pathway (KP): The essential amino acid tryptophan is involved in protein synthesis and is a precursor of many biologically important metabolites (90). The KP is the quantitatively most important metabolic pathway (Figure 5). Here tryptophan is metabolised to kynurenine by the enzyme tryptophan-2,3-dioxygenase (TDO) under homeostatic conditions, accompanied by

indoleamine 2,3-dioxygenases (IDOs) during an inflammatory state (90). IFN-ɣ, in particular, induces IDO, and the ratio between kynurenine and tryptophan (KTR) has been used as an indirect measure of IFN-ɣ-activity (68, 90, 91). The KTR and one of the end products, kynurenic acid (KA), are included in this thesis.

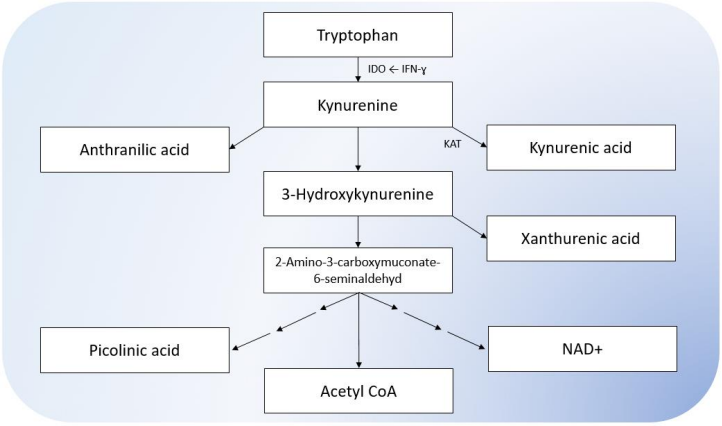

Figure 5: A simplified presentation of the kynurenine pathway. IDO = indoleamine 2,3-dioxygenase. IFN-ɣ = interferon gamma. KAT=kynurenine aminotransferase. Acetyl CoA = acetyl coenzyme A. NAD+ = oxidized nicotinamide-adenine dinucleotide. Adapted from Badawy et al., 2017 (90).

Metabolism of vitamin B6: Another metabolic pathway affected by inflammation is the metabolisation of vitamin B6. The discovery came from the observed association between inflammation and reduced levels of vitamin B6 (92, 93) but without any beneficial effect of B6 supplements on inflammatory biomarkers or clinical outcomes (94-96). Further research implied that the observed association was caused by an increased uptake and metabolisation of vitamin B6 in response to several modes of inflammation independent of vitamin supplement intake (97, 98). A ratio between the different metabolic stages of vitamin B6, the pyridoxic acid ratio index (PAr), has been suggested as an inflammatory biomarker (97). This biomarker seems to capture several modes of inflammation, measured by CRP, markers of cellular immunity such as KTR and neopterin, and white blood cell count and might be useful to gain knowledge of prognosis and pathophysiology (Figure 6) (97).

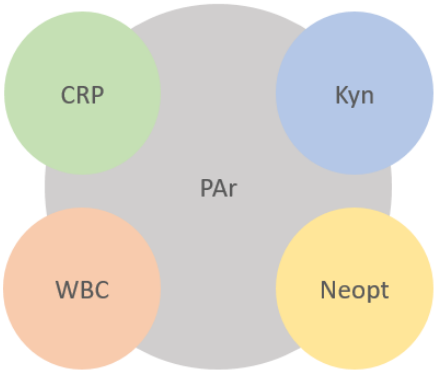

Figure 6: A schematic presentation of the PAr-index. PAr captures several modes of inflammation. CRP, kynurenine, white blood cell count and neopterin can describe 23% of the variation in PAr. CRP=C-reactive protein. Kyn = kynurenine. WBC= white blood cell count. Neopt = neopterin. Based on Ulvik.et al. (97).

The biomarkers' role in inflammation

All the biomarkers described above will in the rest of the thesis collectively be called "biomarkers of inflammation". For ease of the discussion, the biomarkers will be described through their role in the pro-inflammatory interleukin-1β pathway (IL-1β, IL-6, CRP) or interferon-ɣ pathway (IFN-ɣ, neopterin, KTR) (Figure 7). There will also be a description of the biomarkers' role in anti-inflammation (IL-10) and immune tolerance (KA). The rationale for the choice of biomarkers will follow.

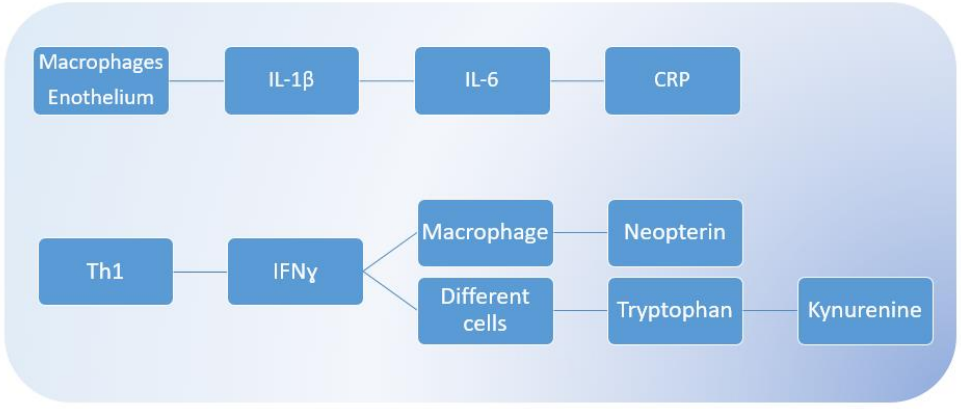

Figure 7: A schematic presentation of the interleukin-1β pathway (IL-1β, IL-6, CRP) and the interferon-ɣ pathway (IFN-ɣ, neopterin, KTR) and their associated biomarkers.

The interleukin-1β pathway: IL-1β is a pro-inflammatory cytokine, mainly produced in macrophages and endothelial cells, with a central role in mediating many inflammatory responses (77, 83, 99). The IL-1β pathway also consists of the two downstream molecules, IL-6 and CRP (85), and is closely linked to the innate immune response through the macrophages (83, 99). IL-1β is not reliably measured in blood, but its activity can be measured by IL-6 and CRP (85). IL-6 is a pro-inflammatory cytokine secreted by macrophages and T helper cells (83) in response to IL-1β and tumour necrosis factor-α (TNF-α) (85, 100). In concert with other cytokines, IL-6 is considered important for disrupting immune tolerance through the balance between T helper cells and T regulatory cells, thus, being a link between the innate and the adaptive immune system (101). Together, IL-1β, TNF-α and IL-6 induce the production of CRP (83). CRP is the most frequently used inflammatory biomarker in the clinical setting but does not have any biological effect in itself (85). The use of the high-sensitive CRP (hs-CRP) technique has enabled research on low-grade chronic inflammation.

In addition to being a cytokine from inflammatory cells, IL-6 is produced as a myokine from muscle cells upon contraction. This will be described in the section *"Anti-inflammation and immune tolerance"*.

The interferon-ɣ pathway

Interferon-ɣ (IFN-ɣ) is mainly produced by activated T helper 1 cells, a subgroup of the T helper cells, and is a central cytokine in the adaptive immune system. The main role of IFN-ɣ is to activate monocytes as part of the defence against viruses (77). Investigations using recombinant IFN-ɣ have shown a short in-vivo halftime, and there is reason to believe that it cannot be measured reliably (102). Hence, IFN-ɣ activity is often measured indirectly by neopterin from activated macrophages (88, 89) or by the kynurenine/tryptophan ratio (KTR) as a measure of IDO activation (103).

Anti-inflammation and immune tolerance

The most important anti-inflammatory cytokine, IL-10, suppresses unnecessary immune responses by inhibiting mononuclear cell functioning and preventing cytokine production in immune and epithelial cells (83, 104). Studies indicate a downregulation of IL-10 associated with IDO-deficiency and the pro-inflammatory M1 macrophage phenotype (105, 106). IL-6 produced as a myokine from muscle cells in response to muscle contraction is associated with beneficial metabolic (100) and anti-inflammatory properties (84). Without a concurrent spike of TNF-α, this spike of IL-6 will not induce CRP but will induce the production of IL-10 (106, 107).

In 1998, Munn et al. published a paper indicating that IDO activity was important for maternal immune tolerance to the fetus by inhibiting T-cell mediated rejection (108). Since then, the importance of the KP in controlling inappropriate immune response has been a significant field of research (75, 90, 109). Kynurenine can be further metabolised to kynurenic acid (KA) by the enzyme kynurenine aminotransferase (KAT) (90). Kynurenine and KA are part of a negative feedback loop of inflammation, inducing immune tolerance, probably through induction of regulatory T cells (56, 90, 91, 110-113). KA might be a useful measure of the body's anti-inflammatory potential (56).

Inflammation and sedentary behaviour

Sedentary behaviour is associated with higher levels of inflammatory biomarkers. IL-6 and CRP are the most studied (18, 114-116). The anti-inflammatory IL-10 has been found to be negatively associated with sedentary behaviour and to increase in response to physical activity (117, 118). The induction of IL-10 by muscle-derived IL-6 might be one of the mechanisms. Additionally, regular exercise has been associated with an increased number of M2 macrophages in muscle and adipose tissue, which also secretes IL-10 (106, 119) (Figure 8).

There are no studies investigating the kynurenine pathway or neopterin and objectively measured sedentary behaviour. However, in a study using questionnaires to investigate

habitual physical activity levels, neopterin was increased in older persons with a reduced physical activity level compared to a group with normal physical activity levels. The KTR was not affected (120). In contrast, acute bouts of exercise have been associated with increased kynurenine and reduced tryptophan (121), hence, a higher KTR.

There seems to be a difference between the impact of acute bouts of exercise and chronic exercise (106). They are both believed to contribute to immune regulation via the kynurenine metabolism, but the evidence for chronic exercise are diverging (56). It is also questioned whether the kynurenine pathway has a role in immune regulation only after an initial activation, for example by inflammation or exercise (122). Exercise has been found to induce the enzyme converting kynurenine to the end product KA, KAT, via peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α1) and the associated transcription factor, peroxisome proliferator-activated receptor alpha (PPARα) (123). KA has never been investigated in the context of sedentary behaviour.

The PAr-index seems to capture the effect of several inflammatory pathways. PAr has never been investigated in the context of sedentary behaviour or physical activity and might be sensitive to the combined effect of the different pathways (97).

The evidence is based on a mixture results from studies of sedentary behaviour, physical activity and exercise, and literature from all three types of studies are used in this thesis (16, 18).

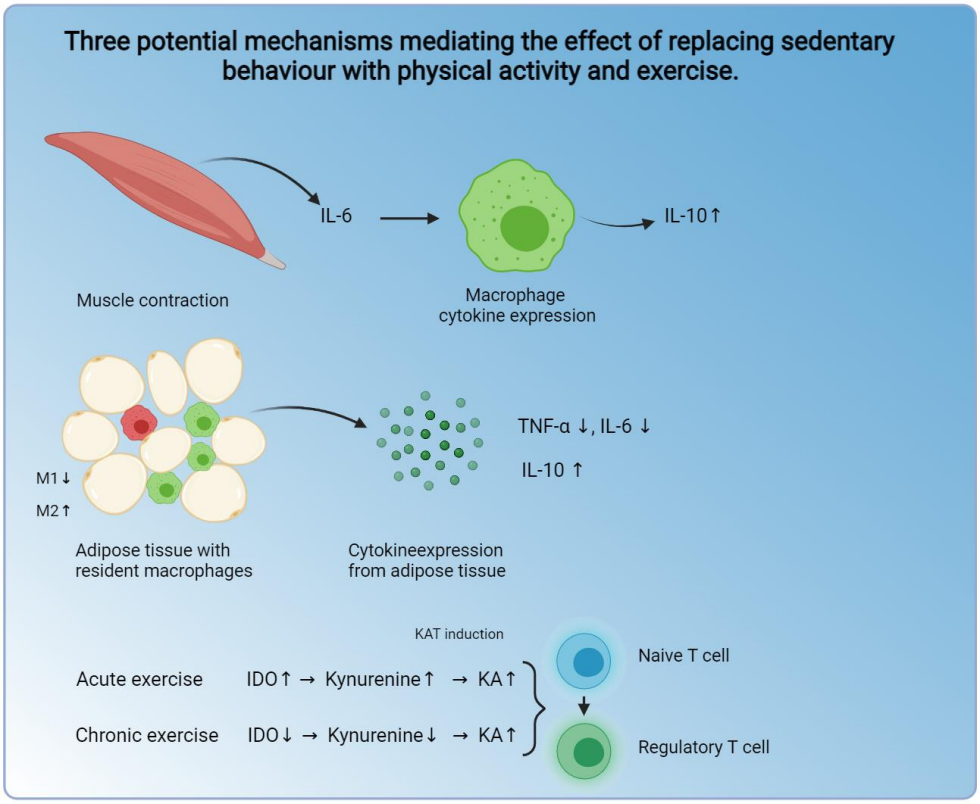

Figure 8: Three potential mechanisms mediating the beneficial effect of replacing sedentary behaviour with physical activity and exercise. 1) Myokine (IL-6) in response to muscle contraction inducing IL-10. 2) Phenotypic change of resident macrophages in adipose tissue to the anti-inflammatory M2 phenotype leading to a change in the cytokine expressed from adipose tissue. 3) Increased production of KA because of KAT induction by muscle contraction, leading to the differentiation of T regulatory cells from naïve T cells. Adapted from Joisten et al., 2020 (56) & Gleeson et al., 2011 (106). Made with Biorender.com.

Inflammation and vascular disease

Almost 25 years ago, Attilio Maseri called the inflammatory hypothesis of vascular risk a "glimpse at the hidden side of the moon" (124). Over the years, this "moon" has revealed itself bit by bit (25, 125-129). In 2010, the Justification for the Use of Statins in Prevention (JUPITER) trial showed that statins reduced the risk of ischemic stroke independent of lipid levels in patients with elevated values for CRP in an apparently healthy population (130). This led to the "proof of concept study", the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS), in which Ridker et al.

targeted inflammation without affecting lipid levels using monoclonal antibodies against IL-1β in a group of patients with prior myocardial infarction and CRP levels above two. They found lower recurrence rates of cardiovascular events, including stroke, in the treatment group (126). The effect of anti-inflammatory drugs has also been shown for colchicine but not for methotrexate, investigated in populations with prior myocardial infarction, with a vascular outcome that included ischemic stroke (131, 132). The differences in effect are probably due to different inflammatory pathways (133). At this time, there is no routine treatment targeting inflammation to lower vascular risk besides statins. Also, anti-inflammatory and immunosuppressive drugs are associated with side effects, such as infections which need to be considered (133).

The CANTOS trial was the final affirmation of the role of the IL-1β pathway in cardiovascular disease (25, 126, 129, 130). IFN-ɣ activity, measured by neopterin and KTR, has also been associated with coronary events and cardiovascular mortality (127, 128, 134, 135). The particular role of KA in disease progression is not fully understood. KA has been found to be associated with coronary events (136-139) but not with stroke (137). PAr has been found to be associated with the risk of future stroke in a population study and long-term mortality risk in patients with CHD (127, 140). The association was independent of CRP, suggesting that CRP does not capture the entire inflammatory process associated with increased stroke risk (127). It is argued that PAr, neopterin and KTR capture overlapping but distinct aspects of inflammation associated with future mortality in the group of patients with vascular disease (140). PAr has never been investigated in a stroke population.

The underlying pathophysiology differs between the stroke subtypes. All of them are associated with inflammation (9, 141, 142), but more evidence exists for atherosclerotic disease. This is, amongst other reasons, because of the research on CHD.

Atherosclerosis and inflammation

Atherosclerosis is a low-grade inflammatory disease (9), and higher levels of inflammation correspond to more unstable disease (9, 143, 144).

A polarisation towards the pro-inflammatory M1 mononuclear cells in the circulation and the atherosclerotic plaque has been found to correspond with the atherosclerotic load (9, 143). Targeting the mononuclear cell polarisation, inducing M2 polarisation, could be a potential therapeutic strategy in atherosclerosis. The effect associated with M2 polarisation on plaque regression is believed to be mediated by, amongst others, the anti-inflammatory cytokine IL-10 (9).

IL-1β, IDO activity measured by gene expression and neopterin have been found to be associated with plaque formation, the complexity of atherosclerotic plaques and plaque instability (9, 145, 146). When the associations between atherosclerosis and IDO activity is investigated using the KTR, the results are more diverse (147, 148)**.** The upregulation of IDO is believed to be protective, and inhibition has been associated with plaque progression (149-152)**.** The downstream molecule, KA, has also been associated with atherosclerosis and is suggested as a potential biomarker of atherosclerotic load (150, 153). As for IDO, KA has also been found to stabilise and decelerate the progression of atherosclerotic disease. Its role in disease development, particularly its role in immune tolerance, is under debate (150, 151).

Cardioembolic strokes and inflammation

After an acute ischemic stroke, no specific cause is found in 32% of the cases (154). At least a subgroup of these patients might have paroxysmal atrial fibrillation not yet captured by any assessment (155). Inflammation is believed to be an important part of the underlying pathology of arrhythmia, and IL-6 has been associated with an increased risk of atrial fibrillation in some studies but not all (32, 141, 156-158). It has been suggested that the use of inflammatory biomarkers could be useful to identify those patients in need of prolonged cardiac monitoring (157).

Inflammation is also believed to be associated with an increased thromboembolic risk in patients with atrial fibrillation (32, 159-161), but the findings are not entirely uniform (162, 163). Assessment of inflammation, anamnestic or through biomarkers, is currently not a part of the workup in patients with atrial fibrillation (33, 34). However, it has been argued that measuring systemic inflammation (e.g. by CRP) when assessing stroke risk and the need for anticoagulant therapy, could be useful (141, 161).

Cerebral small vessel disease and inflammation

Even though inflammation has been established as a risk factor of CSVD, there are some diverging findings (142, 164, 165). IL-6 and CRP have been found to be associated with the presence and the progression of cerebrovascular small vessel disease in two studies (166, 167). The vascular changes were assessed longitudinally, while the biomarkers were taken only at the start or end of follow-up. In another cross-sectional study, no association was found (168). The cross-sectional design is often used, making it difficult to conclude (164). The impact of the IFN-ɣ pathway on CSVD is also diverging in cross-sectional studies, where neopterin has been associated with CSVD, but IFN-ɣ production capacity has not (166, 169).

1.4 Summary and rationale for the thesis

Sedentary behaviour is a known risk factor for stroke, and being sedentary for long periods is believed to be associated with the greatest risk. The hazard is believed to be mediated through the known risk factors of vascular disease, including glucose regulation and inflammation. Still, there are several questions about the details of the underlying molecular mechanisms and the length of a clinically significant sedentary bout.

Disease progression takes several years, and to move forward and to design effective studies of sedentary behaviour and disease progression, valid biomarkers with predictive and explanatory properties are imperative

2. Aims

The primary aim of this thesis was to investigate molecular mechanisms mediating the association between sedentary behaviour and ischemic stroke. The secondary aim was to investigate the impact of bout length of sedentary time on these associations.

Four objectives further defined the aim:

1. To investigate the association between objectively measured habitual daytime sedentary behaviour (total and by bout length) and glucose regulation in a stroke population (Study I).
2. To investigate the association between objectively measured habitual daytime sedentary behaviour (total and by bout length) and traditional and novel biomarkers of inflammation in a stroke population (Study II).
3. To investigate how the index stroke subtype was associated with inflammatory biomarkers at three months (Study III).
4. To investigate the association between inflammatory biomarkers associated with sedentary behaviour measured at three months and the risk of ischemic stroke recurrence and mortality (Study III).

3. Material and methods

3.1 The Nor-COAST study

The Norwegian Cognitive Impairment After Stroke (Nor-COAST) study is a multicentre cohort study including patients admitted to hospital for acute stroke from 18 May 2015 to 31 March 2017. The patient selection is shown in Figure 9. The patients (n=815) were included from five hospitals: St Olavs University Hospital, Trondheim (n=400); Ålesund Hospital, Møre and Romsdal Health Trust, Ålesund (n=33); Haukeland University Hospital, Bergen (n=142); Bærum Hospital, Vestre Viken Hospital Trust, Drammen (n=141); Oslo University Hospital, Ullevål, Oslo (n=99).

The inclusion criteria were 1) hospital admission to one of the participating hospitals in the inclusion period, 2) acute stroke following the World Health Organisation definition or a finding of acute stroke on imaging, 3) being able to communicate in one of the Scandinavian languages, 4) above 18 years of age, 5) living in the catchment area. Patients were excluded if they had a life expectancy of less than three months.

The baseline investigation was on day seven of the stay or at discharge if before day seven. The follow-up was done in the outpatient clinic or interview by phone interview at 3, 18 and 36 months. The assessments were performed by trained research assistants using a standardised case report form (170).

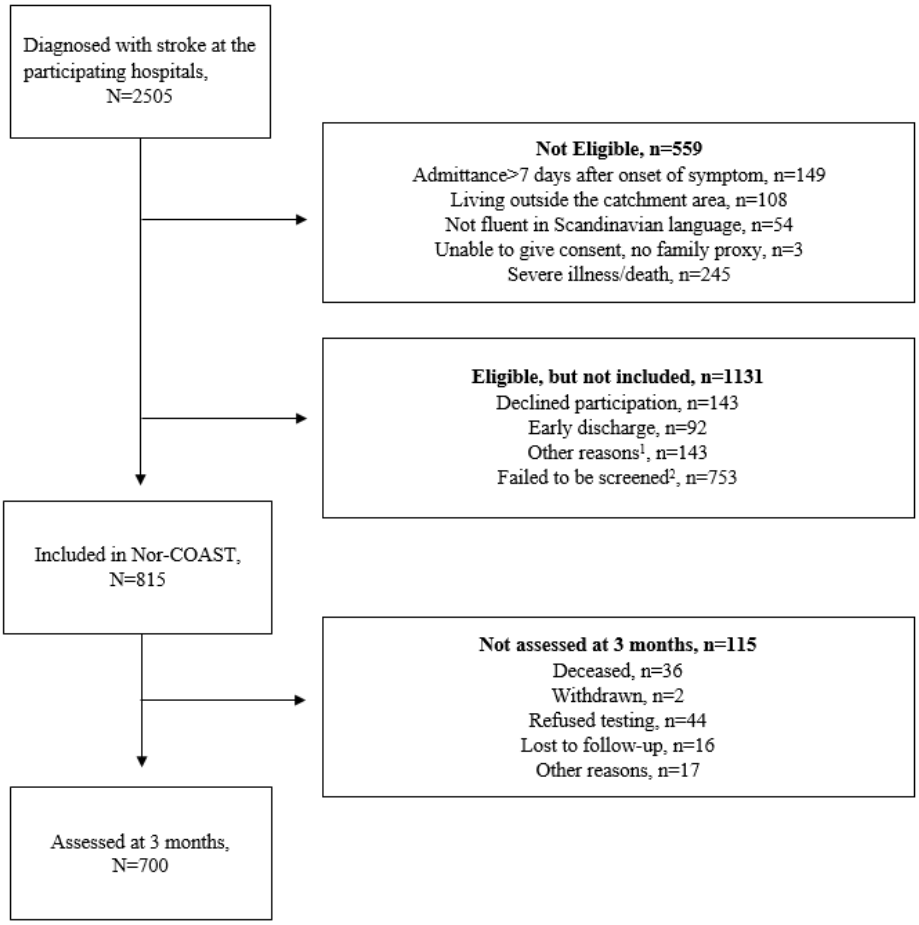

Figure 9: Patient selection for the Nor-COAST study.
[1] Other reasons: delirious patient, hearing impaired, uncertainty about the diagnosis, multi morbid, nursing home resident, other studies.
[2] Failed to screen: infrastructure on the ward, vacation/weekends.

3.1.1 Population for this sub-study

For this sub-study, information from baseline and the three-month follow-up was used, in addition to the information about stroke recurrence and mortality from national registries. Only patients with ischemic stroke at baseline who also attended the three-month follow-up at the outpatient clinic were included in this study. The population varied between the three studies and depended on the availability of activity recordings, blood samples and functional status.

Study I: Sedentary behaviour and HbA1c

This study investigated the association between time in sedentary behaviour and HbA1c measured at the three-month follow-up. Only patients who had valid activity monitoring data for four full days, a value for HbA1c, and were able to walk 50 meters with personal support (Barthel Index item 9 ≥10 points) were included (Figure 10).

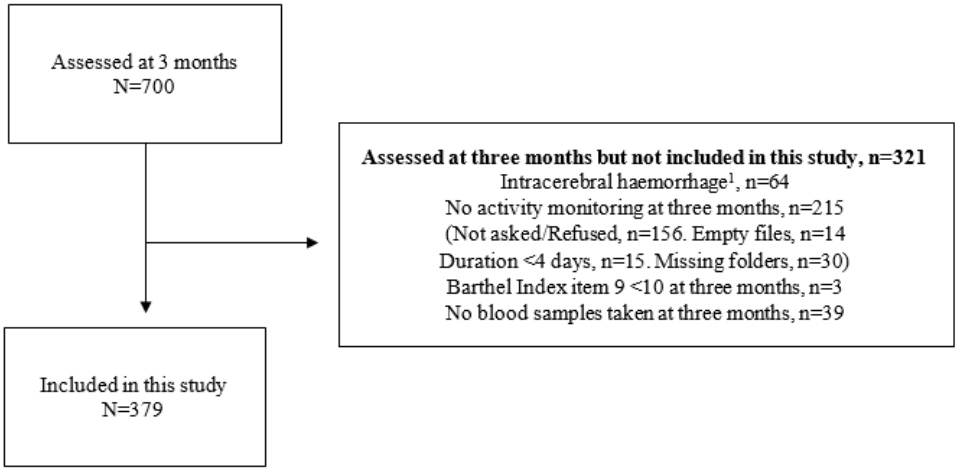

Figure 10: Patient selection for study I. The patients were excluded successively, and the number of patients reported for each reason is based on the remaining population.
[1] Not haemorrhagic transformation.

Study II: Sedentary behaviour and biomarkers of inflammation

This study investigated the association between time in sedentary behaviour and biomarkers of inflammation measured three months after the acute stroke. The patients included had to have activity monitor data for four full days, a value for at least one of the relevant biomarkers, and be able to walk 50 meters with personal support (Barthel Index item 9 ≥10 points) (Figure 11).

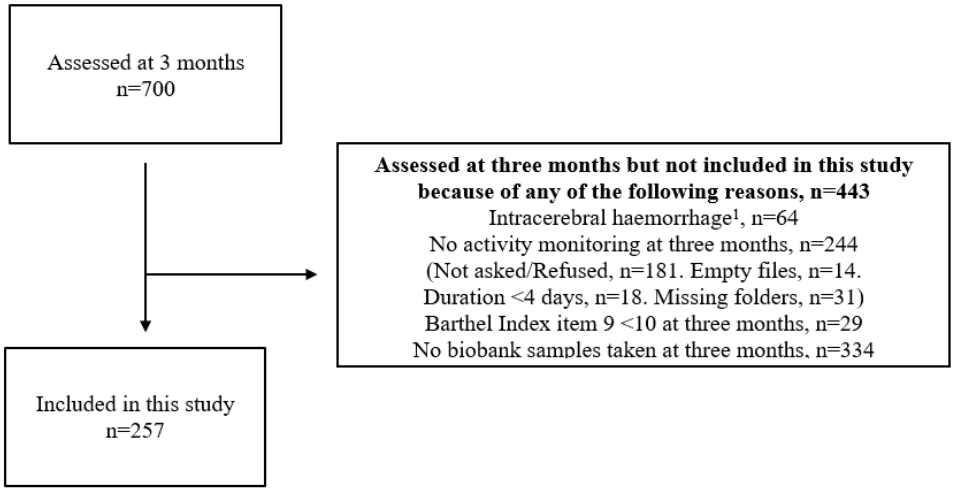

Figure 11: Patient selection for study II.
[1] Not haemorrhagic transformation.

Study III: Biomarkers of inflammation, stroke recurrence and mortality

This study investigated the association between inflammatory biomarkers, found to be significantly associated with sedentary behaviour in study II, and long-term outcomes. Only patients with a value for at least one of the relevant biomarkers taken at the three-month follow-up and who did not have stroke recurrence before the three-month follow-up were included (Figure 12).

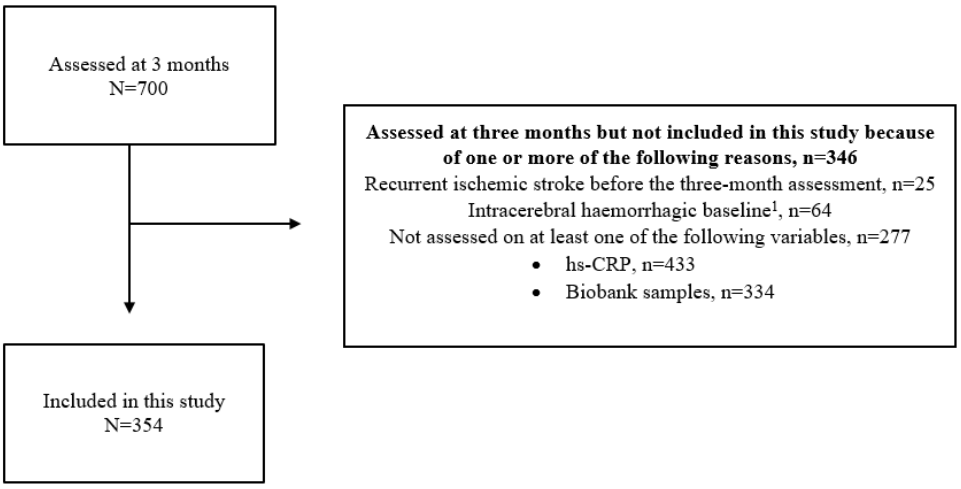

Figure 12: Patient selection for study III.
[1] Not haemorrhagic transformation.

3.1.2 Demographics and medical history

Demographic information, weight, height, medical history (including drug use), information about stroke properties (lesion type, subtype, severity) and function was gathered at baseline. Height, weight, waist circumference, stroke severity, function and drug use were also assessed at three months. The diagnosis of diabetes mellitus was identified at baseline by medical history or/and medication use (Anatomical Therapeutic Chemical Classification: A10) or/and finding of HbA1c ≥6.5 %.

Stroke properties were defined as 1) lesion type; ischemic or haemorrhagic based on findings from imaging; 2) ischemic stroke subgroups, as defined by the Trial of Org 10172 in Acute Stroke Treatment (TOAST)-classification (23); and 3) stroke severity, measured by the NIHSS (41). Ischemic strokes with haemorrhagic transformation were defined as ischemic strokes after evaluation by a trained clinician. Function was assessed using mRS and BI.

3.1.3 Outcomes

Sedentary behaviour

Sedentary behaviour was assessed using a body-worn inclinometer, ActivPAL, attached to the unaffected thigh for up to seven days. Daytime was defined as between 08:00 am and 10:00 pm. The time boundaries were validated by identifying bouts of sedentary behaviour lasting less than 30 minutes during the whole 24-hour period, and 80% of these fell within these time boundaries. The time boundaries were accepted. The monitor was switched on before it was attached to the patients and was still recording after the patient had detached the monitor themselves and returned it by mail. Non-wear time (intermittent or at the end) was identified by visual inspection of the graphical output files. The start of the recording was defined as the first registered activity (position transition), and the end was the last position transition. There were no intermittent periods of non-wear time identified in any of the recordings. The data from the day of attaching the device were excluded, as this was not part of the patient's habitual activity. Recordings from the first four valid days were used. A MATLAB script was developed to identify daytime sedentary time, categorised as

time per day, time per day accumulated through predefined bout lengths (<30 minutes, 30-59 minutes, 60-89 minutes and ≥90 minutes), and as numbers of bouts within each bout-length category.

Laboratory analysis

Non-fasting blood samples were drawn at the three-month follow-up for immediate analysis at the inclusion hospital laboratory and for storing at the biobank. The analyses made at the local hospital laboratory relevant for this project was creatinine, HbA1c and hs-CRP. The hs-CRP was only analysed at Ullevål University Hospital and St Olavs University Hospital because of limited access to the method. The samples for the biobank storage were instantly frozen at -80 °C in aliquots of 0.5 ml. The samples were later sent on dry ice for storage at BioBank1, Central-Norway Health Authority. In 2019, two aliquots of plasma were used to analyse inflammatory biomarkers at two different laboratories. The cytokines were analysed at the Research Laboratory, Nordland Hospital, using Bio-Plex technology kits (Bio-Rad Laboratories, Hercules, CA, USA). The other biomarkers were analysed as part of analytic platform D at Bevital A/S (Bergen, Norway) by liquid chromatography/tandem mass spectrometry. The samples were thawed only once. The biomarkers were part of a predefined kit/platform at the laboratories, and the results included in this study were selected based on the literature and limited to IL-6, IL-10. Pyridoxal 5'-phosphate (PLP), pyridoxal (PL), and 4-pyridoxic acid (PA), tryptophan, kynurenine, kynurenic acid (KA) and neopterin. The PAr-index (PA:(PL+PLP)) and the kynurenine:tryptophan ratio (KTR) were calculated.

Stroke recurrence and mortality

Recurrent stroke was identified by the National Stroke Registry (NSR), and death was identified using the Cause of Death Registry, including data until 31 December 2018.

3.2 Statistical analyses

For continuous variables, Student's t-test, one-way ANOVA, and the Kruskal–Wallis H test as a non-parametric alternative for the skewed variables were used in the descriptive analyses. Pearson's chi-squared test was used for categorical variables. Sedentary behaviour was analysed as mean total sedentary time per day, mean time per day accumulated through the different bout-length categories, and mean number of bouts per bout-length category. Sedentary behaviour was used as a continuous variable. For the laboratory test, the values were logarithmically transformed. Logistic regressions were made for dichotomous outcomes and linear regressions for continuous outcomes. The results were given as standardised beta coefficients. The risk of ischemic stroke recurrence and mortality were investigated using a Cox regression, and for stroke recurrence an additional competing risk regression method was used (171). The variables were standardised and the results presented as hazard ratios (HR) per SD change in the variable. The regression models included confounders based on literature (172-174). A combined endpoint was made by combining the outcomes recurrent ischemic stroke and death. The analyses were performed in Stata/SE 16.1 (Stata Corp.).

Statistical power

The sample size was restricted to the available data from the main study, where it was estimated that approximately 400 would have available activity data. Based on the sample size in prior studies on cytokines in relationship to physical activity, and stroke recurrence rates (17%) a sample size of 400 patients was believed to be sufficient (175, 176).

3.3 Ethical considerations

The project was performed following the institutional guidelines and was approved by the Regional Committee of Medical and Health Research Ethics (For the main study: REK no: 2017/2060/REK Midt. For this study: 2015/171/REK Midt, Ref: 12253). The study was registered with ClinicalTrials.gov (NCT02650531) and first posted on 08 January 2016 (URL: https://clinicaltrials.gov/ct2/show/NCT02650531). Participation was voluntary, and patients gave their written informed consent. Patients who could not consent for themselves were included as long as their caregivers did not decline. Patients with a life expectancy shorter than three months were not included for ethical reasons. The patients received more follow-up than usual by comprehensive assessments at 3 and 18 months. If medical conditions were detected, they were advised to contact their general practitioner, and emergencies were handled immediately. All patients were treated according to national guidelines for acute stroke treatment and secondary prevention.

4. Results – summary of papers

The baseline characteristics of the population in all three studies are shown in Table 1.

Table 1: Baseline characteristics of the population in all three papers

Characteristics	Paper I N= 379	Paper II N=257	Paper III N=354
Sex, male, n (%)	218 (58)	149(58)	201 (57)
Age, mean y (SD)	72 (11)	73 (11)	73 (11)
BMI, mean kg/m^2 (SD)	26 (4.4)	26 (4.2)	26 (4.2)
NIHSS on admission, mean (SD)	3.4 (4.2)	3.7 (4.6)	3.7 (4.7)
Modified Rankin Scale, mean (SD)	1.9 (1.2)	2.0 (1.1)	2.0 (1.2)
Modified Rankin Scale ≤2, n (%)	262 (70)	178 (70)	242 (68)
Barthel Index, mean (SD)	91 (16)	92 (14)	90 (17)

BMI=body mass index (weight/height^2). NIHSS=National Institute of Health Stroke Scale. BI=Barthel Index. SD= standard deviation.

4.1 Paper I: "Is long bout sedentary behaviour associated with long-term glucose levels three months after acute ischemic stroke? A prospective observational cohort study."

Of the 379 ischemic stroke patients included in this study, 74 had diabetes mellitus upon inclusion, and of these 63.5% were using anti-diabetic drugs at three months. The patients with diabetes mellitus had a higher BMI and HbA1c, and they more often had hypercholesterolemia and hypertension. The patients with diabetes mellitus were more sedentary than the patients without diabetes mellitus (Table 2). In patients with diabetes mellitus, sedentary time accumulated through bouts of 90 minutes or more were significantly associated with a higher HbA1c (β=0.32 (CI 0.10, 0.54) p=0.006), after adjustment for age, body mass index (BMI) and the use of anti-diabetic drugs.

Table 2: Mean sedentary time per day (hours) total and by bout length category three months after stroke

	Hours/day, mean (SD)		
	No DM	DM	P-value
Total sedentary time	9.6 (1.2)	10.2 (1.8)	0.008
Bout length category			
<30 minutes	4.0 (1.2)	3.7 (1.0)	0.097
30-59 minutes	2.4 (0.9)	2.7 (0.9)	0.001
60-89 minutes	1.4 (0.9)	1.6 (1.0)	0.118
≥90 minutes	1.8 (1.7)	2.2 (1.6)	0.050

Mean daytime sedentary behaviour per day over a period of four days. *Daytime: 08:00 a.m. to 10:00 p.m. DM= diabetes mellitus.

4.2 Paper II: "Investigating novel biomarkers of immune activation and modulation in the context of sedentary behaviour: a multicentre prospective ischemic stroke cohort study."

A total of 257 ischemic stroke patients were included in this study. A higher level of sedentary time was associated with higher levels of CRP (β=0.25 (CI 0.09, 0.39) p=0.001); IL-6 (β=0.17 (CI 0.04, 0.30) p=0.009); neopterin (β=0.12 (CI 0.00, 0.22) p=0.34); and PAr-index (β=0.21 (CI 0.09 - 0.30) p<0.001), and lower levels of kynurenic acid (β =-0.10 (CI -0.21- -0.02) p=0.045), after adjusting for age, sex, waist circumference and creatinine. In a model, adding all the significant biomarkers to the same model, except CRP because of many missing values, only the PAr-index (β=0.25 (CI 0.10, 0.39) p=0.001) and KA (β =-0.19 (CI -0.35, -0.03) p=0.021) showed independent contributions (Table 3). The inverse association between KA and sedentary behaviour was strengthened after the adjustment for inflammation. There were no associations to IL-10 or KTR.

Table 3: Adjusted multiple regression model of the association between total sedentary time and biomarkers, n=242.

Sedentary time	β	95% CI	P
IL-6	0.12	(-0.01, 0.24)	0.069
Neopterin	0.08	(-0.07, 0.23)	0.280
PAr-index	0.25	(0.10, 0.39)	0.001
Kynurenic acid	-0.19	(-0.35, -0.03)	0.021
Age	0.16	(0.03, 0.29)	0.017
Sex (male)	0.01	(-0.28, 0.30)	0.941
Creatinine	0.02	(-0.17, 0.21)	0.826
Waist circumference	0.21	(0.08, 0.33)	0.002

[1]IL-6=interleukin-6. PAr-index=(4-pyridoxic acid/(pyridoxal 5´-phosphate + pyridoxal).

4.3 Paper III: "Neopterin and kynurenic acid as predictors of stroke recurrence and mortality. A multicentre prospective cohort study on biomarkers of inflammation measured three months after ischemic stroke."

In total, 364 ischemic stroke patients were included in this study. After a mean follow-up of 2.5 years, 16 patients had ischemic stroke recurrence after the three-month follow-up. Of the 28 patients who died, three patients also had ischemic stroke recurrence. The biomarkers included in this study were selected based on the results from paper II. Higher levels of CRP and neopterin were associated with mortality and the combined endpoint (stroke or death), respectively, adjusted for age, sex, prior cerebrovascular disease, and mRS at three months. In a model including both KA and neopterin, KA showed a reduced, and neopterin showed an increased HR for death. There were no associations between the PAr-index or IL-6 and the outcomes. Cardioembolic stroke on baseline corresponded to higher levels of the inflammatory biomarkers CRP, IL-6 and neopterin at three months (Table 4). There were no differences in the PAr-index or KA between the subtypes.

An additional analysis using HbA1c showed no significant associations to stroke recurrence or mortality. This analysis was not included in the paper.

Table 4: Biomarker values at three-month follow-up by ischemic stroke subtype at baseline[1].

	Atherosclerosis (N=26)	Cardioembolic (N=62)	CSVD (N=75)	Unknown (N=141)	P-value
CRP[2], mean (SD)	1.93 (3.29)	3.06 (3.78)	1.88 (2.56)	1.58 (3.71)	0.032
IL-6, mean (SD)	3.71 (1.95)	5.70 (2.18)	3.97 (2.18)	4.26 (2.39)	0.038
Neopterin, mean (SD)	16.0 (1.5)	20.3 (1.7)	16.0 (1.4)	16.0 (1.5)	0.003
PAr, mean (SD)	0.66 (1.77)	0.73 (1.73)	0.60 (1.73)	0.60 (1.56)	0.087
KA, mean (SD)	59.7 (1.5)	67.4 (1.4)	59.7 (1.5)	57.4 (1.5)	0.093

1) The category "other determined" was excluded because of low sample size. The analyses were conducted on log-transformed variables, and the values were transformed back after analysis.
2) For CRP: Atherosclerosis: N=20, cardioembolic: N=46, CSVD: N=50, Unknown: N=100.
CSVD=cerebral small vessel disease. CRP=C-reactive protein, IL-6=interleukin-6, PAr=pyridoxic acid ratio-index=4-pyridoxic acid:(pyridoxal+pyridoxal 5'-phosphate), KA=kynurenic acid. The analysis was done using a one-way ANOVA.

5. Discussion

Main results

In an ischemic stroke cohort, long-bout sedentary behaviour was associated with a higher HbA1c in patients with diabetes mellitus. Total sedentary time was associated with higher levels of pro-inflammatory biomarkers and lower levels of kynurenic acid in the entire study population. Additionally, higher levels of pro-inflammatory biomarkers were associated with the long-term outcomes of mortality and the combined outcome of ischemic stroke recurrence or mortality. Kynurenic acid was negatively associated with mortality after adjusting for inflammation. Finally, patients with cardioembolic strokes at baseline had higher levels of inflammatory biomarkers measured at the three-month follow-up.

The following discussion is organised into three sections. The first section covers how the biomarkers were associated with sedentary behaviour. In the second section, the biomarkers' association to outcomes of disease progression are discussed. Specific methodological issues more or less inseparable from the discussion of the results are reviewed in these two sections. Finally, the third section features a general discussion of methods not addressed previously. Literature from both sedentary behaviour and physical activity/exercise research is referred to in the discussion.

5.1 The association between biomarkers and sedentary behaviour

5.1.1 Glucose regulation and sedentary behaviour

Sedentary behaviour accumulated through bouts ≥90 minutes was associated with a higher HbA1c in stroke patients with a diagnosis of diabetes mellitus, adjusted for age, BMI, and the use of anti-diabetic drugs.

The association between objectively measured habitual sedentary behaviour and glucose regulation has previously been found in the general population (69), in patients with a risk of diabetes mellitus (14), and in a stroke population (9). In this last study, there was no adjustment for age, anthropometric measures or drug use (9). The association can, at least in part, be explained by a reduction in the contraction-

stimulated recruitment of GLUT4 in skeletal muscle tissue (70). Also, exercise has been associated with an increase in the intrinsic capacity of GLUT4. This might be relevant, at least if sedentary behaviour is displacing exercise (177).

Behaviour pattern on glucose regulation

The importance of bout duration

The importance of bout duration is known from the literature (17, 57, 69, 178). In a large population study, Diaz et al. found that mean bout duration was dose-dependent associated with HOMA-IR and 2-hour blood glucose (69). They did not find the same association for HbA1c, but they did not stratify based on the presence of diabetes mellitus (69). In a scoping review by Mackie et al. of intervention studies targeting sedentary behaviour, the overall finding was that breaking up sedentary time with short periods of activity was beneficial for glucose regulation in healthy individuals, patients with overweight, and patients with diabetes mellitus (57).

The novel finding in this study was the apparent 90-minute threshold for the effect of sedentary behaviour on glucose regulation. Corresponding to these results, a basal study found that the contraction-stimulated GLUT4 receptors were re-internalised from the cell membrane between 30 and 85 minutes after the exercise ended (179). The threshold used for intervention studies is mainly 30 minutes (57). The results must be interpreted with caution, but our findings can indicate that 90 minutes might be the threshold for the maximum benefit of breaking up sedentary time in the context of glucose regulation. However, this needs to be confirmed in future research.

The use of bout-length categories is recommended by the SBRN (17), as it captures both time and bout duration in the same variable. In comparison, the number of breaks and mean bout duration does not differentiate between an individual who sits for a long time and then has many short bouts of sedentary behaviour, compared to someone who has medium bouts distributed throughout the day (17). The use of bout-length categories challenges the power of the study by adding more independent variables to the regressions, one for each category. The population included in the analysis

stratified based on the presence of diabetes mellitus was relatively low. Still, the results corresponded to the known physiological mechanisms described above, supporting the notion that the finding was not incidental.

The importance of habit

The review of acute interventions by Mackie et al. mentioned above, demonstrated a tendency towards less effect from breaking up sedentary behaviour with physical activity in the groups of individuals who had a higher habitual physical activity level (57). Sedentary behaviour has been found to have the most significant impact in the most sedentary (14). Studies show that GLUT4 messenger RNA in skeletal muscle returns to basal levels within 18-24 hours after exercise in both patients with and without type II diabetes (180, 181). Consequently, this means that when highly sedentary individuals finally rise, they will have fewer GLUT4 to recruit. This will also be the case for GLUT4 recruitment through the insulin-dependent route. The impact of habit is important when designing studies.

There are two important questions when investigating habitual sedentary behaviour. The first question is what defines a habit? Intervention studies investigating the impact of habitual behaviour change are often conducted over several months (116, 117). On the other hand, studies of the effect of acute behavioural change are often limited to the past 24 hours (13). The transition between acute and chronic is probably gradual and somewhere in between these time limits. By using valid biomarkers associated with sedentary behaviour and physical activity, these thresholds can be identified as a flattening of the curve of the biomarker of interest. This could lead to more time-efficient studies if the results show that the changes happen during the first days or weeks of the behavioural change. This will also vary between outcomes and other confounding factors such as weight and waist circumference in glucose regulation and inflammation. And, of course, for the patient, a good habit should persist for as long as possible.

The second question is how can we get a valid measure of habitual activity? One study of older adults indicated up to three to four days when using a pedometer or

accelerometer (182). A study of healthy adults, where activity was measured using ActivPAL for seven days, they found that five days was enough to give a valid sample. They found an increase in validity when at least one weekend day was included (183). The difference between weekdays and weekends probably depends on the population in question. In the current study, the participants were supposed to wear the monitor for seven days. After excluding the day of attaching and removing the device, the number of days required to be included in the current study was ultimately based on availability and was set to four whole days.

The impact of population and patient characteristics on glucose regulation

Diabetes mellitus

In this study, the association between sedentary behaviour and HbA1c was significant only for patients with diabetes mellitus. In the abovementioned article by Guy et al., the association to HbA1c was only significant in a patient at risk of diabetes mellitus (14). The population study by Diaz et al. found an association to glucose regulation in the whole population when using FPG and OGTT but not for HbA1c (69). The HbA1c is a less sensitive measure compared to FPG and OGTT (184). The contraction-stimulated recruitment of GLUT4 receptors contributes to glucose regulation in all patients. However, being independent of insulin, this pathway might be more crucial for patients with insulin resistance or deficiency (177).

Patients with diabetes mellitus were more sedentary than those without diabetes mellitus, and there might be an unintentional stratification between the least and the most sedentary (14).

Age

The current study population had a high mean age, compared to other studies (14, 69). Even though body composition, physical activity level and nutrition can account for some of the changes in glucose tolerance seen with age, some age-related changes are still unexplained (184). Studies indicate that beta-cell function is preserved with age

but the pulsatile insulin secretion changes (184). In healthy adults, insulin is secreted in pulsations – high frequency for inhibiting hepatic glucose production and low frequency for peripheral glucose consumption (184). Both these pulsation patterns change with age, but the relevance of these changes is not known. It might increase the importance of the insulin independent pathway with age, which makes age relevant in the study design (184). In the current study, age was significantly associated with HbA1c only in patients without diabetes mellitus. The lack of any association in the diabetes group might be because of a lower sample size or that age is less important compared to other factors in patients with diabetes mellitus.

BMI

Sedentary behaviour is associated with higher BMI (185), and higher BMI is associated with higher blood glucose (186). BMI is not a confounder of the association between sedentary behaviour and glucose levels but a potential mediator of the effect of sedentary behaviour on glucose (187). In this study, the analyses were adjusted for BMI to bypass one of the mediators and identify the remaining association.

The importance of glucose-measuring method

In this study, HbA1c, a measure of mean glucose level the past three months, was used as the outcome (64). In the early phases of reduced insulin sensitivity, a pre-diabetic state, there is a compensatory increase in insulin levels without hyperglycaemia (184, 188). HbA1c is not affected because the patient is still normoglycemic. In a review by Chia et al., they argued that the combination of FPG, OGTT and HbA1c would capture the different groups of individuals with reduced glucose tolerance and that combining these methods might be preferred (184). The HOMA-IR method might capture the individuals in a pre-diabetic state with reduced insulin sensitivity, expecting higher values for HOMA-IR to be associated with sedentary behaviour also in normoglycemic patients (65). Hyperinsulinemia can precede diabetes mellitus with more than a decade, leading to metabolic changes associated with an increased vascular risk (189, 190). Identifying these patients when investigating the benefits of

reducing sedentary behaviour is important. In short, the lack of findings in the patients without diabetes mellitus could be due to the method of measuring glucose levels.

To summarise, the results indicate that breaking up sedentary time at least every 90 minutes is beneficial for glucose regulation in patients with diabetes mellitus. The lack of association in patients without diabetes might be caused by methods of measuring glucose regulation or habitual sedentary level. There might also be differences between the type and intensity of the corresponding physical activity between the groups that are not investigated here.

5.1.2 Inflammation and sedentary behaviour

Main results

Sedentary behaviour was associated with increased CRP, IL-6, neopterin, PAr, and reduced KA. The biomarkers were investigated individually, adjusted for age, sex, waist circumference, and creatinine. There were no significant associations between sedentary behaviour and IL-10 or KTR. In a final model, where all the biomarkers significant in the initial analyses except CRP were added, only the association to PAr and KA remained significant. The associations also showed increased strength compared to the initial analyses.

Sedentary behaviour and pro-inflammatory biomarkers

The interleukin-1β pathway: IL-6, CRP

The link between sedentary behaviour and the IL-1β pathway (IL-6 and CRP) is known from previous studies (18, 114-116, 191), as well as when sedentary behaviour is replaced by low-intensity physical activity and not higher intensities (116). In this study, IL-6 had a slightly weaker association with sedentary behaviour compared to CRP. This might be explained by the different populations, as CRP was only measured at two of the five hospitals. It can also be explained by other factors associated with

the physiology and analytical properties of CRP and IL-6. IL-6 rises rapidly in response to inflammation and has a halftime of 15.5 hours (84). Also, when IL-6 is produced as a myokine, this is associated with beneficial metabolic (100) and anti-inflammatory properties (84). IL-6 from muscle cells has been found to return to basal level within an hour (106). The biobank samples were taken at the end of the consultation, which included some physical tests, and there is often some walking distance between the outpatient clinic and the laboratory. The contribution of myocyte derived IL-6 is unclear, but it could reduce the strength of the association between sedentary behaviour and IL-6. Approximately 30% of IL-6 is produced in adipose tissue. Only a small amount comes from adipocytes, and the rest comes from resident macrophages (106). In the regression model, waist circumference was included as an independent variable. Sedentary behaviour is associated with the pro-inflammatory (M1) phenotype of the resident macrophages (106), and changing this polarisation towards the M2 phenotype in adipose tissue is believed to be one of the benefits of reducing sedentary behaviour (106).

IL-6 is a pleiotropic cytokine, and its production and functioning in inflammation and metabolism is a good example of the complexity of cytokine signalling and inflammatory pathways (100). In this setting, CRP, as a measure of the pro-inflammatory activity of IL-6, might be more useful (85).

The IFN-ɣ pathway: neopterin, KTR

We found higher levels of neopterin but not KTR to be associated with sedentary behaviour. This is in line with a study in older adults, where sedentary behaviour assessed by questionnaire was associated with higher neopterin levels but not KTR (120). Neopterin was used as an indirect measure of IFN-ɣ activity and should correspond to a higher level of IFN-ɣ. This seems to be in contrast with a study by Noz et al., where a reduction in the IL-1β pathway after a 16-week physical activity intervention was accompanied by increased IFN-ɣ production capacity. This was interpreted as a compensatory response from the adaptive immune system (116). There was also a relative reduction in circulating monocytes and an increase in the

lymphocyte:monocyte ratio (116). Neopterin is produced by activated macrophages (88, 89). It can be hypothesised that the association found to neopterin is an expression of changes in the mononuclear cells, either quantitatively or as phenotypic changes, followed by an increased response to IFN-ɣ, and not in an increased IFN-ɣ activity per se. This should be investigated further. It is necessary to point out that the confidence interval for the association between neopterin and sedentary behaviour included the number zero. Hence, the results must be interpreted with caution.

The KTR is often used as a measure of IFN-ɣ activity (68, 90, 91). In a review, Joisten et al. argued that KTR should instead be used as a general indicator of kynurenine pathway activation (56). The metabolites from the kynurenine pathway are important contributors to immune tolerance, which might explain the lack of congruence in the results (75, 90). The role of kynurenine as part of a negative feedback loop of inflammation (56) will be described in the discussion of immune tolerance.

There are, to our knowledge, no studies investigating the association between objectively measured sedentary behaviour and neopterin or KTR. This study indicates that neopterin might be a more useful measure of inflammation associated with the IFN-ɣ pathway than KTR.

The PAr-index

The PAr-index was associated with sedentary behaviour at the same level as CRP, but the model seems to explain more of the variation in PAr. When included in a regression model together with IL-6, neopterin and KA, only the PAr-index and KA remained significantly associated with sedentary behaviour, and the associations were strengthened.

The results illustrate and support the prior findings of PAr capturing the effect of several inflammatory pathways (97). In the context of sedentary behaviour, it seems several inflammatory pathways are involved, and PAr could be useful as a sensitive biomarker capturing the combined changes between the pathways. The PAr-index will,

to a lesser extent, be useful for understanding the underlying mechanisms, and more useful in monitoring and prediction.

The regression model (sedentary behaviour, age, sex, waist circumference and kidney function) explained 37% of the variation in PAr compared to 16% of the variation in CRP. This might be due to the role of age, sex and kidney function in the variation of PAr (97, 127). The abovementioned missing data of CRP, must be considered when comparing the biomarkers, even though the missing values were perceived as "missing completely at random" and should not lead to a selection bias. The explanatory value does not change the strength of the association between sedentary behaviour and PAr. It was also somewhat surprising that the association was not stronger compared to CRP.

Sedentary behaviour and anti-inflammation – IL-10

A negative association between IL-10 and sedentary behaviour was expected (116-118). IL-10 is found to be produced following a spike of IL-6 from skeletal muscle cells in response to exercise (106). A higher level of IL-10 was associated with exercise in an intervention study including an older population (117). It was also associated with walking time, investigated in a group of individuals older than 55 years with an elevated vascular risk (116). The lack of association in this study might be explained by the intensity of the corresponding physical activity. A study by Rodas et al. found an independent effect of sedentary behaviour on IL-10 after adjusting for physical activity level (118). The participants in that study were healthier and younger than the stroke patients in the current study, and the measurements were made using questionnaires.

A more thorough discussion of energy expenditure can be found in section 5.3.2.

Sedentary behaviour and immune tolerance

KA has been identified as an important contributor to immune tolerance, increasing the body's anti-inflammatory potential (56). Based on the assumption that sedentary

behaviour increases inflammation, it can be argued that the novel finding in this study of an inverse association between KA and sedentary behaviour is not sporadic but a potential mechanism mediating the hazards of sedentary behaviour.

KA has never been investigated using objective measures of sedentary behaviour, but an increase in KA is known to be associated with exercise (56). The expression and activity of the metabolic enzyme for kynurenic acid, KAT, is induced by muscle contraction (123). The mechanism is via contraction stimulation of peroxisome proliferator-activated receptor-ɣ coactivator-1α1 (PGC-1α1) and the associated transcription factor, peroxisome proliferator-activated receptor-α (PPAR-α) (123). Both acute and chronic exercise is believed to contribute to the induction of the KA pathway. However, the association between chronic exercise and increased levels of KA has been challenging to show in studies. This is either because the blood sample is taken too late after the last activity or obscured by the acute event. This finding of an inverse association supports the impact of chronic physical activity (56).

Kynurenine is believed to induce immune tolerance through the same mechanisms as KA (56, 91). The correlation with inflammation might obscure the effect of KA and kynurenine. This might explain the discrepancies in the results for KTR, compared to neopterin as a measure of IFN-ɣ activity, in the current and other studies described above.

The importance of population must be addressed in this section as well. An intervention study including healthy males older than 65 years found that a 12-week physical exercise intervention increased KAT expression in muscle tissue, but it did not raise the level of KA significantly (122). It has been questioned if the upregulation of KA in response to chronic exercise is more important in a disease population where the KP is already activated due to an inflammatory state (56). Inflammation increases with age, and reducing sedentary behaviour might contribute to reducing inflammageing (75).

To summarise, the findings suggest that CRP, neopterin, the PAr-index and KA are useful biomarkers when investigating inflammation and sedentary behaviour. The results for KA are unique and open exciting paths for sedentary behaviour research.

Going from observations to explanations

At the end of section 1.3.2, three potential mechanisms explaining the association between sedentary behaviour and inflammation, derived from physical activity and exercise research, were described (Figure 8). The results will now be summarised through these three potential mechanisms (56, 106).

Myokine production

As described above, IL-6 from the contracting skeletal muscle (100) induces IL-10 and interleukin-1β receptor antagonist (IL-1βRA) (106). The impact of reducing sedentary behaviour might be mediated by the IL-6 production associated with the corresponding physical activity, but it might depend upon the energy expenditure during this activity. This might explain the lack of association between sedentary behaviour and IL-10 in the current study and the weaker association to IL-6 compared to CRP.

Trained immunity

"Trained immunity", the functional reprogramming of innate immune cells, such as the polarisation of monocytes and macrophages to either a pro- or an anti-inflammatory phenotype, is believed to be an important contributor to chronic low-grade inflammation (79, 80). Inflammageing, the tendency towards low-grade chronic inflammation associated with age, has been described as "the dark side of trained immunity" (9, 75, 76, 79, 143). Studies indicate that physical activity can push the phenotype toward the less inflammatory phenotype, M2 also called the "*healing subtype*" (106, 116). The mechanism for this is not known. A polarisation towards the M1 phenotype might partly cause the increase in inflammatory biomarkers associated

with sedentary behaviour, illustrated by the associations to both the IL-1β pathway and the neopterin response in the IFN-ɣ pathway in the current study.

The kynurenine pathway

The metabolites kynurenine and KA have been found to stimulate the differentiation of naïve T cells to T regulatory cells. The alteration of the ratio between regulatory T cells and the T helper cells increases immune tolerance (56, 106). The link between KA and the T cells is the aryl hydrocarbon receptor (AhR) (56, 123)**.** Kynurenine is also an AhR agonist, but in contrast to KA, kynurenine can be further metabolised to other substances, and KA has a higher affinity to AhR (91). Physical activity induces the enzyme producing KA (123), and downregulation of KA and impaired immune tolerance can be part of the explanation of the association between sedentary behaviour and inflammation.

There are most likely many different and interrelated pathways between sedentary behaviour and inflammation. Likewise, there are known interactions between metabolic risk factors of vascular disease and inflammation.

5.1.3 The interface between inflammation and metabolic risk factors of vascular disease

Nothing in human physiology exists in a vacuum, and discussing inflammation from a vascular disease perspective without mentioning the interaction with metabolic risk factors would be an obvious omission. Even though an exhaustive description of all the crossing pathways is beyond the scope of this thesis, a short outline with some examples of the interaction between inflammation and metabolic risk factors – is required.

Vascular risk factors, such as hypertension, can contribute to increased inflammation (192). Reducing inflammation by targeting the IL-1β-pathway has been beneficial for glucose regulation (184). IL-6 has been found to increase the translocation of GLUT4 receptor to the cell membrane, thus facilitating glucose transportation into the muscle

cells, reducing insulin resistance (100). In basal research, administering KA has reduced hyperlipidemia-induced insulin resistance (193), and KA has been associated with increased energy metabolism (194). The expression of KAT mRNA, the enzyme producing KA, has been found to be modestly reduced in skeletal muscle in patients with diabetes mellitus type II (195).

Another link between metabolism and inflammation is adipose tissue. Adipose tissue is a source of inflammatory signals, and having a healthy diet and physical activity is believed to reduce both the adipose cell size and the number and phenotype of the resident inflammatory cells (106). BMI attenuates but does not eliminate, the relationship between physical activity and inflammation (18). In this study, the regression model included waist circumference, as this is believed to be more closely associated with cardiovascular mortality (196).

5.2 Biomarkers, stroke subtype and long-term outcomes

Main results

Patients with cardioembolic stroke at baseline had higher CRP, IL-6 and neopterin at the three-month follow-up. The biomarkers were investigated individually. CRP and neopterin at three months were associated with mortality and the combined endpoint stroke recurrence and mortality, respectively. In the model where KA and neopterin were included simultaneously, KA was associated with a reduced risk and neopterin with an increased risk for mortality.

We did not find any association between any of the biomarkers and the risk of ischemic stroke recurrence. We did not find any association between HbA1c, the PAr-index or IL-6 and mortality.

5.2.1 Glucose, stroke recurrence and mortality

A large amount of literature supports a causal relationship between circulating glucose and vascular disease development (72, 184). Although not sensitive, the HbA1c is

associated with future CVD and can be used in risk assessment (184). We did not find any association between HbA1c and ischemic stroke recurrence or mortality. HbA1c was primarily included in this project to investigate the association to sedentary time, including the impact of bout length. Because of the existing knowledge on this subject, a thorough discussion of the relationship to long-term outcomes is not made in this thesis. The general discussion about statistical power and sensitivity of the diagnosis of ischemic stroke recurrence outlined in section 5.3.5, also applies to these results.

5.2.2 Inflammation, stroke recurrence and mortality

Pro-inflammatory biomarkers, ischemic stroke recurrence and mortality

There were no associations between the biomarkers and ischemic stroke recurrence. In the literature, the impact of metabolic risk factors, such as glucose and lipids, on vascular disease progression are augmented by the presence of inflammation in general (133). The associations between IL-6, CRP and vascular disease are established (25, 133), and drug interventions targeting IL-1β and IL-6 – and indirectly CRP – have been shown to affect vascular disease recurrence, including stroke recurrence (126, 130, 197, 198). Neopterin and PAr have also been associated with stroke and stroke recurrence (127, 199). Prior studies are often based on a healthy population or populations with prior coronary heart disease. The role of inflammation in stroke recurrence might be different, not least because of the diagnostic workup, including Holter monitoring for paroxysmal atrial fibrillation and the secondary prophylaxis with anticoagulation (24). Because of the low power, a type II error could be made, and the biomarkers should be investigated in a larger study. A more general discussion of power in this project will be made in section 5.3.5.

There was a significant association between CRP and mortality and between neopterin and the combined endpoint. This is in congruence with current literature (25, 128, 163). The concurrent lack of finding to IL-6 was surprising (163), but might be caused by factors associated with test properties and the role of IL-6 in metabolism, outlined in section 5.1.2. Based on the assumption that the PAr-index captures the effect of the other pathways and prior association to mortality in patients with CHD, the lack of

association to PAr was more surprising (140). Because of the relatively low sample size, IL-6 and the PAr-index should not be discarded as predictors in stroke populations. However, the results support that CRP and neopterin are robust predictive biomarkers, representing two different inflammatory pathways through their association to the innate and adaptive immune systems, respectively.

Kynurenic acid and ischemic stroke recurrence and mortality

The novel finding in this study was the negative association between KA and mortality after adjusting for inflammation by adding neopterin to the model. KA has been associated with vascular disease progression in general (153) and with cardiovascular events in patients with stable angina pectoris (136, 150), but not with stroke (137). KA has also been associated with mortality and length of stay in an intensive care unit after cardiac surgery in infants (200). As described in the subsection of *"Anti-inflammation and immune tolerance"* in section 1.3.2., KA is part of a negative feedback loop inducing immune tolerance. Hence, these results from the literature are not in contrast to the finding in the current study (56, 91) but fit the assumed protective effect of a concurrent rise in KA under inflammatory conditions (56, 91). Such effects are easily obscured because of the correlation with inflammation. The model that was adjusted for inflammation was based on the biological rationale described above, in addition to the results from study II, showing that KA was negatively associated with sedentary time with increased strength after adjusting for the other biomarkers of inflammation. To reduce the number of explanatory variables in the regression, only one pro-inflammatory biomarker was added to the model. Neopterin was chosen because of the association to the combined outcome in the primary analyses and because both KA and neopterin are part of the IFN-ɣ pathway and expressions of adaptive immune system activation (77, 88, 89).

The results are in congruence with known physiological mechanisms, supporting that the result is not sporadic. KA might have an important role in chronic low-grade inflammation, disease progression and biological ageing. Studies aiming at increasing

the KA response to inflammation, in particular in the perspective of sedentary behaviour, should be investigated further.

5.2.3 Stroke subtype and inflammatory biomarkers

Patients with cardioembolic stroke at baseline had higher levels of CRP, IL-6 and neopterin. Blood samples were taken three months after the stroke, which is not necessarily representative of the inflammatory status pre-stroke. Still, it says something about the subgroup characteristics.

Based on results from prior studies investigating the inflammatory response in the acute phase of stroke, the further discussion is based on the assumption that the acute inflammatory response associated with the index stroke lesion has been normalised (201).

Besides being sporadic, there can be four possible explanations of the results:

1) The cardioembolic strokes were larger and associated with more complications resulting in an increased unresolved inflammation post stroke.

2) The inflammation is merely a surrogate marker of other comorbidities associated with an increased risk of atrial fibrillation.

3) Inflammation increases the risk of atrial fibrillation.

4) Inflammation increases the risk of thromboembolism in patients with atrial fibrillation.

On the group level, the patients in this study had mild strokes and a high functional level. Even though the initial stroke severity and comorbidity might contribute to the result, the following discussion will be limited to the role of inflammation as a risk factor for atrial fibrillation and thromboembolism.

Inflammation as a risk factor of atrial fibrillation

In a population-based study among patients with transient ischemic attacks or ischemic stroke, 30% of the events were defined as cryptogenic (154). Some of them might be caused by undetected paroxysmal atrial fibrillation. Both randomised trials and observational studies have shown that prolonged or sequential cardiac monitoring can increase the detection rates. The optimal duration of monitoring is not defined (24). Prolonged cardiac monitoring demands considerable resources, and it has been questioned whether blood biomarkers could be used for targeting the patient group in need of prolonged monitoring (157, 202). In a prospective study, Schnabel et al. identified B-type natriuretic peptide and CRP to be associated with future atrial fibrillation (202). In a small study of acute ischemic stroke patients, Lambert et al. found that patients with cardioembolic stroke had higher levels of IL-6 in the acute phase of stroke compared to the other subtypes. They argued that IL-6 might be useful for identifying those in need of long-term cardiac monitoring to capture paroxysmal atrial fibrillation after acute stroke (157). Whether or not the inflammation contributes to the pathophysiology or is merely an indicator of the underlying disease contributing to the atrial fibrillation remains unanswered (141).

Inflammation as a risk factor for thromboembolism

In the context of ischemic stroke, the hazard of atrial fibrillation is the associated risk of thromboembolism. The risk is believed to be caused by the hemodynamic changes of the blood flow in the atriums, endothelial injury, and hypercoagulability (32). At present, the assessment of thromboembolic risk associated with atrial fibrillation is done using a clinical decision-making tool called the CHA2DS2-VASc score (33, 34). Biomarkers associated with altered blood flow, endothelial injury, or hypercoagulability might be useful for additional risk assessment but are not used routinely (32, 33). There is a scientific rationale for using inflammatory biomarkers in the risk prediction of atrial fibrillation-induced thromboembolism (32, 203). In a large study entitled the ARISTOTLE trial investigating the effect of the direct anticoagulant apixaban, the role of inflammatory biomarkers for risk prediction were studied. The

study found that biomarkers of inflammation improved the risk prediction for mortality but not for stroke (162, 163). This population was already treated with anti-coagulation, and it is impossible to draw any conclusion regarding the risk associated with inflammation in an untreated cohort. There are apparent ethical obstacles with investigating the impact of inflammatory biomarkers in patients with known atrial fibrillation in an untreated cohort.

The result supports the importance of inflammation in patients with atrial fibrillation and stroke. Future studies of the role of inflammatory biomarkers should clarify if this is useful as a supplement in the workup for identifying atrial fibrillation and the associated thromboembolic risk. The results also underline the importance of stratifying by stroke subtype when studying stroke risk to avoid overlooking associations to one subtype by the lack of association to the others.

5.3 Discussion of methods

This study has several strengths but also some limitations. Some of the strengths and weaknesses have already been addressed in the discussion of the results above. The following section features a more general methodological discussion of issues not addressed in the previous sections.

5.3.1 Study design and population

Study design

One of the strengths of this study is the prospective longitudinal multicentre design, including patients from both university hospitals and local hospitals. The investigation of the association between sedentary behaviour and biomarkers is cross-sectional, while the analysis of the association to the long-term outcome is longitudinal. The Nor-COAST study was designed to investigate cognitive impairment after stroke, and this sub-study was developed after the main study was established. Hence, the study is prospective for the main aim but not for the current sub-study.

Observational studies in general are prone to confounding, and it is not possible to conclude a direction of the associations. However, there is evidence from intervention studies supporting an association in the direction from sedentary behaviour to glucose regulation and inflammation (57, 135). Drug interventions targeting inflammation showing reduced risk of vascular events supports the causal relationship from inflammation to vascular disease (133, 192), and suggests that the associations found in this study can be an expression of a cause-and-effect relationship.

Population

The Nor-COAST population has been found to have a better pre-stroke health condition than the general stroke population. However, the sample is representative of the group of patients suffering mild strokes, which makes up the largest group (65%) (204). The subpopulation in the current study, which had activity monitoring and/or biobank samples, was more fit than the remaining part of the Nor-COAST population. Hence, the results are not representative of the whole stroke population.

5.3.2. Measuring and analysing sedentary behaviour

The large sample of stroke patients with objectively measured sedentary behaviour is a strength of the study. The algorithm used to estimate the sedentary bouts is also in line with the current recommendation (17) and captures both time and bout length.

Measuring sedentary behaviour using one sensor

When using only one sensor, it is not possible to discern between lying and sitting. Use of two sensors (thigh and back/chest) makes it possible to discern between the positions, but not between active and passive sitting. To differentiate between active and passive sitting, accelerometers are sometimes used. Active sitting is often defined as an accelerometer count >100 counts per minute while in a sitting position. This is typical while working on a computer. Active sitting has been found to have a higher energy expenditure than inactive sitting and lying and has been found to be inversely

associated with inflammation in contrast to non-active sitting and lying (185). The inclusion of active sitting in total sedentary time in our study might have led to a type II error.

The importance of energy expenditure

By using position to identify sedentary behaviour, as is done in this study, the impact of energy expenditure of the corresponding physical activity could not be investigated. Measuring energy expenditure in a stroke population is challenging because of the heterogeneity in the patient group, ranging from hemiparesis to negligible sensory deficits (48). The problem lies in the reliability of the sampling and the validity of the conversion algorithm. Preferred walking speed has been found to be highly correlated with oxygen cost in a stroke population (205). Using walking speed to individualise the energy expenditure equations could increase the validity of the conversion algorithms. Also, adding other parameters to the measure, such as temperature and galvanic skin response, seem to increase the validity (49, 131).

Sleep and non-wear time

In this study, sleep time was predefined as the time between 10.00 pm and 08:00 am. We might have underestimated sedentary behaviour at night that has been falsely defined as sleep and overestimated sedentary behaviour during the daytime that is actually sleep. The low amount of short bout sedentary behaviour in the defined night-time indicates that we have actually captured sleep. Additionally, we might have underestimated long bout sedentary behaviour because sedentary bouts were cut in half when crossing these time boundaries.

Sleeping patterns, including sleep duration, varies in stroke patients (58-60, 206). Algorithms for identifying sleep time (61) should be validated in different populations. Patient characteristics, such as cognitive impairment, nocturia or pain, associated with deviating sleeping patterns might increase the validity of the algorithms. In a study by van der Berg et al., the development of the algorithm was based on machine learning,

with rules and thresholds being set and adjusted by inspecting outputs (61). Machine learning and artificial intelligence might enable more complex algorithms, capturing a greater diversity of activity patterns. This could also be used in the validation and customization of the algorithm to different populations. In addition to algorithms, technical developments, such as enabling the patient to mark the recording when going to bed, rising up from bed or detaching the device, could increase the validity of the data. Also, it might be more valid to investigate the sum of all behaviours during a 24-hour period (sedentary behaviour, physical activity and sleep), and this should probably be preferred in future studies.

5.3.3 Properties and selection of inflammatory biomarkers

The large sample of traditional and novel biomarkers of inflammation carefully selected based on the current literature is a strength of this study. The following section contains a general discussion about cytokine properties, the selection of biomarkers and the role of acute inflammation.

Cytokine properties

Cytokines have a short biological halftime, dynamic secretion, and diurnal variations and are found to be unstable in the sample – some more than others. Standardisation of the blood sampling, handling and storage are important (84). In this study, the blood samples were taken non-fasting at a random time of the day, and the patients were included consecutively throughout the year, which are factors that can affect the results. Consequently, there might be variations in the results due to other factors than the persons' inflammatory state. Beyond that, there has been a standardised protocol for handling the samples, and there is no reason to believe that they have not been treated similarly.

The selection of biomarkers

The selection of biomarkers was based on the current literature describing biomarker properties, such as in vivo and in vitro stability, their role in physiology, and known associations to disease, sedentary behaviour and physical activity. In an effort to try to limit the number of biomarkers and reduce the multiple testing bias, the biomarkers were also prioritized based on their role in different pathways or in a mechanism, such as the IL-1β and IFN-ɣ pathways, anti-inflammation and immune tolerance. Immunology is complex, and it is proper to question whether these forms of simplification and investigation of a limited number of biomarkers individually are useful. In comparison, by adding several biomarkers to the same model, nuances like synergy and feedback mechanisms could have been elucidated. This challenges the power of the sample. In this thesis, such jointed analyses, on a small scale, were made in studies II (KA and PAr) and III (KA and neopterin) by carefully selecting the most important biomarkers based on a combination of prior knowledge, results of initial analyses and availability of data. In this study, the joint analyses seem to have uncovered/strengthened the role of KA in sedentary behaviour and disease progression.

Many relevant biomarkers have not been included in this investigation. TNF-α is central in the inflammatory response, in particular in concert with IL-1β and IL-6. We did not prioritize to include TNF-α based on the sample output where it was not detectable in a large number of samples. This corresponds to the reported biological halftime of 18.2 minutes (84). It has been reported that IL-1β has an in vivo halftime of 21 minutes and that it is not reliably measured in blood (84, 85). Corresponding to this, IL-1β was often not detectable in this study. In comparison, IL-6 has a reported halftime of 15.5 hours and was detectable in all of the patients (84). The half-life of IFN-ɣ is not clear (84); but based on a study of recombinant IFN-ɣ, it is short, and it was often not detectable in the current population (102). TNF-α, IFN-ɣ and IL-1β are also more sensitive to any delay before freezing compared to IL-6 (84). Although relevant, these biomarkers were not included in this study.

The impact of acute inflammation

The aim of this investigation was to identify chronic low-grade inflammation, but there is a possibility that the patients had concomitant subtle acute disease affecting the inflammatory response. Therefore, a modest increase in the acute inflammatory parameters can be an expression of an acute infection rather than chronic low-grade inflammation. This could lead to a type II error because it is independent of their habitual activity level and their future risk of disease. The biomarkers could have been measured several times over a given time period to bypass these random variations. It is also possible to exclude extreme outliers. Extreme outliers were not a big issue in this patient group.

5.3.4 Ischemic stroke recurrence and mortality

The validity of the outcome measure of disease progression is important.

Ischemic stroke recurrence was identified by the NSR, a registry including all patients admitted to hospital for acute stroke (207). The registry has been found to be highly specific; the patients included have actually had stroke but have low sensitivity because only acute strokes in patients admitted to hospital are included (208). Hence, strokes with negligible or non-overt symptoms might not be identified. Also, some patients do not seek medical help, and patients living at nursing homes with adequate secondary prophylaxis and small strokes are sometimes not admitted to hospital. Mortality was measured using the Cause of Death Registry. The registry is highly sensitive for mortality, but the quality of the information about the cause of death is not reliable (209). Some of the patients were registered with stroke as the cause of death. However, it was not possible to determine if the stroke in question was the index stroke or a recurrence, especially without any corresponding registration in the NSR. Patients who died without recurrent stroke registered in the NSR were recorded as having no recurrence.

Also, stroke subtype was not identified upon recurrence. When investigating per subtype, the study needs to be powered to do the stratified analyses. Stroke subtype

was not part of the data from the NSR, and this study was not powered for stratified analyses.

The incidence of ischemic stroke recurrence is most likely too low, increasing the risk of falsely accepting the null hypothesis and making a type II error. Using MRI to identify stroke recurrences is a more sensitive method (36), but it takes a great deal of resources and is demanding for the patients. We also lose the timing of the recurrence. On the other hand, it reduces the sample size needed (36, 166). The use of MRI would enable a more sensitive outcome of CeVD progression other than stroke, such as chronic circulatory dysfunction, appearing as white matter changes in the MRI (19, 35). Assessments to identify other signs of disease progression or risk factors for stroke can also be useful. This could be through ultrasound for identifying carotid intima-media thickness and plaque progression (29) or 24-hour Holter monitoring to identify atrial fibrillation (24, 210). Currently, there is a paucity of evidence regarding the risk of cardiovascular events and incidental findings of atherosclerotic carotid plaques and screening for this to estimate cardiovascular risk in the clinical setting is not recommended (29).

5.3.5 Statistics

Statistical power

The sample size was restricted to the size of the main study. The evaluation of power was done by comparing the size to other studies with similar outcomes. A formal power calculation was not made. To do a post hoc power analysis based on the results is not recommended (211) and has not been done.

Studies I and II

One of the aims of this study was to investigate the impact of bout length on the association between sedentary behaviour and biomarkers. Dividing the sedentary behaviour variable into four subgroups increased the number of independent variables in the regression and challenged the power of the study. As described in section 3.1.1,

papers I and II had slightly different populations because of varying availability of the data for the individual patients. The population in study I was the largest, and it was possible to include the bout-length categories here. However, fewer patients had additional samples for biobank storage, and even fewer had both activity recordings *and* the biobank sample needed for study II. The smaller samples enabled us to investigate sedentary behaviour and the biomarker with sufficient power, only when using total sedentary time as the independent variable. We added the results from the stratified analyses to the supplementary material of paper II, but we have not emphasised the results in the discussion. This was because of a lack of internal congruence in the results.

Study III

The power estimation was based on an estimated recurrence rate of 17% in 12 months (176), and similar studies using cytokines had found significant results with a lower sample size (175). A sample of 400 patients was expected when this study was planned, and this number was perceived as enough for the study question. The overall recurrence rate was lower than expected (all strokes 8.3%; infarction 7.4%), probably due to the Nor-COAST population being more fit compared to the general stroke population (204). Also, the NSR only includes patients admitted to hospital because of stroke. The ischemic stroke recurrence rates in the whole Nor-COAST population and the subsample having biobank samples were similar (7.3%). The goal of this study was to investigate chronic low-grade inflammation, and the blood samples were therefore taken at the three-month follow-up. Many of the stroke recurrences happened between baseline and three-month follow-up and had to be excluded. The final ischemic stroke recurrence rate in the population included in study III was 4.5%. The low power increases the risk of a type II error, and the lack of findings between inflammation and outcomes must be interpreted with caution.

6. Conclusion

The study supports the role of glucose regulation and inflammation as the links between sedentary behaviour and disease progression and mortality. Novel biomarkers can increase the understanding of this link and can be useful when designing studies in the future. In particular, the results for kynurenic acid are unique. The results also illustrate the importance of long-bout sedentary behaviour in disease progression. Finally, the results underline the importance of ischemic stroke as a group of different diseases that need to be investigated separately.

7. Future perspectives

Clinical implications

The results support that reducing – or at least breaking up – sedentary time is beneficial for patients after stroke. For those who do not reach the recommended amount of physical activity, reducing sedentary behaviour could be a more tangible goal, and this should be communicated to the public.

Scientific implications

When designing studies, there is always room for improvement. The strengths and limitations of the study are discussed above, and the following outline will include some of the reflections made throughout the discussion, in addition to the direct impact of the results.

Study design

Compared to observational studies, intervention studies targeting habitual sedentary behaviour will be the most useful design to investigate the current findings further. Sequential biomarker monitoring could help identify the sufficient duration of a behaviour change in the study setting and might enable more time-effective trials in the future. Additionally, it will reduce the bias of intermittent acute disease when

investigating biomarkers of inflammation. The studies should be powered to investigate the subtypes separately.

Measuring and analysing sedentary behaviour

The method for analysing bout duration, including both time and bout length information, used in this study seems feasible for studies with sufficient size. However, more research is needed to achieve consensus on appropriate bout lengths for the different physiological processes. Increased use of machine learning, enabling more complex and individualised algorithms, could be a solution for getting valid measures of energy expenditure and sleep in disease populations. The use of two sensors for details about position in combination with accelerometer could be useful, but conversion algorithms needs to be validated.

The choice of biomarkers

The importance of hyperinsulinemia with or without hyperglycaemia should be acknowledged. Using a combination of fasting glucose, oral glucose tolerance test, or the HOMA-IR technique might be useful for this.

When investigating inflammation, neopterin and the PAr-index seem to be useful biomarkers, adding nuances from other pathways to the already established CRP. They seem to be more reliable compared to cytokines and might be preferred.

The kynurenine pathway should be investigated further. The results in this study illustrate that biomarkers' role in feedback systems, such as immune tolerance, can be obscured when investigated separately. The use of more complex statistical models could be useful. There are obvious problems with using KTR as a measure of inflammation, and this should be addressed in future studies.

Measures of disease progression

The use of other outcome measures of disease progression or risk factors such as MRI, carotid atherosclerosis and atrial fibrillation could give an increased sensitivity compared to clinical stroke recurrence and increase the specificity compared to all-

cause mortality. In particular, MRI will increase the sensitivity of the stroke diagnosis. In addition, MRI will also capture other signs of increased vascular damage and should be the standard for future studies. Functional outcomes could give additional sensitivity, but is unspecific in terms of the underlying cause.

8. References

1. Feigin VL, Norrving B, Mensah GA. Global burden of stroke. Circ Res. 2017;120(3):439-48.
2. Roth GA, Mensah GA, Johnson CO, Addolorato G, Ammirati E, Baddour LM, et al. Global burden of cardiovascular diseases and risk factors, 1990-2019: Update from the GBD 2019 study. J Am Coll Cardiol. 2020;76(25):2982-3021.
3. Li Z, Zhang Z, Ren Y, Wang Y, Fang J, Yue H, et al. Aging and age-related diseases: from mechanisms to therapeutic strategies. Biogerontology. 2021;22(2):165-87.
4. Morris JN, Heady JA, Raffle PA, Roberts CG, Parks JW. Coronary heart-disease and physical activity of work. Lancet. 1953;262(6796):1111-20.
5. Lee IM, Shiroma EJ, Lobelo F, Puska P, Blair SN, Katzmarzyk PT, et al. Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy. Lancet. 2012;380(9838):219-29.
6. Dunstan DW, Howard B, Healy GN, Owen N. Too much sitting - a health hazard. Diabetes research and clinical practice. 2012;97(3):368-76.
7. Bellettiere J, LaMonte MJ, Evenson KR, Rillamas-Sun E, Kerr J, Lee IM, et al. Sedentary behavior and cardiovascular disease in older women: The Objective Physical Activity and Cardiovascular Health (OPACH) Study. Circulation. 2019;139(8):1036-46.
8. English C, Healy GN, Coates A, Lewis L, Olds T, Bernhardt J. Sitting and activity time in people with stroke. Phys Ther. 2016;96(2):193-201.
9. Moore SA, Hallsworth K, Plotz T, Ford GA, Rochester L, Trenell MI. Physical activity, sedentary behaviour and metabolic control following stroke: a cross-sectional and longitudinal study. PLoS One. 2013;8(1):e55263.
10. Tieges Z, Mead G, Allerhand M, Duncan F, van Wijck F, Fitzsimons C, et al. Sedentary behavior in the first year after stroke: a longitudinal cohort study with objective measures. Arch Phys Med Rehabil. 2015;96(1):15-23.
11. Chastin SF, Culhane B, Dall PM. Comparison of self-reported measure of sitting time (IPAQ) with objective measurement (activPAL). Physiol Meas. 2014;35(11):2319-28.
12. Patterson R, McNamara E, Tainio M, de Sa TH, Smith AD, Sharp SJ, et al. Sedentary behaviour and risk of all-cause, cardiovascular and cancer mortality, and incident type 2 diabetes: a systematic review and dose response meta-analysis. Eur J Epidemiol. 2018;33(9):811-29.
13. Benatti FB, Ried-Larsen M. The effects of breaking up prolonged sitting time: a review of experimental studies. Med Sci Sports Exerc. 2015;47(10):2053-61.
14. Gay JL, Buchner DM, Schmidt MD. Dose-response association of physical activity with HbA1c: Intensity and bout length. Prev Med. 2016;86:58-63.
15. Billinger SA, Arena R, Bernhardt J, Eng JJ, Franklin BA, Johnson CM, et al. Physical activity and exercise recommendations for stroke survivors: a statement for healthcare professionals from the American Heart Association/American Stroke Association. Stroke. 2014;45(8):2532-53.
16. Dempsey PC, Matthews CE, Dashti SG, Doherty AR, Bergouignan A, van Roekel EH, et al. Sedentary behavior and chronic disease: mechanisms and future directions. J Phys Act Health. 2020;17(1):52-61.
17. Tremblay MS, Aubert S, Barnes JD, Saunders TJ, Carson V, Latimer-Cheung AE, et al. Sedentary Behavior Research Network (SBRN) - Terminology Consensus Project process and outcome. The international journal of behavioral nutrition and physical activity. 2017;14(1):75.

18. Parsons TJ, Sartini C, Welsh P, Sattar N, Ash S, Lennon LT, et al. Physical activity, sedentary behavior, and inflammatory and hemostatic markers in men. Med Sci Sports Exerc. 2017;49(3):459-65.
19. Rosenberg GA, Wallin A, Wardlaw JM, Markus HS, Montaner J, Wolfson L, et al. Consensus statement for diagnosis of subcortical small vessel disease. J Cereb Blood Flow Metab. 2016;36(1):6-25.
20. Aho K, Harmsen P, Hatano S, Marquardsen J, Smirnov VE, Strasser T. Cerebrovascular disease in the community: results of a WHO collaborative study. Bull World Health Organ. 1980;58(1):113-30.
21. Sacco RL, Kasner SE, Broderick JP, Caplan LR, Connors JJ, Culebras A, et al. An updated definition of stroke for the 21st century: a statement for healthcare professionals from the American Heart Association/American Stroke Association. Stroke. 2013;44(7):2064-89.
22. O'Donnell MJ, Xavier D, Liu L, Zhang H, Chin SL, Rao-Melacini P, et al. Risk factors for ischaemic and intracerebral haemorrhagic stroke in 22 countries (the INTERSTROKE study): a case-control study. Lancet. 2010;376(9735):112-23.
23. Adams HP, Jr., Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. Stroke. 1993;24(1):35-41.
24. Kleindorfer DO, Towfighi A, Chaturvedi S, Cockroft KM, Gutierrez J, Lombardi-Hill D, et al. 2021 Guideline for the prevention of stroke in patients with stroke and transient ischemic attack: a guideline from The American Heart Association/American Stroke Association. Stroke. 2021;52(7):e364-e467.
25. Emerging Risk Factors C, Kaptoge S, Di Angelantonio E, Lowe G, Pepys MB, Thompson SG, et al. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. Lancet. 2010;375(9709):132-40.
26. Boehme AK, Esenwa C, Elkind MS. Stroke risk factors, genetics, and prevention. Circ Res. 2017;120(3):472-95.
27. Nathan C, Ding A. Nonresolving inflammation. Cell. 2010;140(6):871-82.
28. Rucker V, Heuschmann PU, O'Flaherty M, Weingartner M, Hess M, Sedlak C, et al. Twenty-year time trends in long-term case-fatality and recurrence rates after ischemic stroke stratified by etiology. Stroke. 2020;51(9):2778-85.
29. Song P, Fang Z, Wang H, Cai Y, Rahimi K, Zhu Y, et al. Global and regional prevalence, burden, and risk factors for carotid atherosclerosis: a systematic review, meta-analysis, and modelling study. Lancet Glob Health. 2020;8(5):e721-e9.
30. Matsuura Y, Kanter JE, Bornfeldt KE. Highlighting residual atherosclerotic cardiovascular disease risk. Arterioscler Thromb Vasc Biol. 2019;39(1):e1-e9.
31. Arnett DK, Blumenthal RS, Albert MA, Buroker AB, Goldberger ZD, Hahn EJ, et al. 2019 ACC/AHA Guideline on the primary prevention of cardiovascular disease: executive summary: a report of The American College of Cardiology/American Heart Association task force on clinical practice guidelines. J Am Coll Cardiol. 2019;74(10):1376-414.
32. Berg DD, Ruff CT, Morrow DA. Biomarkers for risk assessment in atrial fibrillation. Clin Chem. 2021;67(1):87-95.
33. Freedman B, Potpara TS, Lip GY. Stroke prevention in atrial fibrillation. Lancet. 2016;388(10046):806-17.
34. Lip G, Freedman B, De Caterina R, Potpara TS. Stroke prevention in atrial fibrillation: Past, present and future. Comparing the guidelines and practical decision-making. Thromb Haemost. 2017;117(7):1230-9.
35. Wardlaw JM, Smith EE, Biessels GJ, Cordonnier C, Fazekas F, Frayne R, et al. Neuroimaging standards for research into small vessel disease and its contribution to ageing and neurodegeneration. Lancet Neurol. 2013;12(8):822-38.
36. Zeestraten EA, Lawrence AJ, Lambert C, Benjamin P, Brookes RL, Mackinnon AD, et al. Change in multimodal MRI markers predicts dementia risk in cerebral small vessel disease. Neurology. 2017;89(18):1869-76.

37. Pantoni L. Cerebral small vessel disease: from pathogenesis and clinical characteristics to therapeutic challenges. Lancet Neurol. 2010;9(7):689-701.
38. Iadecola C. The pathobiology of vascular dementia. Neuron. 2013;80(4):844-66.
39. Taylor-Rowan M, Wilson A, Dawson J, Quinn TJ. Functional assessment for acute stroke trials: properties, analysis, and application. Front Neurol. 2018;9:191.
40. World Health Organization. International Classification of Functioning, Disability and Health: ICF. 2001. Available from: https://www.who.int/standards/classifications/international-classification-of-functioning-disability-and-health. Access date: 2021-10-04. [
41. Brott T, Adams HP, Jr., Olinger CP, Marler JR, Barsan WG, Biller J, et al. Measurements of acute cerebral infarction: a clinical examination scale. Stroke. 1989;20(7):864-70.
42. Mahoney FI, Barthel DW. Functional evaluation: the Barthel Index. Md State Med J. 1965;14:61-5.
43. Duffy L, Gajree S, Langhorne P, Stott DJ, Quinn TJ. Reliability (inter-rater agreement) of the Barthel Index for assessment of stroke survivors: systematic review and meta-analysis. Stroke. 2013;44(2):462-8.
44. Banks JL, Marotta CA. Outcomes validity and reliability of the modified Rankin scale: implications for stroke clinical trials: a literature review and synthesis. Stroke. 2007;38(3):1091-6.
45. Thivel D, Tremblay A, Genin PM, Panahi S, Riviere D, Duclos M. Physical activity, inactivity, and sedentary behaviors: definitions and implications in occupational health. Front Public Health. 2018;6:288.
46. Caspersen CJ, Powell KE, Christenson GM. Physical activity, exercise, and physical fitness: definitions and distinctions for health-related research. Public Health Rep. 1985;100(2):126-31.
47. Duncan S, Stewart T, Bo Schneller M, Godbole S, Cain K, Kerr J. Convergent validity of ActiGraph and Actical accelerometers for estimating physical activity in adults. PLoS One. 2018;13(6):e0198587.
48. Kramer S, Johnson L, Bernhardt J, Cumming T. Energy expenditure and cost during walking after stroke: a systematic review. Arch Phys Med Rehabil. 2016;97(4):619-32 e1.
49. Mandigout S, Lacroix J, Ferry B, Vuillerme N, Compagnat M, Daviet JC. Can energy expenditure be accurately assessed using accelerometry-based wearable motion detectors for physical activity monitoring in post-stroke patients in the subacute phase? Eur J Prev Cardiol. 2017;24(18):2009-16.
50. Moore SA, Hallsworth K, Bluck LJ, Ford GA, Rochester L, Trenell MI. Measuring energy expenditure after stroke: validation of a portable device. Stroke. 2012;43(6):1660-2.
51. Taraldsen K, Askim T, Sletvold O, Einarsen EK, Bjastad KG, Indredavik B, et al. Evaluation of a body-worn sensor system to measure physical activity in older people with impaired function. Phys Ther. 2011;91(2):277-85.
52. Mansoubi M, Pearson N, Clemes SA, Biddle SJ, Bodicoat DH, Tolfrey K, et al. Energy expenditure during common sitting and standing tasks: examining the 1.5 MET definition of sedentary behaviour. BMC Public Health. 2015;15:516.
53. Edwardson CL, Winkler EAH, Bodicoat DH, Yates T, Davies MJ, Dunstan DW, et al. Considerations when using the activPAL monitor in field-based research with adult populations. J Sport Health Sci. 2017;6(2):162-78.
54. Lyden K, Kozey Keadle SL, Staudenmayer JW, Freedson PS. Validity of two wearable monitors to estimate breaks from sedentary time. Med Sci Sports Exerc. 2012;44(11):2243-52.
55. Kuster RP, Grooten WJA, Blom V, Baumgartner D, Hagstromer M, Ekblom O. How accurate and precise can we measure the posture and the energyeExpenditure component of sedentary behaviour with one sensor? Int J Environ Res Public Health. 2021;18(11).
56. Joisten N, Walzik D, Metcalfe AJ, Bloch W, Zimmer P. Physical exercise as kynurenine pathway modulator in chronic diseases: implications for immune and energy homeostasis. Int J Tryptophan Res. 2020;13:1178646920938688.

57. Mackie P, Weerasekara I, Crowfoot G, Janssen H, Holliday E, Dunstan D, et al. What is the effect of interrupting prolonged sitting with frequent bouts of physical activity or standing on first or recurrent stroke risk factors? A scoping review. PLoS One. 2019;14(6):e0217981.
58. Mander BA, Winer JR, Walker MP. Sleep and human aging. Neuron. 2017;94(1):19-36.
59. Sivertsen B, Krokstad S, Overland S, Mykletun A. The epidemiology of insomnia: associations with physical and mental health. The HUNT-2 study. J Psychosom Res. 2009;67(2):109-16.
60. Cox SR, Ritchie SJ, Allerhand M, Hagenaars SP, Radakovic R, Breen DP, et al. Sleep and cognitive aging in the eighth decade of life. Sleep. 2019;42(4).
61. van der Berg JD, Willems PJ, van der Velde JH, Savelberg HH, Schaper NC, Schram MT, et al. Identifying waking time in 24-h accelerometry data in adults using an automated algorithm. J Sports Sci. 2016;34(19):1867-73.
62. FDA-NIH Biomarker Working Group. BEST (Biomarkers, EndpointS, and other Tools) Resource. Silver Spring (MD): Food and Drug Administration (US); 2016-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK326791/ .
63. Paynter NP, Kiefe CI, Lewis CE, Loria CM, Goff DC, Lloyd-Jones DM. Accumulation of metabolic cardiovascular risk factors in black and white young adults over 20 years. J Am Heart Assoc. 2015;4(4).
64. Kumar R, Nandhini LP, Kamalanathan S, Sahoo J, Vivekanadan M. Evidence for current diagnostic criteria of diabetes mellitus. World J Diabetes. 2016;7(17):396-405.
65. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985;28(7):412-9.
66. Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes care. 1999;22(9):1462-70.
67. American Diabetes Association. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes - 2020. Diabetes care. 2020;43(Suppl 1):S14-S31.
68. Sarafidis PA, Lasaridis AN, Nilsson PM, Pikilidou MI, Stafilas PC, Kanaki A, et al. Validity and reproducibility of HOMA-IR, 1/HOMA-IR, QUICKI and McAuley's indices in patients with hypertension and type II diabetes. J Hum Hypertens. 2007;21(9):709-16.
69. Diaz KM, Goldsmith J, Greenlee H, Strizich G, Qi Q, Mossavar-Rahmani Y, et al. Prolonged, uninterrupted sedentary behavior and glycemic biomarkers among US Hispanic/Latino adults: The HCHS/SOL (Hispanic Community Health Study/Study of Latinos). Circulation. 2017;136(15):1362-73.
70. Park DR, Park KH, Kim BJ, Yoon CS, Kim UH. Exercise ameliorates insulin resistance via Ca2+ signals distinct from those of insulin for GLUT4 translocation in skeletal muscles. Diabetes. 2015;64(4):1224-34.
71. Kim JY, Yang Y, Sim YJ. Effects of smoking and aerobic exercise on male college students' metabolic syndrome risk factors. J Phys Ther Sci. 2018;30(4):595-600.
72. Emerging Risk Factors C, Sarwar N, Gao P, Seshasai SR, Gobin R, Kaptoge S, et al. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. Lancet. 2010;375(9733):2215-22.
73. Zhang L, Wang M, Zhang R, Zhong Y, Fan H, Wang M, et al. Hemoglobin glycation index in relationship to the risk of cardiovascular complication in patients with type 2 diabetes: a systematic review and meta-analysis. J Diabetes Complications. 2020;34(10):107673.
74. Gu T, Yang Q, Ying G, Jin B. Lack of association between insulin resistance as estimated by homeostasis model assessment and stroke risk: a systematic review and meta-analysis. Med Hypotheses. 2020;141:109700.
75. Sorgdrager FJH, Naude PJW, Kema IP, Nollen EA, Deyn PP. Tryptophan metabolism in inflammaging: from biomarker to therapeutic target. Front Immunol. 2019;10:2565.
76. Ferrucci L, Gonzalez-Freire M, Fabbri E, Simonsick E, Tanaka T, Moore Z, et al. Measuring biological aging in humans: a quest. Aging Cell. 2020;19(2):e13080.
77. Abbas AKL, A. H.; Pillai, S.;. Cellular and molecular immunologi. 9th ed. : Elsevier; 2018.

78. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. Eur J Immunol. 2010;40(7):1830-5.
79. Netea MG, Dominguez-Andres J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining trained immunity and its role in health and disease. Nat Rev Immunol. 2020;20(6):375-88.
80. Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, et al. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. Immunity. 2014;40(2):274-88.
81. Stenken JA, Poschenrieder AJ. Bioanalytical chemistry of cytokines - a review. Anal Chim Acta. 2015;853:95-115.
82. Storjord E, Henno LT, Mollnes TE, Brekke OL. Analysis of cytokines. Tidsskr Nor Laegeforen. 2020;140(1).
83. Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. Biochim Biophys Acta. 2014;1843(11):2563-82.
84. Liu C, Chu D, Kalantar-Zadeh K, George J, Young HA, Liu G. Cytokines: from clinical significance to quantification. Adv Sci (Weinh). 2021;8(15):e2004433.
85. Ridker PM. From C-reactive protein to interleukin-6 to interleukin-1: moving upstream to identify novel targets for atheroprotection. Circ Res. 2016;118(1):145-56.
86. Nelson FR, Zvirbulis RA, Zonca B, Li KW, Turner SM, Pasierb M, et al. The effects of an oral preparation containing hyaluronic acid (Oralvisc(R)) on obese knee osteoarthritis patients determined by pain, function, bradykinin, leptin, inflammatory cytokines, and heavy water analyses. Rheumatol Int. 2015;35(1):43-52.
87. Geiger SS, Fagundes CT, Siegel RM. Chrono-immunology: progress and challenges in understanding links between the circadian and immune systems. Immunology. 2015;146(3):349-58.
88. Fuchs D, Avanzas P, Arroyo-Espliguero R, Jenny M, Consuegra-Sanchez L, Kaski JC. The role of neopterin in atherogenesis and cardiovascular risk assessment. Curr Med Chem. 2009;16(35):4644-53.
89. Fuchs D, Weiss G, Wachter H. Neopterin, biochemistry and clinical use as a marker for cellular immune reactions. Int Arch Allergy Immunol. 1993;101(1):1-6.
90. Badawy AA. Kynurenine pathway of tryptophan metabolism: regulatory and functional aspects. Int J Tryptophan Res. 2017;10:1178646917691938.
91. Wirthgen E, Hoeflich A, Rebl A, Gunther J. Kynurenic acid: the Janus-faced role of an immunomodulatory tryptophan metabolite and its link to pathological conditions. Front Immunol. 2017;8:1957.
92. Morris MS, Sakakeeny L, Jacques PF, Picciano MF, Selhub J. Vitamin B-6 intake is inversely related to, and the requirement is affected by, inflammation status. J Nutr. 2010;140(1):103-10.
93. Gori AM, Sofi F, Corsi AM, Gazzini A, Sestini I, Lauretani F, et al. Predictors of vitamin B6 and folate concentrations in older persons: the InCHIANTI study. Clin Chem. 2006;52(7):1318-24.
94. Bleie O, Semb AG, Grundt H, Nordrehaug JE, Vollset SE, Ueland PM, et al. Homocysteine-lowering therapy does not affect inflammatory markers of atherosclerosis in patients with stable coronary artery disease. J Intern Med. 2007;262(2):244-53.
95. Ebbing M, Bleie O, Ueland PM, Nordrehaug JE, Nilsen DW, Vollset SE, et al. Mortality and cardiovascular events in patients treated with homocysteine-lowering B vitamins after coronary angiography: a randomized controlled trial. JAMA. 2008;300(7):795-804.
96. O'Doherty MG, Gilchrist SE, Young IS, McKinley MC, Yarnell JW, Gey KF, et al. Effect of supplementation with B vitamins and antioxidants on levels of asymmetric dimethylarginine (ADMA) and C-reactive protein (CRP): a double-blind, randomised, factorial design, placebo-controlled trial. Eur J Nutr. 2010;49(8):483-92.
97. Ulvik A, Midttun O, Pedersen ER, Eussen SJ, Nygard O, Ueland PM. Evidence for increased catabolism of vitamin B-6 during systemic inflammation. Am J Clin Nutr. 2014;100(1):250-5.
98. Ulvik A, Midttun O, Pedersen ER, Nygard O, Ueland PM. Association of plasma B-6 vitamers with systemic markers of inflammation before and after pyridoxine treatment in patients with stable angina pectoris. Am J Clin Nutr. 2012;95(5):1072-8.

99. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol. 2009;27:519-50.
100. Munoz-Canoves P, Scheele C, Pedersen BK, Serrano AL. Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword? FEBS J. 2013;280(17):4131-48.
101. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol. 2014;6(10):a016295.
102. Foon KA, Sherwin SA, Abrams PG, Stevenson HC, Holmes P, Maluish AE, et al. A phase I trial of recombinant gamma interferon in patients with cancer. Cancer Immunol Immunother. 1985;20(3):193-7.
103. Taylor MW, Feng GS. Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism. FASEB J. 1991;5(11):2516-22.
104. Wei H, Li B, Sun A, Guo F. Interleukin-10 family cytokines immunobiology and structure. Adv Exp Med Biol. 2019;1172:79-96.
105. Cole JE, Astola N, Cribbs AP, Goddard ME, Park I, Green P, et al. Indoleamine 2,3-dioxygenase-1 is protective in atherosclerosis and its metabolites provide new opportunities for drug development. Proc Natl Acad Sci U S A. 2015;112(42):13033-8.
106. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. Nat Rev Immunol. 2011;11(9):607-15.
107. Leal LG, Lopes MA, Batista ML, Jr. Physical exercise-induced myokines and muscle-adipose tissue crosstalk: a review of current knowledge and the implications for health and metabolic diseases. Front Physiol. 2018;9:1307.
108. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science. 1998;281(5380):1191-3.
109. Durr S, Kindler V. Implication of indolamine 2,3 dioxygenase in the tolerance toward fetuses, tumors, and allografts. J Leukoc Biol. 2013;93(5):681-7.
110. Badawy AA. Hypothesis kynurenic and quinolinic acids: The main players of the kynurenine pathway and opponents in inflammatory disease. Med Hypotheses. 2018;118:129-38.
111. Rothhammer V, Quintana FJ. The aryl hydrocarbon receptor: an environmental sensor integrating immune responses in health and disease. Nat Rev Immunol. 2019;19(3):184-97.
112. Mandi Y, Vecsei L. The kynurenine system and immunoregulation. J Neural Transm (Vienna). 2012;119(2):197-209.
113. de Araujo EF, Loures FV, Preite NW, Feriotti C, Galdino NA, Costa TA, et al. AhR ligands modulate the differentiation of innate lymphoid cells and T helper cell subsets that control the severity of a pulmonary fungal infection. Front Immunol. 2021;12:630938.
114. Wirth K, Klenk J, Brefka S, Dallmeier D, Faehling K, Roque IFM, et al. Biomarkers associated with sedentary behaviour in older adults: a systematic review. Ageing Res Rev. 2017;35:87-111.
115. Swindell N, Rees P, Fogelholm M, Drummen M, MacDonald I, Martinez JA, et al. Compositional analysis of the associations between 24-h movement behaviours and cardio-metabolic risk factors in overweight and obese adults with pre-diabetes from the PREVIEW study: cross-sectional baseline analysis. The international journal of behavioral nutrition and physical activity. 2020;17(1):29.
116. Noz MP, Hartman YAW, Hopman MTE, Willems P, Tack CJ, Joosten LAB, et al. Sixteen-week physical activity intervention in subjects with increased cardiometabolic risk shifts innate immune function towards a less proinflammatory state. J Am Heart Assoc. 2019;8(21):e013764.
117. Abd El-Kader SM, Al-Jiffri OH. Aerobic exercise modulates cytokine profile and sleep quality in elderly. Afr Health Sci. 2019;19(2):2198-207.
118. Rodas L, Riera-Sampol A, Aguilo A, Martinez S, Tauler P. Effects of habitual caffeine intake, physical activity levels, and sedentary behavior on the inflammatory status in a healthy population. Nutrients. 2020;12(8).

119. Leung A, Gregory NS, Allen LH, Sluka KA. Regular physical activity prevents chronic pain by altering resident muscle macrophage phenotype and increasing interleukin-10 in mice. Pain. 2016;157(1):70-9.
120. Valdiglesias V, Sanchez-Flores M, Maseda A, Lorenzo-Lopez L, Marcos-Perez D, Lopez-Corton A, et al. Immune biomarkers in older adults: role of physical activity. J Toxicol Environ Health A. 2017;80(13-15):605-20.
121. Metcalfe AJ, Koliamitra C, Javelle F, Bloch W, Zimmer P. Acute and chronic effects of exercise on the kynurenine pathway in humans - a brief review and future perspectives. Physiol Behav. 2018;194:583-7.
122. Allison DJ, Nederveen JP, Snijders T, Bell KE, Kumbhare D, Phillips SM, et al. Exercise training impacts skeletal muscle gene expression related to the kynurenine pathway. Am J Physiol Cell Physiol. 2019;316(3):C444-C8.
123. Agudelo LZ, Femenia T, Orhan F, Porsmyr-Palmertz M, Goiny M, Martinez-Redondo V, et al. Skeletal muscle PGC-1alpha1 modulates kynurenine metabolism and mediates resilience to stress-induced depression. Cell. 2014;159(1):33-45.
124. Maseri A. Inflammation, atherosclerosis, and ischemic events - exploring the hidden side of the moon. N Engl J Med. 1997;336(14):1014-6.
125. Esenwa CC, Elkind MS. Inflammatory risk factors, biomarkers and associated therapy in ischaemic stroke. Nat Rev Neurol. 2016;12(10):594-604.
126. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, et al. Antiinflammatory therapy with Canakinumab for atherosclerotic disease. N Engl J Med. 2017;377(12):1119-31.
127. Zuo H, Tell GS, Ueland PM, Nygard O, Vollset SE, Midttun O, et al. The PAr index, an indicator reflecting altered vitamin B-6 homeostasis, is associated with long-term risk of stroke in the general population: the Hordaland Health Study (HUSK). Am J Clin Nutr. 2018;107(1):105-12.
128. Pedersen ER, Midttun O, Ueland PM, Schartum-Hansen H, Seifert R, Igland J, et al. Systemic markers of interferon-gamma-mediated immune activation and long-term prognosis in patients with stable coronary artery disease. Arterioscler Thromb Vasc Biol. 2011;31(3):698-704.
129. Boehme AK, McClure LA, Zhang Y, Luna JM, Del Brutto OH, Benavente OR, et al. Inflammatory markers and outcomes after lacunar stroke: levels of inflammatory markers in treatment of stroke study. Stroke. 2016;47(3):659-67.
130. Everett BM, Glynn RJ, MacFadyen JG, Ridker PM. Rosuvastatin in the prevention of stroke among men and women with elevated levels of C-reactive protein: Justification for the Use of statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER). Circulation. 2010;121(1):143-50.
131. Ridker PM, Everett BM, Pradhan A, MacFadyen JG, Solomon DH, Zaharris E, et al. Low-dose methotrexate for the prevention of atherosclerotic events. N Engl J Med. 2019;380(8):752-62.
132. Tardif JC, Kouz S, Waters DD, Bertrand OF, Diaz R, Maggioni AP, et al. Efficacy and safety of low-dose Colchicine after myocardial infarction. N Engl J Med. 2019;381(26):2497-505.
133. Liberale L, Montecucco F, Schwarz L, Luscher TF, Camici GG. Inflammation and cardiovascular diseases: lessons from seminal clinical trials. Cardiovasc Res. 2021;117(2):411-22.
134. Sulo G, Vollset SE, Nygard O, Midttun O, Ueland PM, Eussen SJ, et al. Neopterin and kynurenine-tryptophan ratio as predictors of coronary events in older adults, the Hordaland Health Study. Int J Cardiol. 2013;168(2):1435-40.
135. Li M, Kwok MK, Fong SSM, Schooling CM. Indoleamine 2,3-dioxygenase and ischemic heart disease: a mendelian randomization study. Sci Rep. 2019;9(1):8491.
136. Pedersen ER, Tuseth N, Eussen SJ, Ueland PM, Strand E, Svingen GF, et al. Associations of plasma kynurenines with risk of acute myocardial infarction in patients with stable angina pectoris. Arterioscler Thromb Vasc Biol. 2015;35(2):455-62.
137. Yu E, Ruiz-Canela M, Guasch-Ferre M, Zheng Y, Toledo E, Clish CB, et al. Increases in plasma tryptophan are inversely associated with incident cardiovascular disease in the prevencion con dieta mediterranea (PREDIMED) study. J Nutr. 2017;147(3):314-22.

138. Tomiyama H, Ishizu T, Kohro T, Matsumoto C, Higashi Y, Takase B, et al. Longitudinal association among endothelial function, arterial stiffness and subclinical organ damage in hypertension. Int J Cardiol. 2018;253:161-6.
139. Bonarjee VVS. Arterial stiffness: a prognostic marker in coronary heart disease. Available methods and clinical application. Front Cardiovasc Med. 2018;5:64.
140. Ulvik A, Pedersen ER, Svingen GF, McCann A, Midttun O, Nygard O, et al. Vitamin B-6 catabolism and long-term mortality risk in patients with coronary artery disease. Am J Clin Nutr. 2016;103(6):1417-25.
141. Korantzopoulos P, Letsas KP, Tse G, Fragakis N, Goudis CA, Liu T. Inflammation and atrial fibrillation: a comprehensive review. J Arrhythm. 2018;34(4):394-401.
142. Low A, Mak E, Rowe JB, Markus HS, O'Brien JT. Inflammation and cerebral small vessel disease: a systematic review. Ageing Res Rev. 2019;53:100916.
143. Bekkering S, van den Munckhof I, Nielen T, Lamfers E, Dinarello C, Rutten J, et al. Innate immune cell activation and epigenetic remodeling in symptomatic and asymptomatic atherosclerosis in humans in vivo. Atherosclerosis. 2016;254:228-36.
144. Sugioka K, Naruko T, Matsumura Y, Shirai N, Hozumi T, Yoshiyama M, et al. Neopterin and atherosclerotic plaque instability in coronary and carotid arteries. J Atheroscler Thromb. 2010;17(11):1115-21.
145. Sugioka K, Naruko T, Hozumi T, Nakagawa M, Kitabayashi C, Ikura Y, et al. Elevated levels of neopterin are associated with carotid plaques with complex morphology in patients with stable angina pectoris. Atherosclerosis. 2010;208(2):524-30.
146. Niinisalo P, Oksala N, Levula M, Pelto-Huikko M, Jarvinen O, Salenius JP, et al. Activation of indoleamine 2,3-dioxygenase-induced tryptophan degradation in advanced atherosclerotic plaques: Tampere vascular study. Ann Med. 2010;42(1):55-63.
147. Pertovaara M, Raitala A, Juonala M, Lehtimaki T, Huhtala H, Oja SS, et al. Indoleamine 2,3-dioxygenase enzyme activity correlates with risk factors for atherosclerosis: the Cardiovascular Risk in Young Finns Study. Clin Exp Immunol. 2007;148(1):106-11.
148. Niinisalo P, Raitala A, Pertovaara M, Oja SS, Lehtimaki T, Kahonen M, et al. Indoleamine 2,3-dioxygenase activity associates with cardiovascular risk factors: the Health 2000 study. Scand J Clin Lab Invest. 2008;68(8):767-70.
149. Huang YS, Ogbechi J, Clanchy FI, Williams RO, Stone TW. IDO and kynurenine metabolites in peripheral and CNS disorders. Front Immunol. 2020;11:388.
150. Song P, Ramprasath T, Wang H, Zou MH. Abnormal kynurenine pathway of tryptophan catabolism in cardiovascular diseases. Cell Mol Life Sci. 2017;74(16):2899-916.
151. Baumgartner R, Berg M, Matic L, Polyzos KP, Forteza MJ, Hjorth SA, et al. Evidence that a deviation in the kynurenine pathway aggravates atherosclerotic disease in humans. J Intern Med. 2021;289(1):53-68.
152. Polyzos KA, Ovchinnikova O, Berg M, Baumgartner R, Agardh H, Pirault J, et al. Inhibition of indoleamine 2,3-dioxygenase promotes vascular inflammation and increases atherosclerosis in Apoe-/- mice. Cardiovasc Res. 2015;106(2):295-302.
153. Zapolski T, Kaminska A, Kocki T, Wysokinski A, Urbanska EM. Aortic stiffness - is kynurenic acid a novel marker? Cross-sectional study in patients with persistent atrial fibrillation. PLoS One. 2020;15(7):e0236413.
154. Li L, Yiin GS, Geraghty OC, Schulz UG, Kuker W, Mehta Z, et al. Incidence, outcome, risk factors, and long-term prognosis of cryptogenic transient ischaemic attack and ischaemic stroke: a population-based study. Lancet Neurol. 2015;14(9):903-13.
155. Sposato LA, Cipriano LE, Saposnik G, Ruiz Vargas E, Riccio PM, Hachinski V. Diagnosis of atrial fibrillation after stroke and transient ischaemic attack: a systematic review and meta-analysis. Lancet Neurol. 2015;14(4):377-87.
156. Ma Y, Pan Z, Fan D, Xu S, Pan F. The increased risk of atrial fibrillation in inflammatory arthritis: a systematic review and meta-analysis of cohort studies. Immunol Invest. 2021:1-13.

157. Tancin Lambert A, Kong XY, Ratajczak-Tretel B, Atar D, Russell D, Skjelland M, et al. Biomarkers associated with atrial fibrillation in patients with ischemic stroke: a pilot study from the NOR-FIB study. Cerebrovasc Dis Extra. 2020;10(1):11-20.
158. Lind L, Sundstrom J, Stenemo M, Hagstrom E, Arnlov J. Discovery of new biomarkers for atrial fibrillation using a custom-made proteomics chip. Heart. 2017;103(5):377-82.
159. Li Y, Tan W, Ye F, Wen S, Hu R, Cai X, et al. Inflammation as a risk factor for stroke in atrial fibrillation: data from a microarray data analysis. J Int Med Res. 2020;48(5):300060520921671.
160. Guo Y, Lip GY, Apostolakis S. Inflammation in atrial fibrillation. J Am Coll Cardiol. 2012;60(22):2263-70.
161. Packer M. Characterization, pathogenesis, and clinical Implications of inflammation-related atrial myopathy as an important cause of atrial fibrillation. J Am Heart Assoc. 2020;9(7):e015343.
162. Aulin J, Hijazi Z, Siegbahn A, Andersson U, Alexander JH, Connolly SJ, et al. Serial measurement of interleukin-6 and risk of mortality in anticoagulated patients with atrial fibrillation: insights from ARISTOTLE and RE-LY trials. J Thromb Haemost. 2020;18(9):2287-95.
163. Hijazi Z, Aulin J, Andersson U, Alexander JH, Gersh B, Granger CB, et al. Biomarkers of inflammation and risk of cardiovascular events in anticoagulated patients with atrial fibrillation. Heart. 2016;102(7):508-17.
164. Poggesi A, Pasi M, Pescini F, Pantoni L, Inzitari D. Circulating biologic markers of endothelial dysfunction in cerebral small vessel disease: A review. J Cereb Blood Flow Metab. 2016;36(1):72-94.
165. Jian B, Hu M, Cai W, Zhang B, Lu Z. Update of immunosenescence in cerebral small vessel disease. Front Immunol. 2020;11:585655.
166. Noz MP, Ter Telgte A, Wiegertjes K, Joosten LAB, Netea MG, de Leeuw FE, et al. Trained immunity characteristics are associated with progressive cerebral small vessel disease. Stroke. 2018;49(12):2910-7.
167. van Dijk EJ, Prins ND, Vermeer SE, Vrooman HA, Hofman A, Koudstaal PJ, et al. C-reactive protein and cerebral small-vessel disease: the Rotterdam Scan Study. Circulation. 2005;112(6):900-5.
168. Shoamanesh A, Preis SR, Beiser AS, Vasan RS, Benjamin EJ, Kase CS, et al. Inflammatory biomarkers, cerebral microbleeds, and small vessel disease: Framingham Heart Study. Neurology. 2015;84(8):825-32.
169. Rouhl RP, Damoiseaux JG, Lodder J, Theunissen RO, Knottnerus IL, Staals J, et al. Vascular inflammation in cerebral small vessel disease. Neurobiol Aging. 2012;33(8):1800-6.
170. Thingstad P, Askim T, Beyer MK, Brathen G, Ellekjaer H, Ihle-Hansen H, et al. The Norwegian cognitive impairment after stroke study (Nor-COAST): study protocol of a multicentre, prospective cohort study. BMC Neurol. 2018;18(1):193.
171. Fine JPG, R. J. A proportional hazards model for the subdistribution of a competing risk. Journal of the American Statistical Association 1999;94(443):14.
172. Khanevski AN, Bjerkreim AT, Novotny V, Naess H, Thomassen L, Logallo N, et al. Recurrent ischemic stroke: incidence, predictors, and impact on mortality. Acta Neurol Scand. 2019;140(1):3-8.
173. Mosarrezaii A, Amiri-Nikpour MR, Mehryar HR, Choobi Anzali B, Nourooz-Zadeh S, Babaei S, et al. Investigating the relationship between interleukin-6 serum levels and outcome in acute ischemic CVA. Brain Behav. 2020;10(8):e01668.
174. Gu Y, Gutierrez J, Meier IB, Guzman VA, Manly JJ, Schupf N, et al. Circulating inflammatory biomarkers are related to cerebrovascular disease in older adults. Neurol Neuroimmunol Neuroinflamm. 2019;6(1):e521.
175. Bruunsgaard H. Physical activity and modulation of systemic low-level inflammation. J Leukoc Biol. 2005;78(4):819-35.
176. Wang Y, Xu J, Zhao X, Wang D, Wang C, Liu L, et al. Association of hypertension with stroke recurrence depends on ischemic stroke subtype. Stroke. 2013;44(5):1232-7.
177. Flores-Opazo M, McGee SL, Hargreaves M. Exercise and GLUT4. Exerc Sport Sci Rev. 2020;48(3):110-8.

178. English C, Janssen H, Crowfoot G, Bourne J, Callister R, Dunn A, et al. Frequent, short bouts of light-intensity exercises while standing decreases systolic blood pressure: Breaking Up Sitting Time after Stroke (BUST-Stroke) trial. Int J Stroke. 2018;13(9):932-40.
179. Lauritzen HP. Insulin- and contraction-induced glucose transporter 4 traffic in muscle: insights from a novel imaging approach. Exerc Sport Sci Rev. 2013;41(2):77-86.
180. Hussey SE, McGee SL, Garnham A, McConell GK, Hargreaves M. Exercise increases skeletal muscle GLUT4 gene expression in patients with type 2 diabetes. Diabetes Obes Metab. 2012;14(8):768-71.
181. Kraniou GN, Cameron-Smith D, Hargreaves M. Effect of short-term training on GLUT-4 mRNA and protein expression in human skeletal muscle. Exp Physiol. 2004;89(5):559-63.
182. Hart TL, Swartz AM, Cashin SE, Strath SJ. How many days of monitoring predict physical activity and sedentary behaviour in older adults? The international journal of behavioral nutrition and physical activity. 2011;8:62.
183. Aguilar-Farias N, Martino-Fuentealba P, Salom-Diaz N, Brown WJ. How many days are enough for measuring weekly activity behaviours with the ActivPAL in adults? J Sci Med Sport. 2019;22(6):684-8.
184. Chia CW, Egan JM, Ferrucci L. Age-related changes in glucose metabolism, hyperglycemia, and cardiovascular risk. Circ Res. 2018;123(7):886-904.
185. Beale C, Rauff EL, O'Brien WJ, Shultz SP, Fink PW, Kruger R. Are all sedentary behaviors equal? An examination of sedentary behavior and associations with indicators of disease risk factors in women. Int J Environ Res Public Health. 2020;17(8).
186. Scott RA, Chu AY, Grarup N, Manning AK, Hivert MF, Shungin D, et al. No interactions between previously associated 2-hour glucose gene variants and physical activity or BMI on 2-hour glucose levels. Diabetes. 2012;61(5):1291-6.
187. Inoue K, Goto A, Sugiyama T, Ramlau-Hansen CH, Liew Z. The confounder-mediator dilemma: should we control for obesity to estimate the effect of perfluoroalkyl substances on health outcomes? Toxics. 2020;8(4).
188. McFarlane SI, Banerji M, Sowers JR. Insulin resistance and cardiovascular disease. J Clin Endocrinol Metab. 2001;86(2):713-8.
189. Tabak AG, Jokela M, Akbaraly TN, Brunner EJ, Kivimaki M, Witte DR. Trajectories of glycaemia, insulin sensitivity, and insulin secretion before diagnosis of type 2 diabetes: an analysis from the Whitehall II study. Lancet. 2009;373(9682):2215-21.
190. Kolb H, Kempf K, Rohling M, Martin S. Insulin: too much of a good thing is bad. BMC Med. 2020;18(1):224.
191. Cronin O, Keohane DM, Molloy MG, Shanahan F. The effect of exercise interventions on inflammatory biomarkers in healthy, physically inactive subjects: a systematic review. QJM. 2017;110(10):629-37.
192. Zanoli L, Briet M, Empana JP, Cunha PG, Maki-Petaja KM, Protogerou AD, et al. Vascular consequences of inflammation: a position statement from the ESH Working Group on Vascular Structure and Function and the ARTERY Society. J Hypertens. 2020;38(9):1682-98.
193. Jung TW, Park J, Sun JL, Ahn SH, Abd El-Aty AM, Hacimuftuoglu A, et al. Administration of kynurenic acid reduces hyperlipidemia-induced inflammation and insulin resistance in skeletal muscle and adipocytes. Mol Cell Endocrinol. 2020;518:110928.
194. Agudelo LZ, Ferreira DMS, Cervenka I, Bryzgalova G, Dadvar S, Jannig PR, et al. Kynurenic acid and Gpr35 regulate adipose tissue energy homeostasis and inflammation. Cell Metab. 2018;27(2):378-92 e5.
195. Mudry JM, Alm PS, Erhardt S, Goiny M, Fritz T, Caidahl K, et al. Direct effects of exercise on kynurenine metabolism in people with normal glucose tolerance or type 2 diabetes. Diabetes Metab Res Rev. 2016;32(7):754-61.
196. Ross R, Neeland IJ, Yamashita S, Shai I, Seidell J, Magni P, et al. Waist circumference as a vital sign in clinical practice: a consensus statement from the IAS and ICCR Working Group on Visceral Obesity. Nat Rev Endocrinol. 2020;16(3):177-89.

197. Ridker PM, Libby P, MacFadyen JG, Thuren T, Ballantyne C, Fonseca F, et al. Modulation of the interleukin-6 signalling pathway and incidence rates of atherosclerotic events and all-cause mortality: analyses from the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS). European heart journal. 2018;39(38):3499-507.
198. Coveney S, McCabe JJ, Murphy S, O'Donnell M, Kelly PJ. Anti-inflammatory therapy for preventing stroke and other vascular events after ischaemic stroke or transient ischaemic attack. Cochrane Database Syst Rev. 2020;5:CD012825.
199. Ganz P, Amarenco P, Goldstein LB, Sillesen H, Bao W, Preston GM, et al. Association of osteopontin, neopterin, and myeloperoxidase with stroke risk in patients with prior stroke or transient ischemic attacks: results of an analysis of 13 biomarkers from the Stroke Prevention by Aggressive Reduction in Cholesterol Levels trial. Stroke. 2017;48(12):3223-31.
200. Davidson JA, Pfeifer Z, Frank B, Tong S, Urban TT, Wischmeyer PA, et al. Metabolomic fingerprinting of infants undergoing cardiopulmonary bypass: changes in metabolic pathways and association with mortality and cardiac intensive care unit length of stay. J Am Heart Assoc. 2018;7(24):e010711.
201. Pedersen ED, Waje-Andreassen U, Vedeler CA, Aamodt G, Mollnes TE. Systemic complement activation following human acute ischaemic stroke. Clin Exp Immunol. 2004;137(1):117-22.
202. Schnabel RB, Larson MG, Yamamoto JF, Sullivan LM, Pencina MJ, Meigs JB, et al. Relations of biomarkers of distinct pathophysiological pathways and atrial fibrillation incidence in the community. Circulation. 2010;121(2):200-7.
203. Guo Y, Lip GY, Apostolakis S. Inflammatory biomarkers and atrial fibrillation: potential role of inflammatory pathways in the pathogenesis of atrial fibrillation-induced thromboembolism. Curr Vasc Pharmacol. 2015;13(2):192-201.
204. Kuvas KR, Saltvedt I, Aam S, Thingstad P, Ellekjaer H, Askim T. The risk of selection bias in a clinical multi-center cohort study. Results from the Norwegian COgnitive impairment After STroke (Nor-COAST) Study. Clin Epidemiol. 2020;12:1327-36.
205. Compagnat M, Mandigout S, Chaparro D, Salle JY, Daviet JC. Predicting the oxygen cost of walking in hemiparetic stroke patients. Ann Phys Rehabil Med. 2018;61(5):309-14.
206. Ezeugwu VE, Manns PJ. Sleep duration, sedentary behavior, physical activity, and quality of life after inpatient stroke rehabilitation. J Stroke Cerebrovasc Dis. 2017;26(9):2004-12.
207. Norwegian Stroke Registry. Annual reports from the Norwegian Stroke Registry, 2015-2017. Available from: https://stolav.no/fag-og-forskning/medisinske-kvalitetsregistre/norsk-hjerneslagregister/rapporter. Access date: 2020-05-31
208. Varmdal T, Ellekjaer H, Fjaertoft H, Indredavik B, Lydersen S, Bonaa KH. Inter-rater reliability of a national acute stroke register. BMC Res Notes. 2015;8:584.
209. Pedersen AG, Ellingsen CL. Data quality in the Causes of Death Registry. Tidsskr Nor Laegeforen. 2015;135(8):768-70.
210. Triantafyllou S, Katsanos AH, Dilaveris P, Giannopoulos G, Kossyvakis C, Adreanides E, et al. Implantable cardiac monitoring in the secondary prevention of cryptogenic stroke. Ann Neurol. 2020;88(5):946-55.
211. Lydersen S. Statistical power: before, but not after! Tidsskr Nor Laegeforen. 2019;139(2).

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BMJ Open

Is long-bout sedentary behaviour associated with long-term glucose levels 3 months after acute ischaemic stroke? A prospective observational cohort study

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ABSTRACT

Background and purpose Sedentary behaviour is a risk factor for vascular disease and stroke patients are more sedentary than their age-matched peers. The association with glucose levels, as a potential mediator, is unclear, and we have investigated the association between long-bout sedentary behaviour and long-term glucose levels in stroke survivors.

Methods This study uses data from the Norwegian Cognitive Impairment After Stroke study, a multicentre cohort study. The patients were recruited at hospital admission for acute stroke, and the follow-up was done at the outpatient clinic. Sedentary behaviour—being in a sitting or reclining position—was registered 3 months after stroke using position transition data from the body-worn sensor activPAL attached to the unaffected thigh. A MATLAB script was developed to extract activity data from 08:00 to 10:00 for 4 days and to categorise the data into four bout-length categories. The primary outcome was glycated haemoglobin (HbA1c), analysed at 3 months. Regression models were used to analyse the association between HbA1c and sedentary behaviour in the whole population and stratified based on a diagnosis of diabetes mellitus (DM). Age, body mass index and the use of antidiabetic drugs were added as covariates into the models.

Results From a total of 815 included patients, 379 patients fulfilled the inclusion criteria for this study. We found no association between time in sedentary behaviour and HbA1c in the whole stroke population. We found time in sedentary behaviour in bouts of ≥90 min to be associated with a higher HbA1c in patients with DM.

Conclusion Long-bout sedentary time is associated with a higher HbA1c in patients with DM 3 months after ischaemic stroke. Future research should investigate the benefit of breaking up sedentary time as a secondary preventive measure.

Trial registration number NCT02650531, https://clinicaltrials.gov/ct2/show/NCT02650531

Strengths and limitations of this study

- The study investigates the association between long-bout sedentary behaviour and long-term glucose levels in a large cohort.
- Sedentary behaviour is measured objectively, includes information about bout lenght and the method is valid for the stroke population.
- The outcome is a valid measure for long-term glucose levels.
- We have included information about diabetes mellitus, body mass index and medication use.
- Information about diet, details of medication use and the type of diabetes mellitus including level of insuline resitance would have increased the explanatory abilities of the model, but were not accessible.

INTRODUCTION

Sedentary behaviour is associated with negative health outcomes, especially when accumulated in long bouts and in the least active individuals. Hence, introducing 'sedentary breaks' as an interventional measure has gained interest.[1–3] Sedentary behaviour is associated with vascular disease, like stroke, presumably through metabolic and inflammatory pathways.[4–7] Stroke patients are more sedentary than their age-matched peers from the general population,[8] and targeting sedentary behaviour as a secondary preventive strategy after stroke is recommended.[9] However, details about how different patterns of sedentary behaviour relate to vascular risk factors in different populations are not entirely understood and need to be more thoroughly explored.[9 10]

One unresolved issue is the relationship between sedentary behaviour and glucose metabolism.[4 5 11–14] The relationship is

complex and is dependent on the characteristics of the target population with respect to food intake, habitual activity level, age, body composition, dominating muscle fibre type, diseases (such as diabetes mellitus (DM)) and medication use.[2 4 15–18] Sample size, choice of methods of measuring and analysing sedentary time, and how and when to measure markers of glucose metabolism vary between studies and make it difficult to synthesise the available evidence.[4 11–14 19] Also, many prior studies have relied on questionnaire-based measures of sedentary behaviour.[13] More recently, the use of accelerometer-based technology is increasing, and this allows for more reliable and detailed information about sedentary behaviour, as opposed to self-reported activity.[13]

In this study, we have investigated habitual sedentary behaviour and the association to long-term glucose levels, measured by glycated haemoglobin (HbA1c), in a stroke population, using objective measures of activity. The primary aim was to investigate the association between long-bout sedentary behaviour and glucose levels in stroke survivors. The secondary aim was to investigate how this association was altered in the presence of prestroke DM.

Our hypotheses were that sedentary behaviour in long bouts was associated with long-term glucose levels in an unselected stroke population and that the association would be more pronounced in the subgroup of patients suffering from DM.

MATERIALS AND METHODS

The patients were part of the Norwegian Cognitive Impairment After Stroke (Nor-COAST) study, a prospective cohort study recruiting acute stroke patients from five contributing hospitals from May 2015 to March 2017.[20] The patients were assessed at hospital admission and after 3, 18 and 36 months. Inclusion criteria were (1) acute stroke, according to the WHO criteria, arriving at hospital within 1 week after symptom onset; (2) above 18 years of age; (3) ability to understand Norwegian; and (4) ability to give informed consent. For patients unable to provide consent for themselves, the next of kin may give oral consent. Exclusion criteria were (1) not living in the catchment area of one of the inclusion hospitals, (2) the symptoms explained by other diagnosis than stroke, (3) short life expectancy (<3 months) or a modified Rankin Scale (mRS) score of 5, except for patients included at the main centre, St. Olavs Hospital.

Some additional criteria were made for this substudy: patients were included only if they attended the 3-month follow-up, had ischaemic stroke (including those with haemorrhagic transformation), were able to walk 50 m with a walker or personal support (Barthel Index (BI) item 9: ≥10 points), had blood samples taken and valid activity data, all at 3 months.

Assessment at baseline was performed on day 7 after symptom onset or at discharge from hospital if this occurred earlier. The assessments were performed by trained research assistants at the outpatient clinic, using a standardised case report form.[20]

Sedentary behaviour was measured at 3 months by registering position transition with a single thigh-worn sensor (activPAL3, Model 20.2; PAL Technologies, Glasgow, UK) on the unaffected thigh for seven consecutive days. Only patients with recordings from at least four full days were included. Activity was analysed during daytime, defined as between 08:00 am and 10:00 pm. Sedentary events were divided if they crossed these time boundaries. Manual inspection of the output to identify non-wear time was performed. Sedentary behaviour was defined as sitting or lying. The threshold for noise was 1.5 s and sedentary events were merged if they were broken by events of standing of ≤3 s.

A custom-made MATLAB script (V.R2016b MathWorks, Natick, Massachusetts, USA) was developed to extract frequency and duration of sedentary bouts into predefined bout categories (see Statistics section).

Stroke severity was measured by the National Institutes of Health Stroke Scale on admission and at the three month follow-up, global function by the mRS[21] and basic activities of daily living by the BI.[22] Non-fasting blood samples were analysed for HbA1c %. Body mass index (BMI, kg/m^2) was calculated from height and weight. Medications were analysed based on the Anatomical Therapeutic Chemical (ATC) classification system. The diagnosis of DM was defined at baseline by medical history or medication use (ATC: A10) or finding of HbA1c≥6.5% at baseline. Hypercholesterolaemia at baseline was defined by medication use (ATC: C10) or total cholesterol of >6.2 mmol/L or/and low-density lipoprotein of ≥4.1 mmol/L at baseline. Hypertension at baseline was based on medication use (ATC: C02, C03, C04, C07, C08 and C09) on admission.

Statistics

Differences between patients who were included and not included, and between those with and without DM with respect to characteristics at baseline and 3-month follow-up and sedentary behaviour were analysed using t-test and χ^2 test. The results are shown as frequency and per cent or mean and SD. The use of the t-test was based on the central limit theorem.

Sedentary behaviour is displayed as daily averages (hours/day) of total sedentary time and number of bouts by bout length (<30, 30–59, 60–89 and ≥90 min).

A linear regression was used to analyse the association between the dependent variable, HbA1c and the independent variables total sedentary time, sedentary time at different bout lengths, BMI, age and the use of antidiabetic drugs. A multiple linear regression model was made using the same dependent and independent variables, except total sedentary time because of collinearity. The model was tested for multicollinearity. The covariates were added by forced entry based on literature.[17] The analysis was done using the whole population and stratified based on the presence of a diagnosis of DM, because

of the alterations in glucose metabolism in the patients with DM.[17] A standardised regression coefficient, CI and p value are presented. The residuals of the regressions were not normally distributed; thus, for the significance test, we used a cubic transformed version of the dependent variable, giving a normal distribution of the residuals of the regressions.

Missing data were not imputed as this was limited to 10.8% of the population (HbA1c missing in 4.5% of the patients and in BMI for 6.9% of the patients).

The significance level was set to 0.05, but since we have not made any formal correction for multiple comparison, p values above 0.01 should be interpreted with caution.

The power calculation was made for the main study. For this study, we made a post hoc power calculation for the stratified multiple regression. For the smallest group, n=70, R^2=0.37; probability level was 0.05; and calculated beta was 0.99.

Collinearity was checked using the Pearson product–moment correlation coefficient with a cut-off of ≥0.6. The significance level was set to p<0.05. Multicollinearity was checked by investigating the variance inflation factor (VIF), with a tolerance level of 1/VIF>0.1.

The statistical analyses were conducted in Stata/SE V.16.0 for Windows, revision 01 Aug 2019.

Patient and public involvement

The Nor-COAST study has included one stroke patient and three spouses representing the national unions for patients with stroke and dementia. The user representatives have been actively participating in the planning and performance of the Nor-COAST study, including the choice of analytical approach and the dissemination of results to the users. They have been invited to meetings for the Nor-COAST research group, and we have held separate meetings with them two to three times per year where substudies, such as this study, are presented.

RESULTS

A total of 815 patients were included in the study, 700 assessed at 3 months, 636 who had ischaemic stroke. Of these, 379 fulfilled the inclusion criteria for this study (figure 1).

Details are shown in table 1. There were 218 men (57.8%); the mean age was 71.5 (SD 11.4) years, and they had mean BI scores of 90.9 (SD 15.8) and 97.5 (SD 6.7) points at baseline and 3 months, respectively.

At baseline, a diagnosis of DM was registered in 74 (19.5%) patients, and these patients had a higher mean BMI (27.6 kg/m^2 (SD 4.3) vs 25.8 kg/m^2 (SD 4.3), p=0.002) and more often had hypercholesterolaemia (73.0% vs 50.8%, p=0.001) and hypertension (79.7% vs 48.5%, p<0.001). The groups were otherwise similar at baseline. At 3 months, patients with DM still had a higher mean BMI (28.3 kg/m^2 (SD 4.7) vs 26.3 kg/m^2 (SD 4.3), p=0.001) and HbA1c (7.0% (SD 1.6) vs 5.6% (SD 0.4), p<0.001). Of the patients with DM, 47 (63.5%) used antidiabetic drugs, and of these, 16 (21.6%) used insulin.

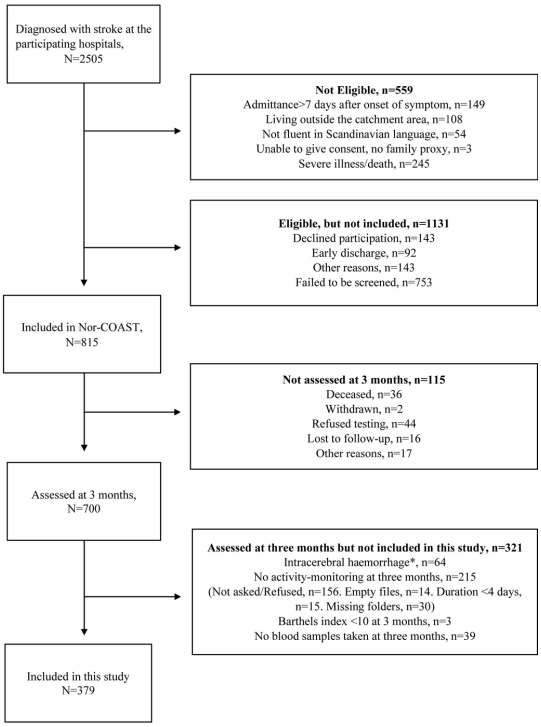

Figure 1 Flowchart for inclusion to the Norwegian Cognitive Impairment After Stroke-study (Nor-COAST).*Not haemorrhagic transformation.

The distribution of sedentary behaviour is presented in table 2 and figure 2. Patients with DM spent more time (mean hours/day) in sedentary behaviour (10.2 (SD 1.8) vs 9.6 (SD 1.2), p=0.008), more time (mean hours/day) in sedentary bouts of 30–59 min (2.7 (SD 0.9) vs 2.4 (SD 0.9), p=0.001) and a higher number of bouts of 30–59 min (3.8 (SD 1.2) vs 3.4 (SD 1.2), p=0.017) and ≥90 min (1.0 (SD 0.7) vs 0.8 (SD .7), p=0.026) per day, respectively.

The results from the unadjusted regressions are found in table 3 and figure 3. The adjusted analysis is found in table 4. There was a significant association between total sedentary time and sedentary time in bouts lasting 60–89 min and ≥90 min and HbA1c in the whole population. It was also a significant association between total sedentary time and sedentary time in bouts of ≥90 min in the subgroup of patients with DM in the unadjusted analysis. In the adjusted analyses, sedentary time in bouts of ≥90 min (β=0.43, CI 0.17 to 0.72, p=0.008) was associated with a higher HbA1c in patients with DM. The model explained 38% of the variance in HbA1c (R^2=0.38).

Table 1 Baseline and 3 months characteristics

Characteristics	Not included (n=321)	Included (n=379)	P value	No DM (n=305)	DM (n=74)	P value
Gender, male, n (%)	181 (56.4)	218 (57.8)	0.710	174 (57.1)	45 (60.8)	0.557
Age at baseline (years), mean (SD)	74.0 (12.2)	71.5 (11.4)	0.005	71.3 (11.8)	72.0 (9.4)	0.625
At 3 months	74.8 (12.2)	72.3 (11.4)	0.004	72.1 (11.8)	72.8 (9.3)	0.656
BMI (kg/m^2), mean (SD)	25.6 (4.0)	26.2 (4.4)	0.101	25.8 (4.3)	27.6 (4.3)	0.002
At 3 months	26.4 (3.8)	26.7 (4.4)	0.370	26.3 (4.3)	28.3 (4.7)	0.001
NIHSS score on admission, mean (SD)	4.8 (6.0)	3.4 (4.2)	<0.001	3.4 (4.3)	3.4 (4.0)	0.981
At 3 months	1.3 (2.3)	0.7 (1.3)	<0.001	0.7 (1.3)	0.6 (1.1)	0.579
mRS, mean (SD)	2.5 (1.4)	1.9 (1.2)	<0.001	2.0 (1.3)	1.9 (1.1)	0.728
At 3 months	2.2 (1.5)	1.4 (0.9)	0.000	1.4 (0.5)	1.4 (0.9)	0.448
mRS score ≤2, n (%)	154 (48.1)	262 (69.5)	<0.001	207 (68.1)	55 (74.3)	0.297
At 3 months	194 (60.4)	334 (88.4)	<0.001	267 (87.5)	67 (91.8)	0.310
BI, mean (SD)	79.4 (26.6)	90.9 (15.8)	<0.001	90.3 (16.4)	93.4 (12.5)	0.130
At 3 months	86.8 (22.9)	97.5 (6.7)	<0.001	97.5 (7.1)	97.4 (5.1)	0.908
BI score ≥95, n (%)	165 (51.4)	260 (68.6)	<0.001	203 (66.6)	57 (77.0)	0.082
At 3 months	211 (65.7)	341 (90.0)	<0.001	275 (90.5)	66 (89.2)	0.802
BI item 9≥10 points at baseline, n (%)	273 (85.3)	366 (96.6)	<0.001	293 (96.1)	73 (98.7)	0.598
At 3 months	292 (91.0)	379 (100.0)	<0.001	–	–	–
Living conditions before stroke, n (%)			0.000			0.427
At home	314 (97.8)	379 (100)		304 (99.7)	74 (100)	
Without home nursing care	261 (81.3)	358 (94.5)		290 (95.1)	68 (91.9)	
With home nursing care	49 (15.3)	20 (5.3)		14 (4.6)	6 (8.1)	
Residential care*	4 (1.3)	1 (0.3)		1 (0.3)	0	
Nursing home	7 (2.2)	0		0	0	
DM at baseline, n (%)	54 (16.8)	74 (19.5)	0.357	–	–	–
HbA1c %, mean (SD)						
At 3 months, mean (SD)	5.7 (0.8)	5.9 (0.8)	0.102	5.6 (0.4)	7.0 (1.6)	<0.001
Antidiabetic drugs, (%)						
All at 3 months, n (%)	35 (10.9)	47 (12.4)	0.529	0	47 (63.5)	<0.001
Insulin use at 3 months, n (%)	15 (4.7)	16 (4.2)	0.772	0	16 (21.6)	<0.001
Hypercholesterolaemia baseline, n (%)	145 (45.2)	209 (55.2)	0.009	155 (50.8)	54 (73.0)	0.001
Hypertension baseline, n (%)	175 (54.5)	207 (54.6)	0.979	148 (48.5)	59 (79.7)	<0.001
Prior cerebrovascular disease, n (%)	84 (26.2)	80 (21.1)	0.115	64 (21.0)	16 (21.6)	0.904

BMI=weight/height^2.
*Residental care: own customised apartment with home nursing care.
BI, Barthel Index; BMI, body mass index; DM, diabetes mellitus; HbA1C, glycated haemoglobin; mRS, modified Rankin Scale; NIHSS, National Institute of Health Stroke Scale.

For the other covariates in the unadjusted analyses (table 3), BMI and antidiabetic drugs were seen to be significantly associated with a higher HbA1c in the whole population. BMI and age were associated with HbA1c in the non-diabetic group, while in the group of patients with DM, only antidiabetic drugs were associated with HbA1c. In the adjusted analyses (table 4), we found the same pattern except that the BMI was not being significantly associated with HbA1c in the whole population.

DISCUSSION

The primary aim of this study was to investigate the association between long-bout sedentary behaviour and long-term glucose levels, measured by the HbA1c in stroke survivors. We found no significant associations. The secondary aim was to investigate any differences in the association between the patients with or without DM. We found sedentary behaviour in bouts of ≥90 min to be associated with a higher HbA1c in patients with DM.

Table 2 Mean sedentary time per day (hours) total and by bout length category 3 months after stroke

	Hours/day, mean (SD)			Bouts/day (n), mean (SD)		
	No DM	DM	P value	No DM	DM	P value
Total sedentary time	9.6 (1.2)	10.2 (1.8)	0.008	43.3 (14.3)	40.4 (12.0)	0.107
Bout-length category (min)						
<30	4.0 (1.2)	3.7 (1.0)	0.097	37.8 (15.0)	34.2 (12.8)	0.056
30–59	2.4 (0.9)	2.7 (0.9)	0.001	3.4 (1.2)	3.8 (1.2)	0.017
60–89	1.4 (0.9)	1.6 (1.0)	0.118	1.2 (0.7)	1.3 (0.8)	0.142
≥90	1.8 (1.7)	2.2 (1.6)	0.050	0.8 (0.7)	1.0 (0.7)	0.026

Mean daytime sedentary behaviour per day over a period of 4 days.
Daytime: 08:00–10:00.
DM, diabetes mellitus.

Results from studies investigating the associations between level of activity and glucose metabolism in general have varied.[5 11 12 14] For stroke in particular, Moore *et al* found an association between energy expenditure and glucose and insulin sensitivity (HOMA), not adjusted for BMI, age, DM or medication use.[23] Beyond that, high quality studies on the impact of sedentary behaviour in the stroke population are scarce. Much of the discrepancies seen in the literature can be explained by differences in applied methodology. We will therefore make an outline of the results in the context of (1) study population, (2) methods for measuring and analysing sedentary behaviour and (3) methods for measuring glucose metabolism.

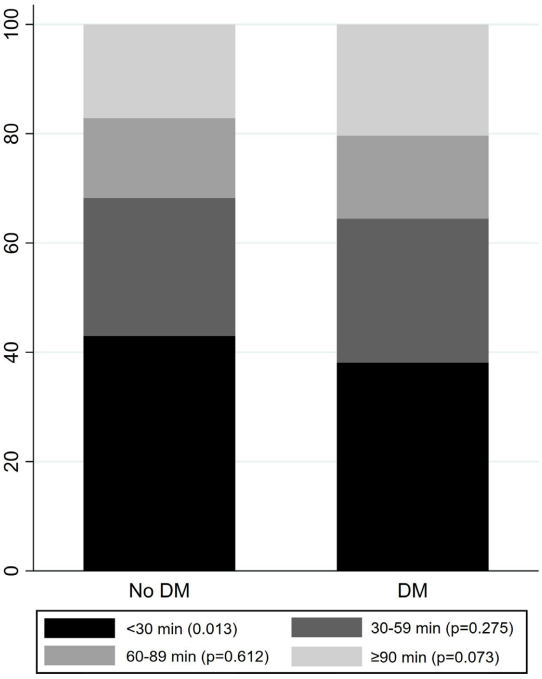

Figure 2 Differences in accumulation patterns of sedentary behaviour by bout length categories in per cent of mean sedentary time between patients with or without DM. DM, diabetes mellitus.

The population in this study are people with ischaemic stroke who are relatively old. We also have a subgroup of patients with DM. All these factors are relevant in the context of sedentary behaviour and glucose metabolism. As expected from our knowledge of sedentary behaviour patterns in stroke patients, we find that our patients are more sedentary than their age-matched peers from the general population.[8 24] The negative impact of sedentary behaviour is found to be stronger for the most sedentary.[2] It is also important to keep in mind the age related changes in glucose metabolism and altered glucose metabolism due to DM when interpreting our results.[17 25] Physical activity is known to increase both the transcription of and the translocation of glucose transporter type 4 (GLUT-4), responsible for the transportation of glucose into muscle and fat cells. Contraction-stimulated GLUT-4 reallocation can, in part, counter-act the down-regulation seen in patients with reduced insulin sensitivity, such as patients with diabetes type 2.[25 26] Hence, the association between sedentary behaviour and a higher HbA1c in the participants with DM might be partly explained by these mechanisms.[26 27]

Historically, sedentary behaviour has been measured and analysed using a variety of tools and methods.[1 5 11 13 28] In this study, we used time in sedentary behaviour in a given bout-length category, as recommended by the Sedentary Behaviour Research Network (SBRN).[13] The combined effect of time and bout length gives a more nuanced measure of the exposure compared with mere bout frequency, number of breaks or mean bout duration.

In a consensus guideline, the SBRN in 2017 presented a phenomenological definition of sedentary behaviour as 'any waking behaviour characterised by an energy expenditure ≤1.5 metabolic equivalents (METs) while in a sitting or reclining posture'.[13] How to measure energy expenditure is not defined, but body-worn sensors are preferred.[13] One common method is to convert accelerometer counts into metabolic equivalents (METs).[13] This conversion is based on healthy norms and the method

Table 3 Unadjusted linear regressions investigating the associations between HbA1c and sedentary time*, BMI, age and use of antidiabetic drugs in patients with or without DM

	All patients (n=379)			No DM (n=305)			DM (n=74)		
	β	CI	P value	β	CI	P value	β	CI	P value
Total sedentary time	0.18	0.08 to 0.29	<0.001	−0.03	−0.15 to 0.08	0.598	0.38	0.16 to 0.60	0.001
Bout-length category (min)									
<30	−0.07	−0.20 to 0.00	0.059	−0.06	−0.18 to 0.05	0.273	−0.08	−0.31 to 0.15	0.510
30–59	0.09	−0.01 to 0.20	0.075	0.03	−0.09 to 0.14	0.616	0.04	−0.20 to 0.27	0.754
60–89	0.14	0.04 to 0.25	0.005	0.06	−0.06 to 0.17	0.326	0.24	−0.02 to 0.44	0.067
≥90	0.07	−0.03 to 0.17	0.007	−0.03	−0.15 to 0.08	0.595	0.32	0.10 to 0.54	0.006
BMI	0.15	0.04 to 0.25	0.002	0.16	0.04 to 0.28	0.009	−0.10	−0.34 to 0.14	0.427
Age	0.08	−0.02 to 0.19	0.110	0.17	0.06 to 0.29	0.003	0.05	−0.18 to 0.29	0.657
Antidiabetic drugs	0.72	0.50 to 0.93	<0.001	–	–	–	0.51	0.10 to 0.93	<0.001

BMI=weight/height^2.
Antidiabetic drugs are defined as Anatomical Therapeutic Chemical A10.
*Sedentary time is analysed as total time (mean hours/day) and by bout-length category (mean hours/day).
.BMI, body mass index; DM, diabetes mellitus; HbA1c, glycated haemoglobin.

is not validated for the older and frailer population in general. Stroke patients in particular have been shown to have a higher energy expenditure when walking,[29] and there is no valid conversion norm for this patient group. In a healthy population, the energy expenditure of standing has been estimated to be 1.59 METs,[30] and data regarding position change from sitting to standing has shown to be accurate for the stroke population.[31] We have therefore chosen to use sitting and lying position as an approximation for sedentary behaviour.

Finally, methods for measuring glucose metabolism have changed, following the revised diagnostic criteria for DM, towards using HbA1c instead of fasting and 2 hour glucose. In this study we have used HbA1c as it represents the mean glucose level in a 3 month period and is not affected by recent changes in diet or activity. Compared with other measures of glucose metabolism, HbA1c is more convenient in regard to fasting state, with better analytic stability and less day-to-day variation. Any potential difference in regards to predictive value for future vascular disease is not entirely clear.[32] The homeostasis model assessment of insulin resistance could have been a useful supplement, as one might suspect the relative importance of sedentary behaviour on glucose

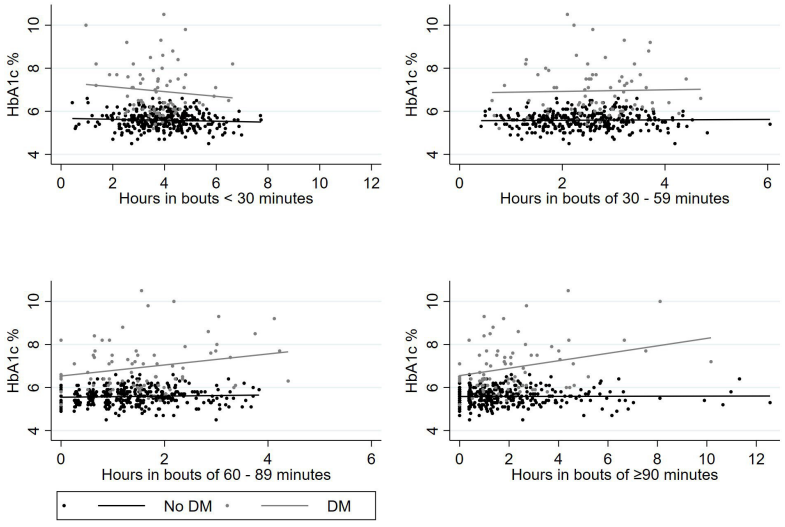

Figure 3 Association between the amount of sedentary time (hours) and HbA1c (%) by different sedentary time bout-length categories and HbA1c value shown for patients with our without a diagnosis of DM. DM, diabetes mellitus; HbA1c, glycated haemoglobin.

Table 4 Adjusted relationship between HbA1c and sedentary time by bout-length categories (mean hours/day), BMI, age and use of antidiabetic drugs in patients with or without DM

	All patients (n=338)			No DM (n=270)			DM (n=68)		
	β	CI	Pvalue	β	CI	P value	β	CI	P value
Bout-length category (min)									
<30	0.02	−0.08 to 0.12	0.725	−0.08	−0.25 to 0.08	0.311	0.24	−0.03 to 0.50	0.052
30–59	0.01	−0.06 to 0.09	0.734	0.01	−0.13 to 0.12	0.910	0.06	−0.15 to 0.27	0.292
60–89	0.04	−0.05 to 0.13	0.363	0.01	−0.15 to 0.13	0.899	0.16	−0.05 to 0.38	0.214
≥90	0.04	−0.05 to 0.13	0.417	−0.12	−0.27 to 0.02	0.105	0.43	0.17 to 0.72	0.008
BMI	0.06	0.02 to 0.14	0.127	0.20	0.08 to 0.33	0.001	−0.2	−0.37 to 0.04	0.202
Age	0.05	−0.03 to 013	0.198	0.20	0.07 to 0.33	0.002	−0.1	−0.28 to 0.15	0.978
Antidiabetic drugs	0.71	0.48 to 0.93	<0.001	–	–	–	0.4	−0.04 to 0.84	<0.001

BMI=weight/height^2.
Antidiabetic drugs are defined as Anatomical Therapeutic Chemical A10.
BMI, body mass index; DM, diabetes mellitus; HbA1C, glycated haemoglobin.

metabolism to be higher in those with insulin resistance than in those with insulin deficiency. This was not included in the laboratory work-up in the study, among other reasons because fasting blood samples were not feasible.[33]

In our study, the diagnosis of hypercholesterolaemia and hypertension is, among others, based on the use of medications. Thus, in line with national guidelines[34] for primary protective strategies in DM, we found a higher rate of patients defined as having hypercholesterolaemia and hypertension in patients with DM.

The aim of this study was to investigate daytime sedentary behaviour. Hence, sleep time, predefined as 10 hours from 10:00 pm until 08:00 am, was excluded from the analysis. A study by Ezeugwu and Manns has shown an average sleep duration of 8.9 hours (range 6.6–11.6) in stroke patients .[35] By making this assumption with regard to sleep patterns, we might have underestimated sedentary behaviour. However, a quality check of the daytime data against the 24-hour data showed that more than 80% of the short sedentary bouts (<30 min) occurred between 08:00 pm and 10:00 am, indicating that we actually have succeeded in capturing daytime activity and excluding sleep time. Nevertheless, future research should focus on developing algorithms that are able to extract sleep time from the 24-hour data in order to capture a greater diversity of activity patterns.

With the exception of one hospital, patients with a mRS score of 5 were not included in the NorCOAST study. In this subsample, patients had to be able to walk 50 m with walking aids or personal support and be fit enough to come to the outpatient clinic. Thus, this population is healthier and fitter than the general stroke population, reducing the generalisability of our results.

Strengths and limitations

This study has several strengths. It was done on a large sample of stroke patients, and all of the assessments were done at 3 months poststroke. We have objective registrations of sedentary behaviour, reflecting the habitual level of physical activity of the patient. We have considered the contribution of potential confounders, factors associated with glucose metabolism, such as age, medication use, DM and BMI. We have investigated the impact of sedentary behaviour at different bout lengths, hence getting a more nuanced evaluation of sedentary behaviour.

There are some limitations to our study. Diet, details about medication, the relative contribution of insulin deficiency versus insulin resistance and the intensity of physical activity are not accounted for. These factors would be associated with the outcome, but not with the explanatory variable, and hence were not real confounders. Information about these factors would have increased the explanatory abilities of the model, but would not have changed the association.

SUMMARY

This study did not find an association between sedentary behaviour and HbA1c in a stroke population 3 months after stroke. However, we identified an association between long-bout sedentary behaviour and a higher HbA1c in patients with DM. The results are in agreement with knowledge about glucose consumption in general and in patients with DM in particular. Reducing long-bout sedentary behaviour in patients with DM might be an important target for secondary prevention, but the results need to be verified by experimental studies. If confirmed, this will increase our understanding of the causative pathways between sedentary behaviour and vascular risk.

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Competing interests A-BK: principal investigator in two drug trials (Roche BN29553 and Boehringer-Ingelheim 1346-0023) at the memory clinic, Oslo University Hospital. Others: none declared.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Materials and methods section for further details.

Patient consent for publication Not required.

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REFERENCES

1 Healy GN, Dunstan DW, Salmon J, *et al*. Breaks in sedentary time: beneficial associations with metabolic risk. *Diabetes Care* 2008;31:661–6.
2 Gay JL, Buchner DM, Schmidt MD. Dose-response association of physical activity with HbA1c: intensity and bout length. *Prev Med* 2016;86:58–63.
3 Dunstan DW, Thorp AA, Healy GN. Prolonged sitting: is it a distinct coronary heart disease risk factor? *Curr Opin Cardiol* 2011;26:412–9.
4 Benatti FB, Ried-Larsen M. The effects of breaking up prolonged sitting time: a review of experimental studies. *Med Sci Sports Exerc* 2015;47:2053–61.
5 Healy GN, Winkler EAH, Owen N, *et al*. Replacing sitting time with standing or stepping: associations with cardio-metabolic risk biomarkers. *Eur Heart J* 2015;36:2643–9.
6 Wirth K, Klenk J, Brefka S, *et al*. Biomarkers associated with sedentary behaviour in older adults: a systematic review. *Ageing Res Rev* 2017;35:87–111.
7 Dunstan DW, Howard B, Healy GN, *et al*. Too much sitting-a health hazard. *Diabetes Res Clin Pract* 2012;97:368–76.
8 Tieges Z, Mead G, Allerhand M, *et al*. Sedentary behavior in the first year after stroke: a longitudinal cohort study with objective measures. *Arch Phys Med Rehabil* 2015;96:15–23.
9 Billinger SA, Arena R, Bernhardt J, *et al*. Physical activity and exercise recommendations for stroke survivors: a statement for healthcare professionals from the American heart Association/American stroke association. *Stroke* 2014;45:2532–53.
10 Young DR, Hivert M-F, Alhassan S, *et al*. Sedentary behavior and cardiovascular morbidity and mortality: a science Advisory from the American heart association. *Circulation* 2016;134:e262–79.
11 Diaz KM, Goldsmith J, Greenlee H, *et al*. Prolonged, uninterrupted sedentary behavior and glycemic biomarkers among US Hispanic/Latino adults: the HCHS/SOL (Hispanic community health Study/Study of Latinos). *Circulation* 2017;136:1362–73.
12 English C, Janssen H, Crowfoot G, *et al*. Breaking up sitting time after stroke (BUST-stroke). *Int J Stroke* 2018;13:921–31.
13 Tremblay MS, Aubert S, Barnes JD, *et al*. Sedentary behavior research network (SBRN) - terminology consensus project process and outcome. *Int J Behav Nutr Phys Act* 2017;14:75.
14 Kallings LV, Sierra Johnson J, Fisher RM, *et al*. Beneficial effects of individualized physical activity on prescription on body composition and cardiometabolic risk factors: results from a randomized controlled trial. *Eur J Cardiovasc Prev Rehabil* 2009;16:80–4.
15 Bey L, Akunuri N, Zhao P, *et al*. Patterns of global gene expression in rat skeletal muscle during unloading and low-intensity ambulatory activity. *Physiol Genomics* 2003;13:157–67.
16 Bailey DP, Maylor BD, Orton CJ, *et al*. Effects of breaking up prolonged sitting following low and high glycaemic index breakfast consumption on glucose and insulin concentrations. *Eur J Appl Physiol* 2017;117:1299–307.
17 Chia CW, Egan JM, Ferrucci L. Age-related changes in glucose metabolism, hyperglycemia, and cardiovascular risk. *Circ Res* 2018;123:886–904.
18 James DE, Jenkins AB, Kraegen EW. Heterogeneity of insulin action in individual muscles in vivo: euglycemic clamp studies in rats. *Am J Physiol* 1985;248:E567–74.
19 Healy GN, Matthews CE, Dunstan DW, *et al*. Sedentary time and cardio-metabolic biomarkers in US adults: NHANES 2003-06. *Eur Heart J* 2011;32:590–7.
20 Thingstad P, Askim T, Beyer MK, *et al*. The Norwegian cognitive impairment after stroke study (Nor-COAST): study protocol of a multicentre, prospective cohort study. *BMC Neurol* 2018;18:193.
21 Banks JL, Marotta CA. Outcomes validity and reliability of the modified Rankin scale: implications for stroke clinical trials: a literature review and synthesis. *Stroke* 2007;38:1091–6.
22 Duffy L, Gajree S, Langhorne P, *et al*. Reliability (inter-rater agreement) of the Barthel index for assessment of stroke survivors: systematic review and meta-analysis. *Stroke* 2013;44:462–8.
23 Moore SA, Hallsworth K, Plötz T, *et al*. Physical activity, sedentary behaviour and metabolic control following stroke: a cross-sectional and longitudinal study. *PLoS One* 2013;8:e55263.
24 English C, Healy GN, Coates A, *et al*. Sitting and activity time in people with stroke. *Phys Ther* 2016;96:193–201.
25 Chen R, Ovbiagele B, Feng W. Diabetes and stroke: epidemiology, pathophysiology, pharmaceuticals and outcomes. *Am J Med Sci* 2016;351:380–6.
26 Park D-R, Park K-H, Kim B-J, *et al*. Exercise ameliorates insulin resistance via Ca2+ signals distinct from those of insulin for GLUT4 translocation in skeletal muscles. *Diabetes* 2015;64:1224–34.
27 Dohm GL. Invited review: regulation of skeletal muscle GLUT-4 expression by exercise. *J Appl Physiol* 2002;93:782–7.

28 Bellettiere J, Healy GN, LaMonte MJ, *et al*. Sedentary behavior and prevalent diabetes in 6,166 older women: the objective physical activity and cardiovascular health study. *J Gerontol: Series A* 2019;74:387–95.
29 Kramer S, Johnson L, Bernhardt J, *et al*. Energy expenditure and cost during walking after stroke: a systematic review. *Arch Phys Med Rehabil* 2016;97:619–32.
30 Mansoubi M, Pearson N, Clemes SA, *et al*. Energy expenditure during common sitting and standing tasks: examining the 1.5 Met definition of sedentary behaviour. *BMC Public Health* 2015;15:516.
31 Taraldsen K, Askim T, Sletvold O, *et al*. Evaluation of a body-worn sensor system to measure physical activity in older people with impaired function. *Phys Ther* 2011;91:277–85.
32 Kumar R, Nandhini LP, Kamalanathan S, *et al*. Evidence for current diagnostic criteria of diabetes mellitus. *World J Diabetes* 2016;7:396–405.
33 Matthews DR, Hosker JP, Rudenski AS, *et al*. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;28:412–9.
34 The Norwegian directorate of health: national guidelines for diabetes mellitus, helsedirektoratet.No/retningslinjer/diabetes 2018.
35 Ezeugwu VE, Manns PJ, duration S. Sleep duration, sedentary behavior, physical activity, and quality of life after inpatient stroke rehabilitation. *J Stroke Cerebrovasc Dis* 2017;26:2004–12.

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RESEARCH Open Access

Investigating novel biomarkers of immune activation and modulation in the context of sedentary behaviour: a multicentre prospective ischemic stroke cohort study

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Abstract

Background: Sedentary behaviour is associated with disease, but the molecular mechanisms are not understood. Valid biomarkers with predictive and explanatory properties are required. Therefore, we have investigated traditional and novel biomarkers of inflammation and immune modulation and their association to objectively measured sedentary behaviour in an ischemic stroke population.

Methods: Patients admitted to hospital with acute ischemic stroke were included in the multicentre Norwegian Cognitive Impairment After Stroke (Nor-COAST) study ($n = 815$). For this sub-study ($n = 257$), sedentary behaviour was registered 3 months after stroke using position transition data from the body-worn sensor, ActivPal®. Blood samples were analysed for high sensitive C-reactive protein (hsCRP), the cytokines interleukin-6 (IL-6) and 10 (IL-10), neopterin, tryptophan (Trp), kynurenine (kyn), kynurenic acid (KA), and three B6 vitamers, pyridoxal 5′-phosphate (PLP), pyridoxal (PL), and pyridoxic acid (PA). The kynurenine/tryptophan ratio (KTR) and the pyridoxic acid ratio index (PAr = PA: PL + PLP) were calculated.

Results: Of the 815 patients included in the main study, 700 attended the three-month follow-up, and 257 fulfilled the inclusion criteria for this study. Sedentary time was significantly associated with levels of hsCRP, IL-6, neopterin, PAr-index, and KA adjusted for age, sex, waist circumference, and creatinine. In a fully adjusted model including all the significant biomarkers except hsCRP (because of missing values), sedentary time was independently positively associated with the PAr-index and negatively with KA. We did not find an association between sedentary behaviour, IL-10, and KTR.

Conclusions: The PAr-index is known to capture several modes of inflammation and has previously shown predictive abilities for future stroke. This novel result indicates that the PAr-index could be a useful biomarker in future studies on sedentary behaviour and disease progression. KA is an important modulator of inflammation, and this finding opens new and exciting pathways to understand the hazards of sedentary behaviour.

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Trial registration: The study was registered at Clinicaltrials.gov (NCT02650531). First posted 08/01/2016.

Keywords: Sedentary behavior, Inflammation, Immune modulation, Vascular disease, Kynurenine pathway, Stroke

Background

Sedentary behaviour is associated with an increased risk of vascular disease, amongst others, through inflammatory pathways [1–5]. The impact depends on the properties of the sedentary behaviour and the corresponding physical activity, which can vary in bout length, intensity, and habit [1, 6–9]. To increase the understanding of the link between sedentary behaviour and vascular disease, valid biomarkers with predictive and explanatory properties are required.

Cytokines can be predominantly pro- or anti-inflammatory, but their downstream effect also depends on other contextual factors. Also, cytokines vary in their biological and analytical properties, and some of them, such as interleukin 1β (IL-1β) and interferon ɣ (IFN-ɣ), are therefore often measured indirectly by other more stable downstream molecules [5, 10, 11]. The inflammatory biomarker C-reactive protein (CRP) is a downstream marker of interleukin 6 (IL-6) and IL-1β [10]. The inflammation associated with this pathway has been shown to increase with age and to be associated with sedentary behaviour and disease development [1, 2, 10, 12–14]. Interleukin-10 (IL-10) is a predominantly anti-inflammatory cytokine found to be induced by acute bouts of moderate to vigorous physical activity (MVPA) and in response to chronic exercise [4, 15–17]. The association to sedentary behaviour is not clear.

The inflammatory pathway associated with IFN-ɣ can be measured by the ratio between kynurenine and tryptophan—the kynurenine tryptophan ratio (KTR)—and neopterin secreted by activated macrophages. Kynurenine is a metabolite of the amino acid tryptophan and is the first metabolic step of the kynurenine pathway (KP) [18, 19]. KTR and neopterin have been shown to predict future coronary events [11, 20], but the association to sedentary behaviour is unclear [21, 22].

A novel and sensitive biomarker of inflammation, the pyridoxal acid ratio index (PAr-index = (pyridoxic acid (PA): (pyridoxal (PL) + pyridoxal 5-phosphate (PLP)), represents several modes of inflammation and captures the effect of the inflammatory pathways associated with CRP, white blood cell count, neopterin, and KTR [23, 24]. The PAr-index has been shown to predict future stroke with better precision than high sensitive C-reactive protein (hsCRP) [25] but has never been studied in the context of sedentary behaviour.

Kynurenic acid (KA), a side-product in the KP, is not an inflammatory biomarker but part of a negative feedback mechanism inducing immune tolerance [26, 27]. The role of KA in health and disease is not fully understood [26] and KA has never been investigated in relation to sedentary behaviour. KA has been found to increase following physical exercise and KA can potentially be one of the molecular links between sedentary behaviour and inflammation [22, 28–32].

The primary objective of the present study was to investigate the association between novel biomarkers of inflammation and immune modulation and objectively measured habitual sedentary behaviour in a stroke population. The secondary objective was to investigate the impact of bout duration on these associations. Our hypothesis was that sedentary behaviour would be positively associated with a pro-inflammatory profile.

Material and methods

Subjects

The prospective cohort study, the Norwegian Cognitive Impairment After Stroke Study (Nor-COAST), included 815 adults admitted for acute stroke at one of five contributing hospitals from May 2015 through March 2017. Details of the Nor-COAST study, including inclusion and exclusion criteria and patient selection, have been published elsewhere [33, 34]. In the current study, only patients with ischemic stroke who attended the three-month follow-up, had blood samples drawn for direct analyses at the local laboratory and for storage in the bio-bank, were able to walk 50 m independently or with support from a person/walker (Barthel index item 9, ≥10 points), and had valid activity data were included.

Clinical data and laboratory analyses

Demographic information, as well as information about risk factors of vascular disease, stroke severity, and functional outcomes were collected during the index stay and at the three-month follow-up. Stroke severity was measured by the National Institute of Health Stroke Scale (NIHSS) at the time of hospital arrival and at three-month follow-up [35]. Functional state was measured by the Barthel index and the modified Rankin scale at discharge or day seven. The Barthel index is a 10-item assessment for basal activities of daily living, with a maximum score of 100 points indicating no functional deficits [36]. The modified Rankin scale is a five-point scale measuring global disability, where 0–2 is defined as 'good outcomes', 3–5 indicates increasingly disability, and 6 is death [37].

Laboratory markers

Non-fasting blood samples were collected at the three-month follow-up at the outpatient clinic.

Routine clinical-chemical analyses The blood samples were analysed for high sensitive C-reactive protein (hsCRP, mg/L) and creatinine (μmol/L) at the local laboratory directly. The estimated glomerular filtration rate (eGFR) was calculated with the Chronic Kidney Disease Epidemiology Collaboration formula [38] using a dedicated STATA software package.

Sample collection of research analyses Aliquots of serum and plasma were immediately frozen at − 80 °C at the inclusion hospital. The samples were transported on dry ice and stored at BioBank1, Central Norway Health Authority. In 2019, two aliquots of plasma were transported on dry ice to the Research Laboratory Nordland Hospital (Bodø, Norway) and Bevital A/S (Bergen, Norway), respectively, and were thawed only once.

Cytokines Interleukin (IL)-6 and IL-10 were measured as the classical cytokines previously shown to be of interest in these patients (see Introduction). They were analysed at the Research Laboratory Nordland Hospital using the Bio-Plex technology kits obtained from Bio-Rad Laboratories Inc., Hercules, CA. The assay was performed according to the manufacturer's procedure. Values below the lower detecting limit ($n = 36$ for IL-10, none for IL-6) were replaced by random values below the lower detecting limit when statistics were performed.

Other biomarkers The three vitamin B6 forms pyridoxal 5′-phosphate (PLP), pyridoxal (PL), and 4-pyridoxic acid (PA) were measured along with tryptophan, the two tryptophan metabolites kynurenine and kynurenic acid (KA), and neopterin as novel biomarkers of interest (see Introduction) as part of analytic platform D at Bevital A/S (Bergen, Norway) by liquid chromatography/tandem mass spectrometry using EDTA-plasma. The PAr-index was calculated as PA:(PL + PLP) [23].

Sedentary behaviour

Sedentary behaviour was defined as sitting or lying. Time in sedentary behaviour was given as total time per day and as time accumulated through pre-defined bout-length categories (< 30 min, 30–59 min, 60–89 min, and ≥ 90 min). Sedentary behaviour was measured by registering position transition with a single thigh-worn sensor (ActivPal3®, Model 20.2, PAL Technologies Ltd., Glasgow, United Kingdom); in patients with hemiparesis, the sensor was attached to the unaffected side. The monitor was attached when the patient visited the outpatient clinic and returned by mail.The threshold for noise was 1.5 s, and sedentary events were merged if they were interrupted by events lasting ≤3 s. Measurements were performed for seven consecutive days/nights and analysed using a custom-made MATLAB® script (version R2016b, MathWorks, Natick, MA, USA) to extract periods of sedentary behaviour from the recordings. The recordings were analysed for daytime sedentary behaviour, defined as occurring from 08:00 am through 10:00 pm, and a registered period of sedentary behaviour—a sedentary bout—was divided into two periods if it crossed these time boundaries. Only patients with recordings from at least four full 24-h periods starting from 08:00 am were included. As a final control, manual inspection of the output to identify non-wear time was performed.

Statistics

Laboratory results and characteristics at baseline and three months were given in means and standard deviations, numbers with percentages, and medians with interquartile range. Differences between groups were tested with the chi-squared test, t-test, and Wilcoxon-Mann-Whitney test.

Sedentary behaviour was given as a continuous variable of mean hours per day, both as total time of sedentary behaviour and as time accumulated through different bout-length categories (< 30 min, 30–59 min, 60–89 min, and ≥ 90 min).

Associations between variables were assessed using multiple linear and Tobit regressions using the biomarker as the dependent variable and sedentary behaviour as the independent variable adjusted for age, sex, waist circumference, and kidney function [27, 39–41]. The results were given as standardised beta coefficients with confidence intervals and *p*-values. One final regression was made to investigate the independent association between the biomarkers significant in the initial analyses and sedentary behaviour. Here sedentary behaviour was used as the dependent variable, while age, sex, waist circumference, and kidney function were independent variables, in addition to the biomarkers with significant results in the primary analyses, with the exception of hsCRP because of missing values (see below). A sensitivity analysis including only those patients with values for hsCRP was added. The residuals of the regressions did not follow a normal distribution, and the value of the biomarkers were log-transformed. For the cytokines, the significance test was performed using a Tobit regression with left censoring [41], applying the highest value amongst the lower detection limits as cut-off (see laboratory analyses for details). The analyses were performed in STATA/SE 16.1.

In the final population, 88 cases had missing values for hsCRP because this analysis was not available at two of

the inclusion centres; 12 had missing values for waist circumference and 4 for eGFR.

Results

Patient characteristics and laboratory values

Out of 700 patients attending three-month follow-up, 257 fulfilled the inclusion criteria (Fig. 1). Of the included patients, 149 (58%) were men; mean age was 73 (±11) years; they had a mean body mass index (BMI) of 27 (±4.3) and a waist circumference of 95 (±13). Compared to the rest of the Nor-COAST population, our subgroup had a lower NIHSS on admission (3.7 (4.6) vs. 5.0 (6.5), $p = 0.004$) and at 3 months (0.7 (1.3) vs. 1.0 (2.0), $p = 0.022$), had more favourable functional outcomes (Barthel index 98 (6.1) vs. 90 (21), $p < 0.001$) and scores for global disability (modified Rankin scale (1.5 (0.9) vs. 2.0 (1.4), $p < 0.001$) at 3 months. There were no differences in the laboratory results between the patient groups, except for the PAr-index, which was higher in included patients (0.64 (0.44, 0.91) vs. 0.52 (0.39, 0.73), $p = 0.005$) (Table 1).

Sedentary behaviour

The patients in our study had a mean total sedentary time of 9.7 (1.9) hours per day. Of the total time spent sedentary, 4.0 (1.1) hours were accumulated through bouts of less than 30 min, 2.5 (0.9) hours through bouts of 30–59 min, 1.4 (0.9) hours through bouts of 60–89 min, and 1.3 (1.5) hours through bouts ≥90 min (Fig. 2).

Regression analyses

In the adjusted analyses we found hsCRP (0.25, $p = 0.001$), IL-6 (0.17, $p = 0.009$), neopterin (0.12, $p = 0.034$), and the PAr-index (0.21, $p < 0.001$) to be positively associated with total sedentary time (Table 2). The KA (−0.10, $p = 0.045$) showed an inverse association with sedentary time. No significant associations were found for IL-10 and KTR.

In the final model, where all biomarkers significantly associated with sedentary time were added, only the PAr-index (0.25, $p = 0.001$) and KA (− 0.19, $p = 0.021$) remained independently associated with total sedentary time (Table 3). In a sensitivity analyses (Table 4) restricted to those with data on hsCRP, only the PAr-index (0.31, p = 0.001) were significantly associated. Adding hsCRP to this model did not change the results (numbers not shown).

There was a tendency towards an increased association with longer sedentary bout lengths for hsCRP, IL-6, neopterin, and PAr-index (Supplementary Table 1).

Discussion

In this study, we have explored novel biomarkers associated with inflammation and immune modulation and their association to objectively measured sedentary time

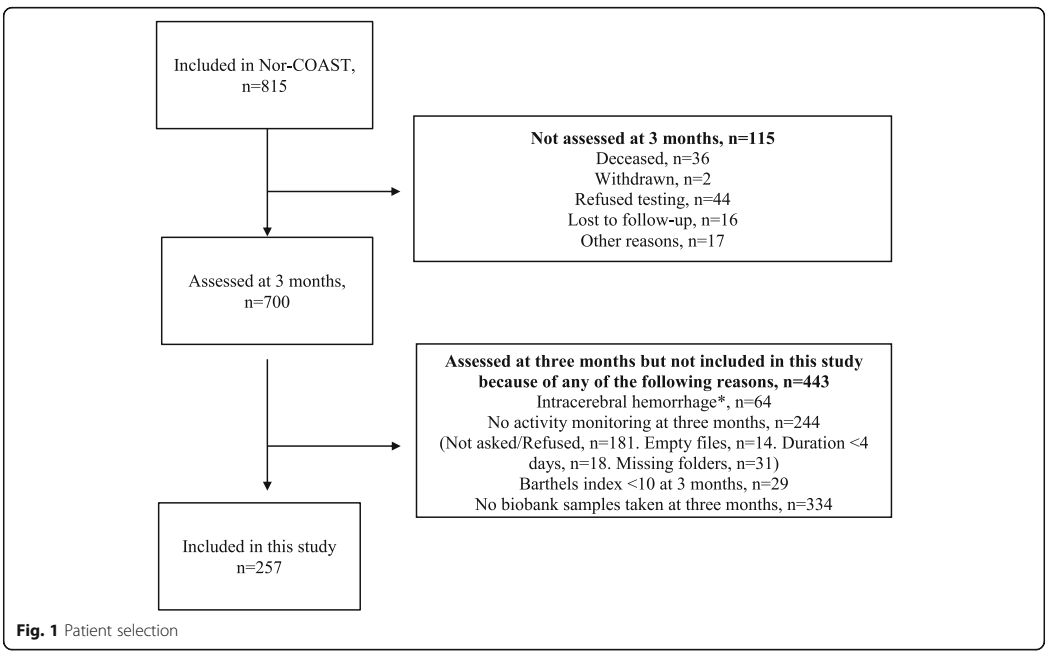

Fig. 1 Patient selection

Table 1 Baseline and three-month characteristics of the study population compared to the remaining Nor-COAST population

	Total *n* = 700		
	Not included (*n* = 443)	**Included (*n* = 257)**	**p**
Sex, male, n (%)	251 (57)	149 (58)	0.734
Age at three months, mean y (SD)	74 (12)	73 (11)	0.162
BMI at three months, mean kg/m^2 (SD)	27 (4.2)	27 (4.3)	0.797
Waist at three months, mean cm (SD)	97 (13)	95 (13)	0.283
NIHSS on admission, mean (SD)	5.0 (6.5)	3.7 (4.6)	0.004
At three months	1.0 (2.0)	0.7 (1.3)	0.022
Modified Rankin scale, mean (SD)	2.5 (1.5)	2.0 (1.1)	< 0.001
At three months	2.0 (1.4)	1.5 (0.9)	< 0.001
Modified Rankin scale ≤2, n (%)	269 (49)	178 (70)	< 0.001
At three months	304 (68)	224 (88)	< 0.001
Barthel index, mean (SD)	79 (28)	92 (14)	< 0.001
At three months	90 (21)	98 (6.1)	< 0.001
Laboratory values at three months*, median (IQR)			
eGFR (*N* = 272/253)	77 (60, 89)	76 (63, 88)	0.696
hsCRP (*N* = 98/169)	1.9 (0.7, 3.6)	1.6 (0.8, 3.7)	0.513
IL6 (*N* = 111/257)	4.6 (2.5, 6.7)	4.6 (2.9, 7.4)	0.438
IL10 (*N* = 111/257)	18 (8.6, 32)	20 (8.6, 36)	0.304
Neopterin (*N* = 109/257)	16 (12, 22)	16 (12, 22)	0.998
PAr-index (*N* = 109/257)	0.52 (0.39, 0.73)	0.64 (0.44, 0.91)	0.005
KTR (*N* = 109/257)	32 (28, 40)	34 (29, 42)	0.062
KA (*N* = 109/257)	55 (44, 75)	59 (46, 76)	0.276

*Number of patients attending baseline and three months: A total of 558 patients had blood samples taken at three months. Of these, 368 had biobank samples taken. The N for each individual biomarker is shown for each group (not included/included)

in an ischemic stroke population 3 months after the acute stroke. We have also replicated known associations between traditional biomarkers of inflammation and sedentary behaviour. We found hsCRP, IL-6, neopterin, PAr-index and KA (invers) to be significantly associated with sedentary time. The PAr-index and KA also showed an independent association in a model including IL-6 and neopterin.

The PAr-index has never been studied in the context of sedentary behaviour. The PAr-index reflects several modes of inflammation and has been shown to be associated with CRP, white blood cell count, and markers of cellular immune activation such as neopterin and KTR [23]. In accordance to this, the independent association in the fully adjusted model illustrates that the PAr-index captures the effect of the other pathways by attenuating the association to IL-6 and neopterin when included in the same model. As the PAr-index has recently found to be associated with the risk of future stroke, it might be a valid and clinically relevant biomarker for future intervention studies on sedentary behaviour [25].

The association between sedentary time, hsCRP and IL-6 confirms prior findings, both from observational and intervention studies [1, 2, 8, 40, 42]. The results from intervention studies imply a causal relationship between sedentary behaviour and inflammation, and Noz et al. found a phenotypic shift in the innate immune system towards a less inflammatory phenotype of circulating monocytes in vitro when sedentary time was replaced with physical activity [1]. Other studies have found a change in the inflammatory phenotype of infiltrating inflammatory cells in adipose tissue in addition to a reduction in total adipose tissue volume in response to exercise [4, 5, 8, 40].

Neopterin and KTR are indirect measures of IFN-ɣ activity, which plays a central role in activating cellular immune response [19, 43]. Neopterin and KTR have been found to be associated with future major vascular events and all-cause mortality [11]. We found a significant association between sedentary behaviour and neopterin, but not KTR. A less convincing association between sedentary behaviour and biomarkers associated with IFN-ɣ-mediated inflammation might be explained by findings from the above-mentioned intervention study by Noz et al., where increased walking time altered innate immune function towards a less pro-inflammatory state in

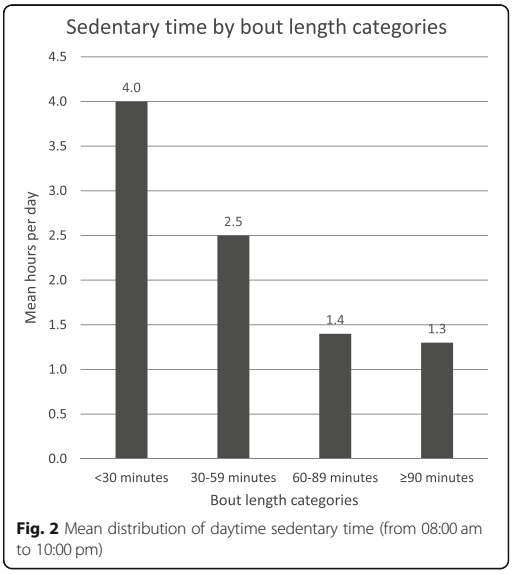

Fig. 2 Mean distribution of daytime sedentary time (from 08:00 am to 10:00 pm)

combination with an increased IFN-ɣ production capacity upon stimulation [1]. The implication of this in the context of inflammation and vascular disease, is not clear. This is the first study to investigate sedentary behaviour and its association with neopterin and KTR using objective measures for monitoring daytime activity. Prior investigations were predominantly based on questionnaire information and investigated the association to physical activity rather than to sedentary behaviour. The results were inconclusive [21, 22, 31].

We found an inverse association between KA and sedentary behaviour, and the association was strengthened after adjustment for inflammation. KA is metabolised from kynurenine by kynurenine amino transferases (KATs) [27] and is part of a negative feedback loop where chronic inflammation induces immune tolerance via the aryl hydrocarbon receptor (AhR) and the G-protein-coupled receptor 35 [18, 26, 27, 44, 45]. In a recent review, Joisten et al. argued for the relevance of KA in disease prevention [26]. KAT expression and activity has been found to be induced by chronic exercise via peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1α) and the associated transcription factor, peroxisome proliferator activated receptor alpha (PPARα) [28–31]. The inverse relationship found in our study suggests a reduced KAT expression during sedentary behaviour. PPARα has been found to be negatively associated with IL-6 in older adults, and Lustgarten et al. observed that PPARα activation was related to decreased inflammation [12]. Kynurenic acid could be a molecular link between sedentary behaviour, inflammation, and vascular diseases such as stroke. The KA association was significant only after adjustment for kidney function. KA is eliminated in urine, and lower muscle mass is associated with lower creatinine and higher levels of sedentary

Table 2 The association between biomarkers and total sedentary time

	Crude			Adjusted[a]		
	β	95%CI	P	β	95%CI	P
hsCRP						$R^2 = 0.16$
Total sedentary time	0.32	(0.18, 0.47)	< 0.001	0.25	(0.09, 0.39)	0.001
IL-6[b]						$R^2 = 0.12$
Total sedentary time	0.26	(0.14, 0.38)	< 0.001	0.17	(0.04, 0.30)	0.009
IL-10[b]						$R^2 = 0.04$
Total sedentary time	0.04	(−0.09, 0.16)	0.509	0.01	(− 0.12, 0.14)	0.841
Neopterin						$R^2 = 0.35$
Total sedentary time	0.24	(0.12, 0.36)	< 0.001	0.12	(0.00, 0.22)	0.034
PAr-index						$R^2 = 0.37$
Total sedentary time	0.33	(0.22, 0.45)	< 0.001	0.21	(0.09, 0.30)	< 0.001
KTR						$R^2 = 0.45$
Total sedentary time	0.21	(0.09, 0.33)	0.001	0.06	(−0.05, 0.15)	0.238
KA						$R^2 = 0.48$
Total sedentary time	0.04	(−0.08, 0.16)	0.512	−0.10	(−0.21, − 0.02)	0.045

hsCRP = high sensitive C-reactive protein. IL-6 = interleukin-6. IL-10 = interleukin-10, PAr-index = (4-pyridoxic acid/(pyridoxal 5′-phosphate + pyridoxal). KTR = kynurenine/tryptophan ratio = (kynurenine nM/tryptophan μM), KA = kynurenic acid

[a]Model: biomarker is the dependent variable. Sedentary time, age, sex, waist circumference, and creatinine are independent variables

[b]For IL-6 and IL-10, Tobit regressions were used for the significance test and regular linear regressions to calculate the beta coefficients

Table 3 Adjusted multiple regression model of the association between total sedentary time and biomarkers, $n = 242$

Sedentary time	β	95% CI	P
IL-6	0.12	(−0.01, 0.24)	0.069
Neopterin	0.08	(−0.07, 0.23)	0.280
PAr-index	0.25	(0.10, 0.39)	0.001
Kynurenic acid	−0.19	(−0.35, − 0.03)	0.021
Age	0.16	(0.03, 0.29)	0.017
Sex (male)	0.01	(−0.28, 0.30)	0.941
Creatinine	0.02	(−0.17, 0.21)	0.826
Waist circumference	0.21	(0.08, 0.33)	0.002

IL-6 = interleukin-6. PAr-index = (4-pyridoxic acid/(pyridoxal 5′-phosphate + pyridoxal)

behaviour [46]. This can explain the observed change after the adjustments. In the sensitivity analyses restricted to those with a value for hsCRP, the association to KA was no longer significant, probably due to lower sample size. The strengthened association between sedentary behaviour and KA, independent of inflammation in the final model, can be seen as a 'proof of concept' of the connection between sedentary behaviour and immune modulation.

Prior studies have found increased levels of the anti-inflammatory cytokine IL-10 in response to exercise [4, 15–17]. A single bout of MVPA has been associated with an increased expression of IL-6 from the myocytes, and this spike of IL-6 is believed to induce IL-10 [4]. We expected an inverse association between sedentary behaviour and IL-10. The lack of association between sedentary time and IL-10 in our study might be explained by the distinction between physical activity in general (the inverse of sedentary behaviour) and MVPA, which has a higher level of energy expenditure. This also illustrates how duration and intensity of cytokine expression is important for the downstream effects. IL-6 falls to basal levels within an hour after exercise [4], and this dual role of IL-6 prompts caution when designing intervention studies.

Table 4 Adjusted multiple regression model of the association between total sedentary time and biomarkers restricted to those with a value for hsCRP, $n = 161$

Sedentary time	β	95% CI	P
IL-6	0.10	(−0.06, 0.26)	0.229
Neopterin	0.09	(−0.11, 0.29)	0.374
PAr-index	0.31	(0.13, 0.49)	0.001
Kynurenic acid	−0.17	(−0.38, 0.04)	0.110
Age	0.11	(−0.05, 0.27)	0.195
Sex (male)	0.05	(−0.31, 0.40)	0.803
Creatinine	−0.05	(−0.30, 0.20)	0.692
Waist circumference	0.15	(−0.01, − 0.31)	0.062

IL-6 = interleukin-6. PAr-index = (4-pyridoxic acid/(pyridoxal 5′-phosphate + pyridoxal)

From our results, it seems that all sedentary behaviour was associated with increased inflammation, but that there was a trend towards stronger associations to sedentary time accumulated through longer bouts. In some cases, the association between sedentary behaviour and the biomarker was no longer significant when stratified for bout length because of reduced statistical power, which also potentially explain the spread in the results.

The strength of this study lies in the sample size, the carefully characterised population of older patients with vascular disease, and the objective measure of sedentary behaviour over several days in a habitual setting. The Nor-COAST population is representative of the group of patients who have suffered mild strokes [34]. The use of both traditional and novel biomarkers has enabled us to confirm prior findings, contributing to filling in the gaps of uncertainties and to opening new and interesting paths and new perspectives. Information about other explanatory variables such as age, waist circumference, and kidney function have increased the validity of the results.

Because of the observational nature of the study, we can only identify associations and not any causal direction. Methods of measuring, defining, and analysing sedentary behaviour were thoroughly discussed in a prior study [47], however, because sedentary bouts were cut if they crossed the day/night timeline, some overrepresentation of shorter bout lengths and concordant underestimation/to the expense of longer bout lengths may have occurred. As shown in the baseline table, the stroke patients in this study were fitter compared to the entire Nor-COAST population, and the findings cannot be generalised to the entire stroke population.

Conclusion

This novel result indicates that the PAr-index is a potentially useful biomarker in future studies on sedentary behaviour and disease progression. The association to KA opens fresh interesting pathways to understanding disease progression in general, the hazards of sedentary behaviour in particular, and should be further investigated.

Abbreviations

AhR: Aryl hydrocarbon receptor; BMI: Body mass index; CRP: C-reactive protein; eGFR: Estimated glomerular filtration rate; hsCRP: High sensitive C-reactive protein; IFN-γ: interferon gamma; IL-6: Interleukin-6; IL-10: Interleukin-10; KA: Kynurenic acid; KAT: Kynurenine aminotransferases; KP: Kynurenine pathway; KTR: Kynurenine/tryptophan ratio; MVPA: Moderate to vigorous physical activity; NIHSS: National Institute of Stroke Scale; Nor-COAST: Norwegian Cognitive Impairment After Stroke Study; PAr-index: Pyridoxal acid ratio index; PLP: Pyridoxal-5-phosphate; PL: Pyridoxal; PA: Pyridoxal acid; PGC1α: Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; PPARα: Peroxisome proliferator-activated receptor alpha; REK: Regional ethics committee

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12883-021-02343-0.

Additional file 1: Supplementary Table 1. Crude and adjusted linear regression analyses of the association between biomarkers and time in sedentary behaviour by bout-length category.

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Authors' contributions

KNA has planned this study. She has planned analyses, performed the statistical analyses and interpreted the results. She has written the article. TA has contributed to the design of this study and was responsible for the physical activity data from the main study. She has contributed in revising the manuscript. JA has supervised the process of developing and describing the statistical method and contributed in the interpretation and the presentation of the data. TEM has analysed the cytokines used in this study. Also contributed in the post analytical work and defined the method of handling the data and to interpret the results. He has also contributed to revising the manuscript. MN has contributed to the development of the study and revised the manuscript. HN has contributed to the development of the study and revised the manuscript. He has also contributed to the development of the statistical method and the interpreting of the results. IS has designed and lead the work of the main project and contributed in the revision of the manuscript. PMU has been in charge of analysing the biomarkers from Bevital. He has also contributed in the preparation phase of designing the study and selecting the analytic platform and in interpreting the results and the revision of the manuscript. AU has contributed with the statistical analyses and the interpretation of the results. He has contributed with detailed knowledge of the biomarkers from Bevital and to the process of writing the manuscript. ABK has contributed to the development of the study and to the selection of biomarkers and interpretation of the results. She has also contributed to the writing process. All authors have read and agreed to the published version of the manuscript and are accountable for the content.

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Availability of data and materials

Due to Norwegian regulations and conditions for informed consent, the dataset is not publicly available.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with institutional guidelines and was approved by the Regional Committee of Medical and Health Research Ethics (REK no: 2017/2060/REK Midt, 2015/171/REK Nord). The study was registered at Clinicaltrials.gov (NCT02650531). The participation required that the patient were able to give informed consent. For patients unable to consent for themselves, next of kin may give oral consent.

Consent for publication

Not applicable. The publication does not contain individual data.

Competing interests

The authors declare that they have no competing interests.

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References

1. Noz MP, Hartman YAW, Hopman MTE, Willems P, Tack CJ, Joosten LAB, et al. Sixteen-week physical activity intervention in subjects with increased Cardiometabolic risk shifts innate immune function towards a less Proinflammatory state. J Am Heart Assoc. 2019;8(21):e013764. https://doi.org/10.1161/JAHA.119.013764.
2. Wirth K, Klenk J, Brefka S, Dallmeier D, Faehling K, Roque IFM, et al. Biomarkers associated with sedentary behaviour in older adults: a systematic review. Ageing Res Rev. 2017;35:87–111. https://doi.org/10.1016/j.arr.2016.12.002.
3. Lavie CJ, Ozemek C, Carbone S, Katzmarzyk PT, Blair SN. Sedentary behavior, exercise, and cardiovascular health. Circ Res. 2019;124(5):799–815. https://doi.org/10.1161/CIRCRESAHA.118.312669.
4. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. Nat Rev Immunol. 2011;11(9):607–15. https://doi.org/10.1038/nri3041.
5. Tir AMD, Labor M, Plavec D. The effects of physical activity on chronic subclinical systemic inflammation. Arh Hig Rada Toksikol. 2017;68(4):276–86. https://doi.org/10.1515/aiht-2017-68-2965.
6. Tremblay MS, Aubert S, Barnes JD, Saunders TJ, Carson V, Latimer-Cheung AE, et al. Sedentary behavior research network (SBRN) - terminology consensus project process and outcome. Int J Behav Nutr Phys Act. 2017;14(1):75. https://doi.org/10.1186/s12966-017-0525-8.
7. Benatti FB, Ried-Larsen M. The effects of breaking up prolonged sitting time: a review of experimental studies. Med Sci Sports Exerc. 2015;47(10):2053–61. https://doi.org/10.1249/MSS.0000000000000654.
8. Parsons TJ, Sartini C, Welsh P, Sattar N, Ash S, Lennon LT, et al. Physical activity, sedentary behavior, and inflammatory and hemostatic markers in men. Med Sci Sports Exerc. 2017;49(3):459–65. https://doi.org/10.1249/MSS.0000000000001113.
9. Dunstan DW, Thorp AA, Healy GN. Prolonged sitting: is it a distinct coronary heart disease risk factor? Curr Opin Cardiol. 2011;26(5):412–9. https://doi.org/10.1097/HCO.0b013e3283496605.
10. Ridker PM. From C-reactive protein to Interleukin-6 to Interleukin-1: moving upstream to identify novel targets for Atheroprotection. Circ Res. 2016;118(1):145–56. https://doi.org/10.1161/CIRCRESAHA.115.306656.
11. Pedersen ER, Midttun O, Ueland PM, Schartum-Hansen H, Seifert R, Igland J, et al. Systemic markers of interferon-gamma-mediated immune activation and long-term prognosis in patients with stable coronary artery disease. Arterioscler Thromb Vasc Biol. 2011;31(3):698–704. https://doi.org/10.1161/ATVBAHA.110.219329.
12. Lustgarten MS, Fielding RA. Metabolites associated with circulating Interleukin-6 in older adults. J Gerontol A Biol Sci Med Sci. 2017;72(9):1277–83.
13. Ferrucci L, Gonzalez-Freire M, Fabbri E, Simonsick E, Tanaka T, Moore Z, et al. Measuring biological aging in humans: a quest. Aging Cell. 2020;19(2):e13080. https://doi.org/10.1111/acel.13080.
14. Henson J, Yates T, Edwardson CL, Khunti K, Talbot D, Gray LJ, et al. Sedentary time and markers of chronic low-grade inflammation in a high risk population. PLoS One. 2013;8(10):e78350. https://doi.org/10.1371/journal.pone.0078350.

15. Leal LG, Lopes MA, Batista ML Jr. Physical exercise-induced Myokines and muscle-adipose tissue crosstalk: a review of current knowledge and the implications for health and metabolic diseases. Front Physiol. 2018;9:1307. https://doi.org/10.3389/fphys.2018.01307.
16. Abd El-Kader SM, Al-Jiffri OH. Aerobic exercise modulates cytokine profile and sleep quality in elderly. Afr Health Sci. 2019;19(2):2198–207. https://doi.org/10.4314/ahs.v19i2.45.
17. Sallam N, Laher I. Exercise modulates oxidative stress and inflammation in aging and cardiovascular diseases. Oxidative Med Cell Longev. 2016;2016:7239639.
18. Mandi Y, Vecsei L. The kynurenine system and immunoregulation. J Neural Transm (Vienna). 2012;119(2):197–209. https://doi.org/10.1007/s00702-011-0681-y.
19. Schrocksnadel K, Wirleitner B, Winkler C, Fuchs D. Monitoring tryptophan metabolism in chronic immune activation. Clin Chim Acta. 2006;364(1–2):82–90. https://doi.org/10.1016/j.cca.2005.06.013.
20. Eussen SJ, Ueland PM, Vollset SE, Nygard O, Midttun O, Sulo G, et al. Kynurenines as predictors of acute coronary events in the Hordaland health study. Int J Cardiol. 2015;189:18–24. https://doi.org/10.1016/j.ijcard.2015.03.413.
21. Valdiglesias V, Sanchez-Flores M, Maseda A, Lorenzo-Lopez L, Marcos-Perez D, Lopez-Corton A, et al. Immune biomarkers in older adults: role of physical activity. J Toxicol Environ Health A. 2017;80(13–15):605–20. https://doi.org/10.1080/15287394.2017.1286898.
22. Theofylaktopoulou D, Midttun O, Ulvik A, Ueland PM, Tell GS, Vollset SE, et al. A community-based study on determinants of circulating markers of cellular immune activation and kynurenines: the Hordaland health study. Clin Exp Immunol. 2013;173(1):121–30. https://doi.org/10.1111/cei.12092.
23. Ulvik A, Midttun O, Pedersen ER, Eussen SJ, Nygard O, Ueland PM. Evidence for increased catabolism of vitamin B-6 during systemic inflammation. Am J Clin Nutr. 2014;100(1):250–5. https://doi.org/10.3945/ajcn.114.083196.
24. Ulvik A, Midttun O, Pedersen ER, Nygard O, Ueland PM. Association of plasma B-6 vitamers with systemic markers of inflammation before and after pyridoxine treatment in patients with stable angina pectoris. Am J Clin Nutr. 2012;95(5):1072–8. https://doi.org/10.3945/ajcn.111.029751.
25. Zuo H, Tell GS, Ueland PM, Nygard O, Vollset SE, Midttun O, et al. The PAr index, an indicator reflecting altered vitamin B-6 homeostasis, is associated with long-term risk of stroke in the general population: the Hordaland health study (HUSK). Am J Clin Nutr. 2018;107(1):105–12. https://doi.org/10.1093/ajcn/nqx012.
26. Joisten N, Walzik D, Metcalfe AJ, Bloch W, Zimmer P. Physical exercise as kynurenine pathway modulator in chronic diseases: implications for immune and energy homeostasis. Int J Tryptophan Res. 2020;13:1178646920938688.
27. Wirthgen E, Hoeflich A, Rebl A, Gunther J. Kynurenic acid: the Janus-faced role of an immunomodulatory tryptophan metabolite and its link to pathological conditions. Front Immunol. 2017;8:1957.
28. Agudelo LZ, Femenia T, Orhan F, Porsmyr-Palmertz M, Goiny M, Martinez-Redondo V, et al. Skeletal muscle PGC-1alpha1 modulates kynurenine metabolism and mediates resilience to stress-induced depression. Cell. 2014;159(1):33–45. https://doi.org/10.1016/j.cell.2014.07.051.
29. Schlittler M, Goiny M, Agudelo LZ, Venckunas T, Brazaitis M, Skurvydas A, et al. Endurance exercise increases skeletal muscle kynurenine aminotransferases and plasma kynurenic acid in humans. Am J Physiol Cell Physiol. 2016;310(10):C836–40. https://doi.org/10.1152/ajpcell.00053.2016.
30. Cervenka I, Agudelo LZ, Ruas JL. Kynurenines: Tryptophan's metabolites in exercise, inflammation, and mental health. Science. 2017;357(6349):eaaf9794.
31. Metcalfe AJ, Koliamitra C, Javelle F, Bloch W, Zimmer P. Acute and chronic effects of exercise on the kynurenine pathway in humans - a brief review and future perspectives. Physiol Behav. 2018;194:583–7. https://doi.org/10.1016/j.physbeh.2018.07.015.
32. Baumgartner R, Berg M, Matic L, Polyzos KP, Forteza MJ, Hjorth SA, et al. Evidence that a deviation in the kynurenine pathway aggravates atherosclerotic disease in humans. J Intern Med. 2021;289(1):53–68. https://doi.org/10.1111/joim.13142.
33. Thingstad P, Askim T, Beyer MK, Brathen G, Ellekjaer H, Ihle-Hansen H, et al. The Norwegian cognitive impairment after stroke study (nor-COAST): study protocol of a multicentre, prospective cohort study. BMC Neurol. 2018;18(1):193. https://doi.org/10.1186/s12883-018-1198-x.
34. Kuvas KR, Saltvedt I, Aam S, Thingstad P, Ellekjaer H, Askim T. The risk of selection Bias in a clinical multi-center cohort study. Results from the Norwegian cognitive impairment after stroke (nor-COAST) study. Clin Epidemiol. 2020;12:1327–36. https://doi.org/10.2147/CLEP.S276631.
35. Brott T, Adams HP Jr, Olinger CP, Marler JR, Barsan WG, Biller J, et al. Measurements of acute cerebral infarction: a clinical examination scale. Stroke. 1989;20(7):864–70. https://doi.org/10.1161/01.STR.20.7.864.
36. Duffy L, Gajree S, Langhorne P, Stott DJ, Quinn TJ. Reliability (inter-rater agreement) of the Barthel index for assessment of stroke survivors: systematic review and meta-analysis. Stroke. 2013;44(2):462–8. https://doi.org/10.1161/STROKEAHA.112.678615.
37. Banks JL, Marotta CA. Outcomes validity and reliability of the modified Rankin scale: implications for stroke clinical trials: a literature review and synthesis. Stroke. 2007;38(3):1091–6. https://doi.org/10.1161/01.STR.0000258355.23810.c6.
38. Stockmann H, Neves AA, Stairs S, Ireland-Zecchini H, Brindle KM, Leeper FJ. Development and evaluation of new cyclooctynes for cell surface glycan imaging in cancer cells. Chem Sci. 2011;2(5):932–6. https://doi.org/10.1039/c0sc00631a.
39. Sorgdrager FJH, Naude PJW, Kema IP, Nollen EA, Deyn PP. Tryptophan metabolism in Inflammaging: from biomarker to therapeutic target. Front Immunol. 2019;10:2565. https://doi.org/10.3389/fimmu.2019.02565.
40. Cronin O, Keohane DM, Molloy MG, Shanahan F. The effect of exercise interventions on inflammatory biomarkers in healthy, physically inactive subjects: a systematic review. QJM. 2017;110(10):629–37. https://doi.org/10.1093/qjmed/hcx091.
41. Wang W, Griswold ME. Estimating overall exposure effects for the clustered and censored outcome using random effect Tobit regression models. Stat Med. 2016;35(27):4948–60. https://doi.org/10.1002/sim.7045.
42. Swindell N, Rees P, Fogelholm M, Drummen M, MacDonald I, Martinez JA, et al. Compositional analysis of the associations between 24-h movement behaviours and cardio-metabolic risk factors in overweight and obese adults with pre-diabetes from the PREVIEW study: cross-sectional baseline analysis. Int J Behav Nutr Phys Act. 2020;17(1):29. https://doi.org/10.1186/s12966-020-00936-5.
43. Teixeira LK, Fonseca BP, Barboza BA, Viola JP. The role of interferon-gamma on immune and allergic responses. Mem Inst Oswaldo Cruz. 2005;100(Suppl 1):137–44. https://doi.org/10.1590/S0074-02762005000900024.
44. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol. 2014;6(10):a016295. https://doi.org/10.1101/cshperspect.a016295.
45. Jung TW, Park J, Sun JL, Ahn SH, Abd El-Aty AM, Hacimuftuoglu A, et al. Administration of kynurenic acid reduces hyperlipidemia-induced inflammation and insulin resistance in skeletal muscle and adipocytes. Mol Cell Endocrinol. 2020;518:110928. https://doi.org/10.1016/j.mce.2020.110928.
46. Smith L, Tully M, Jacob L, Blackburn N, Adlakha D, Caserotti P, et al. The Association Between Sedentary Behavior and Sarcopenia Among Adults Aged >/=65 Years in Low- and Middle-Income Countries. Int J Environ Res Public Health. 2020;17(5):1708.
47. Alme KN, Knapskog AB, Naess H, Naik M, Beyer M, Ellekjaer H, et al. Is long-bout sedentary behaviour associated with long-term glucose levels 3 months after acute ischaemic stroke? A prospective observational cohort study. BMJ Open. 2020;10(11):e037475. https://doi.org/10.1136/bmjopen-2020-037475.

Publisher's Note

Supplementary file

Title page

Investigating novel biomarkers of immune activation and modulation in the context of sedentary behaviour: a multicentre prospective ischemic stroke cohort study.

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Supplementary table 1: Crude and adjusted linear regression analyses of the association between biomarkers and time in sedentary behaviour by bout-length category.

	Crude			Adjusted		
	β	95% CI	P	β	95% CI	P
hsCRP			R^2=0.12			R^2=0.19
<30 minutes	0.15	(-0.02, 0.33)	0.092	0.12	(-0.05, 0.30)	0.171
30-59 minutes	0.21	(0.06, 0.36)	0.006	0.11	(-0.04, 0.27)	0.143
60-89 minutes	0.00	(-0.16, 0.17)	0.979	-0.06	(0.06, -0.18)	0.493
>90 minutes	0.32	(0.15, 0.49)	<0.001	0.34	(0.17, 0.51)	<0.001
IL6			R^2=0.07			R^2=0.13

<30 minutes	0.17	(0.01, 0.32)	0.032	0.15	(-0.01, 0.30)	0.053
30-59 minutes	0.12	(0.00, 0.25)	0.044	0.07	(-0.06, 0.20)	0.231
60-89 minutes	0.06	(-0.07, 0.20)	0.338	0.00	(-0.14, 0.14)	0.985
>90 minutes	0.27	(0.12, 0.41)	0.001	0.22	(0.07, 0.37)	0.005
IL10			R^2=0.01			R^2=0.05
<30 minutes	0.11	(-0.05, 0.27)	0.204	0.13	(-0.03, 0.29)	0.114
30-59 minutes	-0.02	(-0.15, 0.11)	0.876	-0.04	(-0.18, 0.09)	0.837
60-89 minutes	0.04	(-0.11, 0.18)	0.947	0.03	(-0.11, 0.18)	0.888
>90 minutes	0.06	(-0.09, 0.21)	0.331	0.04	(-0.13, 0.19)	0.535
Neopterin			R^2=0.07			R^2=0.38
<30 minutes	0.22	(0.06, 0.37)	0.006	0.20	(0.07, 0.33)	0.002
30-59 minutes	0.16	(0.04, 0.29)	0.008	0.07	(-0.07, 0.22)	0.178
60-89 minutes	0.08	(-0.06, 0.21)	0.285	-0.03	(-0.15, 0.09)	0.610
>90 minutes	0.20	(0.05, 0.34)	0.008	0.16	(0.04, 0.29)	0.012
PAr-index			R^2=0.12			R^2=0.38
<30 minutes	0.21	(0.06, 0.36)	0.007	0.20	(0.07, 0.33)	0.003
30-59 minutes	0.16	(0.04, 0.28)	0.008	0.07	(-0.04, 0.18)	0.201
60-89 minutes	0.27	(0.13, 0.40)	<0.001	0.17	(0.05, 0.29)	0.005
>90 minutes	0.18	(0.04, 0.32)	0.013	0.14	(0.01, 0.26)	0.032
Kynurenine/tryptophan ratio			R^2=0.06			R^2=0.46
<30 minutes	0.07	(-0.09, 0.22)	0.405	0.03	(-0.09, 0.15)	0.596
30-59 minutes	0.21	(0.08, 0.33)	0.001	0.11	(0.01, 0.21)	0.041
60-89 minutes	0.11	(-0.02, 0.25)	0.105	0.00	(-0.11, 0.11)	0.978
>90 minutes	0.07	(-0.08, 0.22)	0.345	0.01	(-0.10, 0.13)	0.823
Kynurenic acid			R^2=0.01			R^2=0.49
<30 minutes	-0.01	(-0.17, 0.15)	0.907	-0.02	(-0.14, 0.10)	0.724

30-59 minutes	-0.00	(-0.13, 0.12)	0.945	-0.13	(-0.22, -0.03)	0.012
60-89 minutes	0.12	(-0.03, 0.26)	0.112	0.02	(-0.09, 0.13)	0.744
>90 minutes	-0.03	(-0.18, 0.12)	0.658	-0.07	(-0.19, 0.04)	0.209

[1]hsCRP=high sensitive C-reactive protein. IL6=interleukin-6. IL10=interleukin-10. PAr-index= 4-pyridoxic acid divided/(pyridoxal 5´-phosphate + pyridoxal).
[2]Model: biomarker is the dependent variable. Sedentary time, age, sex, waist circumference, and creatinine are independent variables.
[3]For IL-6 and IL-10, Tobit regressions were used for the significance test and regular linear regressions to calculate the beta coefficients.

III

Title page

Neopterin and kynurenic acid as predictors of stroke recurrence and mortality. A multicentre prospective cohort study on biomarkers of inflammation measured three months after ischemic stroke.

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Abstract (max. 300 words)

Background

Chronic low-grade inflammation is associated with both ischemic stroke and sedentary behaviour. The aim of this study was to investigate the predictive abilities of biomarkers of inflammation and immune modulation associated with sedentary behaviour for ischemic stroke recurrence and mortality in a stroke population.

Methods

Patients admitted to hospital for acute stroke were recruited to the prospective multicentre cohort study, the Norwegian Cognitive Impairment After Stroke (Nor-COAST) study, from May 2015 until March 2017. Patients with ischemic stroke, blood samples available from the three-month follow-up, and no stroke recurrence before the three-month follow-up were included. Serum was analysed for C-reactive protein (CRP) with high-sensitive technique, and plasma for interleukin-6 (IL-6), neopterin, pyridoxic acid ratio index (PAr-index: 4-pyridoxic acid: [pyrioxal+pyridoxal-5´-phosphate]) and kynurenic acid (KA). Ischemic stroke recurrence and death were identified by the Norwegian Stroke Registry and the Cause of Death Registry until 31 December 2018.

Results

The study included 354 patients, 57% male, mean age 73 (SD 11) years, mean observation time 2.5 (SD 0.6) years, and median National Institute of Health Stroke Scale of 0 (IQR 1) at three months. CRP was associated with mortality (HR 1.40, CI 1.01, 1.96, p=0.046), and neopterin was associated with the combined endpoint (recurrent ischemic stroke or death) (HR 1.52, CI 1.06, 2.20, p=0.023), adjusted for age, sex, prior cerebrovascular disease, modified Rankin Scale, and creatinine. When adding neopterin and KA to the same model, KA was negatively associated (HR 0.57, CI 0.33, 0.97, p=0.038), and neopterin was

positively associated (HR 1.61, CI 1.02, 2.54, p=0.040) with mortality. Patients with cardioembolic stroke at baseline had higher levels of inflammation at three months.

Conclusion

Neopterin might be a valuable prognostic biomarker in stroke patients. The use of KA as a measure of anti-inflammatory capacity should be investigated further.

Trial registration

The study was registered at Clinicaltrials.gov (NCT02650531). First posted on 08/01/2016. URL: https://clinicaltrials.gov/ct2/show/NCT02650531

Keywords: sedentary behaviour, inflammation, immune modulation, vascular disease, kynurenine pathway, stroke

Background

Ischemic stroke is associated with chronic low-grade inflammation, and there is a need for more knowledge about mechanisms and management (1-4). Prognosis, outcomes, and management of stroke depends on stroke subtype, defined by the assumed aetiology of atherosclerosis, cardioembolism, or small vessel disease (5, 6). In the context of inflammation, atherosclerosis is the most studied subtype, but inflammation has been found to contribute to all three categories (7-10). Despite the evidence, clinical trials using drugs targeting inflammation have so far been inconclusive, and there are to date no established preventive treatment strategies that target inflammation in particular (4, 11), possibly related to the challenge of obtaining an optimal balance between potential benefits and an increased risk of (fatal) infections (4). Obviously, treatment strategies that minimise severe side effects are desired.

Reducing chronic low-grade inflammation by targeting sedentary behaviour has shown promising results (12). Still, there is a need for valid biomarkers associated with inflammation with explanatory and predictive properties for outcomes like vascular disease and mortality (3). In a prior study, we investigated plasma biomarkers of inflammation and immune modulation in a stroke population measured three months after the acute ischemic event and their association to objectively measured sedentary time. We identified the biomarkers C-reactive protein (CRP), interleukin-6 (IL-6), neopterin, and the pyridoxal acid ratio-index (PAr-index) to be positively associated, and kynurenic acid (KA) to be negatively associated with objectively measured sedentary behaviour (13). CRP (14), IL-6 (15), neopterin (16, 17) and PAr-index (18) are inflammatory biomarkers associated with vascular disease. KA is part of a negative feedback loop inducing immune tolerance (19, 20). Hence, even though it will rise in response to inflammation, this response is believed to be beneficial for disease

progression during an inflammatory state (20). The association to vascular disease progression is unclear.

In this study, we investigated the associations between biomarkers of inflammation and stroke recurrence and all-cause mortality. Finally, given the above-mentioned clinical importance of the stroke subtypes, we also investigated the association to the stroke subtype at baseline.

Material and methods

Subjects

The patients were included in the prospective multicentre cohort study, the Norwegian Cognitive Impairment After Stroke (Nor-COAST) study, at admission for acute stroke between May 2015 and March 2017. The inclusion criteria were 1) hospital admission to one of the five participating hospitals, 2) acute stroke following the World Health Organisation definition or finding of acute stroke on imaging 3) being able to communicate in one of the Scandinavian languages, 4) above 18 years of age, 5) living in the catchment area. Patients were excluded if they had a life expectancy of less than three months. Details about the Nor-COAST population are described in prior studies (21, 22). For this study, only patients with ischemic stroke at baseline who participated at the three-month follow-up and with data on at least one of the relevant biomarkers were included. To avoid the acute inflammatory response associated with the acute stroke event, we chose the three-month follow-up after the acute stroke as the baseline for this study, assuming the inflammatory biomarkers were not affected by the acute event at this time point (Figure 1) (23).

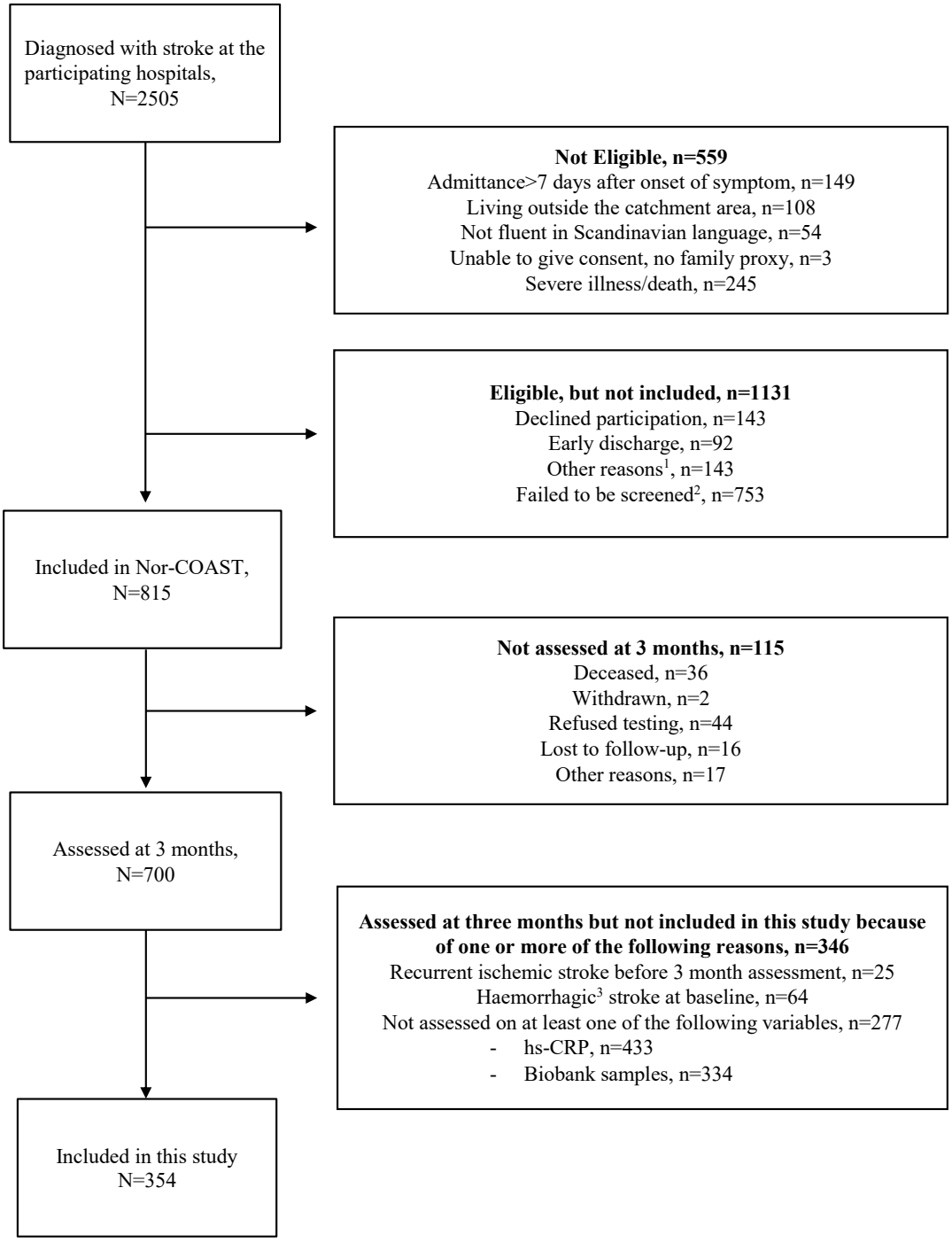

Figure 1: Patient selection. [1] Other reasons: i.e.: delirious patient, hearing, uncertainty about the diagnosis, multi morbid, nursing home resident, infrastructure on the ward, vacation/weekends, other studies.
[2] Failed to screen: infrastructure on the ward, vacation/weekends. [3] Not haemorragic transformation.

Clinical data

Demographic data and information about stroke properties, namely lesion type and stroke subtype, and stroke risk factors, were collected at baseline. Waist circumference, smoking status, stroke severity and functional outcomes were assessed at the three-month follow-up. Stroke severity was measured using the National Institute of Health Stroke Scale (NIHSS), global function by use of the modified Rankin Scale (mRS), and basic activities of daily living (ADL) by the Barthel Index (BI). The diagnosis of diabetes mellitus was defined at baseline by medical history and/or medication use (Anatomical therapeutical chemical Classification (ATC): A10) and/or HbA1c ≥6.5% at baseline. Hypercholesterolemia at baseline was defined by medication use (ATC: C10) and/or total cholesterol >6.2 mmol/L and/or LDL≥4.1 mmol/L at baseline. Hypertension at baseline was defined by medication use (ATC: C02, C03, C04, C07, C08, C09).

Laboratory analyses

At the three-month follow-up, non-fasting blood samples were collected for routine analysis and for storage in the biobank.

Routine clinical–chemical analyses: serum C-reactive protein (CRP) – measured using a high-sensitive technique (mg/L) – and serum creatinine (μmol/L) were analysed at the local laboratories of the inclusion centres.

Sample collection of research analyses: Aliquots of serum and plasma were immediately frozen at -80°C, and sent on dry ice to BioBank1, Central Norway Regional Health Authority for storage. The inflammatory biomarkers were analysed in 2019. Two aliquots of plasma were used, one for each of the two laboratories. The samples were thawed only once. The selection of biomarkers for this study was based on prior findings (13) and included CRP (mg/L), interleukin-6 (IL-6, pg/ml), neopterin (nmol/L), 4-pyridoxic acid (nmol/L), pyridoxal,

pyridoxal 5´-phosphate (nmol/L), and kynurenic acid (KA, nmol/L). The pyridoxic acid ratio index – PAr-index: 4-pyridoxic acid (pyrioxal+pyridoxal 5´-phosphate) (24) was calculated. The cytokines were analysed at Research Laboratory Nordland Hospital using the Bio-Plex technology kits obtained from Bio-Rad Laboratories Inc., Hercules, CA.. The other biomarkers were analysed as part of analytic platform D at Bevital A/S (Bergen, Norway) by liquid chromatography/tandem mass spectrometry. Because of the sample size, IL-6 was analysed using multiple trays. The upper and lower detection limit varied among the trays according to the standard curve. There were no values above or below the detection limits for IL-6. For the biomarkers from BeVital AS (neopterin, kynurenic acid, and the B6 vitamers), the performance of the method has been published previously (25). There were no values out of range.

Outcomes

Recurrent ischemic stroke was identified by the Norwegian Stroke Registry. The coverage was 84–87% in the period from 2015–2018, and stroke was defined according to the WHO's definition (26). Death was identified by the Norwegian Cause of Death Registry, which has a general coverage of 98% of deaths of Norwegian citizens within the country or abroad (27)(Pedersen 2015). The data extraction was performed on 31 December 2018.

Patient outcomes were defined based on the first event: recurrent ischemic stroke, death or no event. Some of the patients died during the follow-up period after first having a stroke recurrence. In the regression analyses, these patients were included in both groups. A third outcome was defined as the composite of recurrent ischemic stroke and death. The patients with both outcomes were counted only once.

Statistics

For the baseline and three-month descriptions, patients were stratified by first event and analysed using the chi-squared test for categorical variables (numbers and percentages), one-way ANOVA for continuous variables, and Kruskal–Wallis H-test for the continuous variables that were not normally distributed. Data were presented as means with standard deviations (SD) and medians with interquartile range (IQR).

One-way ANOVA were used for the analyses of laboratory values according to stroke subtype at baseline. The subgroup "other determined" was excluded.

The associations between the individual biomarkers and the outcomes stroke, mortality and the composite endpoint were analysed using Cox regressions. The start of the follow-up time was defined as the date of the three-month visit to the hospital. For the outcome "recurrent ischemic stroke", an additional analysis using a competing-risk Cox regression based on the method of Fine and Grey (28) as an alternative to the regular Cox regression was used (29), with death as a competing outcome for ischemic stroke. All the biomarkers were right-skewed. After log-transformation, regression residuals approached normal distribution. The variables were standardised, and the hazard ratios are given per standard deviation change of the variable value. To account for potential confounders, we added modified Rankin scale and prior cerebrovascular disease as independent variables, because they have been found to be associated with both stroke recurrence (30) and with inflammation (mRS (31), prior cerebrovascular disease (before the baseline stroke in this study) (32)). We also added age and sex. Creatinin was included because some of the biomarkers had renal clearance (Model 1). As the blood samples were taken at the three-month follow-up, we used the results of the mRS recorded at this time point. To account for the different pathophysiology of the stroke subtypes, we did a second analysis where we added the Trial of Org 10172 in Acute Stroke

Treatment (TOAST) criteria to the model (Model 2), defining five stroke subtypes (large artery atherosclerosis, cardioembolic, cerebral small vessel disease, other determined, undetermined). The stroke subtype "other determined" is used when the cause of stroke is determined (i.e. dissection, vasculitis). This group was excluded from this analysis because of low sample size. Because KA is part of a feedback loop in response to inflammation, a third regression model including both KA and neopterin in addition to the same covariates as in Model 1 was made.

The significance level was set to 0.05, but p-values >0.01 should be evaluated with caution due to a high number of tests. The analyses were performed in STATA/SE 16.1 (Stata Corp LLC, College Station, TX, USA).

Results

Patient characteristics and laboratory values

Of the 700 patients attending the three-month follow-up, 354 were included in this sub-study. The remaining 346 patients were excluded due to at least one of the following reasons: stroke before three months (n=25), haemorrhagic stroke at baseline (n=64), or no relevant biomarker available (n=277).

The mean follow-up time from baseline for the whole group was 2.5 (0.6) years, and the mean time-to-event was 1.5 (1.0) and 1.6 (0.7) years for stroke and death, respectively. From the three-month follow-up and throughout the follow-up time, 16 (4.5%) patients had ischemic stroke recurrence. In total, 28 (7.9 %) patients died, and 25 (7.1 %) of them died without being registered as having stroke recurrence.

The baseline and three-month data are presented in Table 1, stratified by the first event. The patients who died were older compared to those with no event. Patients with no events had a higher Barthels Index (p 0.007) and a lower mRS ($p<0.001$). There were overall differences between the groups in the presence of prior ischemic stroke ($p<0.001$), prior coronary artery disease ($p=0.007$), and atrial fibrillation at baseline ($p=0.034$), the patients experiencing no events showing the lowest prevalence. There were overall differences between the groups in the values for IL-6, neopterin, and the PAr-index, where the patients who died had the highest values.

Table 1: Baseline and three-month characteristics according to the first event after three-month follow-up.

	All N=354	New IS N=16	Death N=25[1]	No event N=313	*P*-value
Age[2,3], mean years (SD)	73 (11)	73 (10)	82 (9)	73 (11)	<0.001
Male[4] sex, n (%)	201 (57)	11 (69)	16 (64)	174 (56)	0.439
Observation time[3], mean years (SD)	2.5 (6)	1.5 (1.0)	1.6 (0.7)	2.6 (0.5)	<0.001
Baseline					
TOAST[4] subtype[6], n (%)					0.661
Large artery	30 (9)	1 (6)	0	29 (10)	
Cardioembolic	72 (21)	5 (31)	6 (24)	61 (20)	
Small vessel disease	83 (24)	4 (25)	6 (7)	73 (24)	
Other determined	10 (3)	1 (6)	0	9 (3)	
Undetermined	128 (43)	5 (31)	13 (52)	128 (43)	
Prior IS[4], n (%)	72 (20)	7 (44)	13 (52)	52 (17)	<0.001
Prior CHD[4], n (%)	61 (17)	3 (19)	10 (40)	48 (13)	0.007
Atrial fibrillation[4], n (%)	89 (25)	6 (37)	11 (44)	72 (23)	0.034
Hypertension[4], n (%)	202 (57)	11 (69)	19 (76)	172 (60)	0.077
Hypercholesterolemia[4], n (%)	178 (50)	8 (50)	16 (64)	154 (46)	0 363
Diabetes mellitus[4], n (%)	60 (17.0)	0	6 (24)	54 (17)	0.124
Three-months					
NIHSS[5] median (IQR)	0 (1)	1 (1)	0.5(3)	0 (1)	0.122
BI[5] median (IQR)	100 (0)	100 (0)	95 (17)	100 (0)	0.017
mRS[5] median (IQR)	1 (1)	2 (1)	3 (2)	1 (1)	<0.001
Waist[2, 3], mean cm (SD)	95 (13)	89 (15)	98 (15)	95 (13)	0.137
Current smoking[2, 4], n (%)	31 (9)	1 (7)	4 (17)	26 (9)	0.597
Laboratory measures[2,5]					
CRP, median (IQR), n=267	1.6 (2.9)	1.1 (5.3)	3.0 (26.5)	1.6 (2.9)	0.053
IL-6, median (IQR), n=334	4.6 (4.5)	4.8 (4.1)	6.1 (6.1)	4.2 (4.2)	0.038
Neopterin, median (IQR), n=334	16 (10)	17 (12)	24 (17)	16 (9)	<0.001
PAr-index, median (IQR), n=334	0.62 (0.46)	0.57 (0.80)	1.0 (0.8)	0.60 (0.42)	0.002
Kynurenic acid, n=334	58 (30)	56 (35)	65 (52)	59 (29)	0.522

IS=ischemic stroke. TOAST=Trial of Org 10172 in Acute Stroke Treatment. CHD=coronary heart disease. NIHSS=National Institute of Stroke Scale. IQR=interquartile range. BI=Barthels Index. mRS=modified Rankin Scale. CRP= C-reactive protein. IL-6=Interleukin-6. PAr-index=pyridoxic acid ratio-index=4-pyridoxic acid:(pyridoxal+pyridoxal 5'-phosphate).

1) Additionally, three patients died after having recurrent strokes.
2) At three months.
3) The one-way ANOVA was used for the continuous variables.
4) The chi-squared test was used for the categorical variables.
5) For the continuous variables with non-normal distribution, the non-parametric Kruskal–Wallis H-test was used.
6) TOAST subtype of index stroke.

Stroke subtype

The biomarker levels by stroke subtype at baseline are presented in Table 2. There was a significant overall difference between the groups in the level of CRP, IL-6, and neopterin. Patients with cardioembolic stroke at baseline showed the highest values.

Table 2: Biomarker values at three-month follow-up by ischemic stroke subtype at baseline[1].

	Atherosclerosis (N=26)	**Cardioembolic (N=62)**	**CSVD (N=75)**	**Unknown (N=141)**	***P*-value**
CRP[2], mean (SD)	1.93 (3.29)	3.06 (3.78)	1.88 (2.56)	1.58 (3.71)	0.032
IL-6, mean (SD)	3.71 (1.95)	5.70 (2.18)	3.97 (2.18)	4.26 (2.39)	0.038
Neopterin, mean (SD)	16.0 (1.5)	20.3 (1.7)	16.0 (1.4)	16.0 (1.5)	0.003
PAr, mean (SD)	0.66 (1.77)	0.73 (1.73)	0.60 (1.73)	0.60 (1.56)	0.087
KA, mean (SD)	59.7 (1.5)	67.4 (1.4)	59.7 (1.5)	57.4 (1.5)	0.093

1) The category "other determined" was excluded because of low sample size. The analyses were conducted on log-transformed variables, and the values were transformed back after analysis.
2) For CRP: Atherosclerosis: N=20, cardioembolic: N=46, CSVD: N=50, Unknown: N=100.
CSVD=cerebral small vessel disease. CRP=C-reactive protein, IL-6=interleukin-6, PAr=pyridoxic acid ratio-index=4-pyridoxic acid:(pyridoxal+pyridoxal 5'-phosphate), KA=kynurenic acid, The analyses were done using a one-way ANOVA.

Regression analyses

The crude and adjusted hazard ratios (HR) for the outcome's ischemic stroke recurrence, death, and the composite endpoint of stroke and death, investigated for each of the biomarkers individually, are presented in Table 3. We did not find any associations with stroke recurrence. In the crude analyses, all biomarkers except for KA were significantly associated with the risk of death or the composite endpoint. In the adjusted analyses, we found that CRP was associated with death (Model 1: HR 1.40, (CI 1.01, 1.96), p=0.046) and neopterin with the combined endpoint (Model 1: HR 1.52, (CI 1.06, 2.20), p=0.023. Model 2: HR 1.55, (CI 1.06, 2.27), p=0.025).

Table 3: Crude and adjusted Cox regressions of the association between the individual biomarkers and ischemic stroke recurrence, death and the composite endpoint (ischemic stroke recurrence and death).

	Crude			Model 1			Model 2		
	HR	CI	*P*	HR	CI	*p*	HR	CI	*P*
Recurrent ischemic stroke, N=16									
CRP[1)]	0.90	(0.44, 1.83)	0.772	0.86	(0.41, 1.77)	0.674	0.92	(0.44, 1.90)	0.816
IL-6	1.11	(0.67, 1.86)	0.686	1.19	(0.52, 1.97)	0.578	1.10	(0.30, 2.02)	0.754
Neopterin	1.34	(0.85, 2.11)	0.209	1.45	(0.82, 2.57)	0.201	1.39	(0.80, 2.41)	0.244
PAr	1.16	(0.71, 1.90)	0.553	1.13	(0.61, 2.10)	0.692	1.24	(0.67, 2.31)	0.494
KA	0.91	(0.55, 1.50)	0.710	0.82	(0.41, 1.61)	0.563	0.89	(0.43, 1.86)	0.761
Death[2], N=28									
CRP[1)]	1.81	(1.29, 2.53)	0.001	1.40	(1.01, 1.96)	0.046	1.41	(1.00, 1.99)	0.053
IL-6	1.74	(1.20, 2.51)	0.003	1.34	(0.87, 2.07)	0.181	1.36	(0.87, 2.12)	0.173
Neopterin	1.93	(1.43, 2.61)	<0.001	1.44	(0.89, 2.33)	0.136	1.55	(0.91, 2.64)	0.104
PAr	1.69	(1.15, 2.49)	0.008	1.11	(0.68, 1.82)	0.666	1.13	(0.67, 1.90)	0.641
KA	1.12	(0.75, 1.66)	0.577	0.64	(0.38, 1.07)	0.088	0.69	(0.41, 1.14)	0.145
Stroke and death, N=41									
CRP[1)]	1.59	(1.16, 2.18)	0.004	1.27	(0.92, 1.74)	0.140	1.27	(0.93, 1.74)	0.135
IL-6	1.47	(1.08, 2.02)	0.016	1.23	(0.85, 1.77)	0.274	1.23	(0.85, 1.77)	0.275
Neopterin	1.79	(1.39, 2.31)	<0.001	1.52	(1.06, 2.20)	0.023	1.55	(1.06, 2.27)	0.025
PAr	1.62	(1.19, 2.22)	0.002	1.28	(0.86, 1.90)	0.224	1.34	(0.89, 2.01)	0.162
KA	1.27	(0.58, 2.79)	0.555	0.55	(0.19, 1.54)	0.252	0.60	(0.21, 1.71)	0.339

Each biomarker was investigated individually.
Model 1: Age, sex, prior cerebrovascular disease, modified Rankin Scale at three months, creatinine.
Model 2: As in model 1 + TOAST-classification at baseline (TOAST=Trial of Org 10172 in Acute Stroke Treatment)
1) For CRP, N=212, 8 and 13 for all, recurrent IS and death, respectively.
2) Includes both those with and without ischemic stroke recurrence prior to death.
HR=hazard ratio. CI=confidence interval. CRP=C-reactive protein. IL-6=Interleukin-6. PAr=PAr-index=pyridoxic acid ratio-index=4-pyridoxic acid:(pyridoxal+pyridoxal-5`-phosphate). KA=kynurenic acid.

In the additional competing risk regression analyses, where we used death as a competing event for stroke recurrence, the risk of ischemic stroke recurrence did not change significantly (see Supplementary Table 1).

The regression analyses, including both KA and neopterin in addition to the variables in Model 1, are shown in Table 4. KA was negatively associated with mortality (HR 0.57, (0.33, 0.97), 0.038), and neopterin was positively associated with mortality (HR 1.61, (1.02, 2.54), 0.040). Neopterin was also associated with the composite endpoint (HR 1.59, (1.11, 2.27), 0.011).

Table 4: Adjusted Cox regression (Model 1) of the combined contributions of kynurenic acid and neopterin in stroke, mortality and the composite endpoint (stroke and death).

	Stroke recurrence			Death			Stroke and death		
	HR	CI	p	HR	CI	p	HR	CI	p
KA	0.78	(0.39, 1.57)	0.492	0.57	(0.33, 0.97)	0.038	0.72	(0.46, 1.10)	0.131
Neopterin	1.48	(0.83, 1.57)	0.177	1.61	(1.02, 2.54)	0.040	1.59	(1.11, 2.27)	0.011
Age	0.98	(0.51, 2.61)	0.949	1.11	(0.60, 2.06)	0.746	1.09	(0.68, 1.76)	0.720
Sex	1.39	(0.73, 2.66)	0.314	1.61	(1.00, 2.62)	0.052	1.43	(0.97, 2.10)	0.071
Prior CeVD	1.37	(0.87, 2.17)	0.175	1.56	(1.10, 2.22)	0.012	1.44	(1.08, 1.91)	0.012
mRS	0.97	(0.46, 2.05)	0.945	3.25	(1.77, 5.96)	<0.001	2.24	(1.40, 3.57)	0.001
Creatinine	1.31	(0.20, 3.38)	0.583	2.23	(1.12, 4.42)	0.022	1.65	(0.93, 2.92)	0.085

The analyses includes KA, neopterin and Model 1: Age, sex, prior cerebrovascular disease, modified Rankin Scale at three months, creatinine.
HR=hazard ratio. CI=confidence interval. KA=kynurenic acid. CeVD=cerebrovascular disease. mRS=modified Rankin Scale.

Discussion

In this study, we showed that higher levels of C-reactive protein (CRP) measured three months after acute ischemic stroke were associated with an increased risk of death. Neopterin was associated with the composite endpoint of stroke and death. In a model including both neopterin and kynurenic acid, neopterin and kynurenic acid were associated with an increased and a reduced risk of death, respectively. Furthermore, patients with cardioembolic stroke at baseline and those who died during follow-up had higher levels of pro-inflammatory biomarkers at three months.

Stroke recurrence

We did not find any association between inflammatory biomarkers and stroke recurrence. This could be due to low sample size, the relatively short time of follow-up, or not all recurrent strokes being clinically acknowledged leading to a statistical type II error. The role of inflammation in stroke recurrence, investigated by traditional biomarkers such as IL-6 and CRP, has been well established (14, 15, 33, 34). The novel biomarkers; neopterin, PAr-index, and KA, are less studied. Neopterin has shown to predict future coronary events (16, 17) and stroke recurrence (35). Neopterin has also been associated with complex carotid plaques in patients with stable angina pectoris (36), with the extent of cerebral small vessel disease (CSVD) in stroke patients (37) and with the presence, but not the future risk, of atrial fibrillation (38). The PAr-index has been associated with future stroke in a population study (18) but has never been studied in a stroke population nor in the context of stroke recurrence. The role of KA in stroke recurrence is not clear, but KA has been found to be associated with coronary events (39, 40) and aortic stiffness, which in turn is associated with atrial fibrillation (41). In a recent study, Baumgarten et al. found that the branch of the kynurenine pathway, leading to the formation of KA, was downregulated in atherosclerotic plaques, and this downregulation was associated with an increased risk of cerebrovascular events (19).

Mortality

We found that inflammation, in general, was significantly associated with mortality in the unadjusted analyses. In the adjusted analyses, only CRP and neopterin showed added value as predictors compared to traditional parameters such as prior vascular disease and mRS (30). The lack of association to the remaining biomarkers could again be explained by statistical type II error due to the low sample size and short time of follow-up.

Several inflammatory biomarkers have been found to be independent predictors of all-cause and cardiovascular mortality. Both IL-6 and CRP have been found to be associated with increased mortality (34, 42), and neopterin has been identified as an independent predictor of all-cause and cardiovascular mortality (16). The PAr-index has been associated with all-cause mortality in patients with coronary artery disease (43).

KA was negatively associated with mortality first after adjusting for inflammation. We did this analysis based on the increasing evidence of KA as a contributor to the body's anti-inflammatory capacity, as part of a negative feedback loop (19, 20, 44). KA increases in response to inflammation, and our hypothesis, that a higher level of KA in response to inflammation, measured by neopterin (45) would be associated with a beneficial outcome, was supported as we found a higher value of KA to be associated with reduced risk of mortality when adjusted for inflammation. In fact, both risk estimates were strengthened after adjustment, indicating that they mutually camouflaged some of their individual contributions.

Our current finding is in concordance with KA's role in vascular disease, amongst others, by inhibiting leukocyte recruitment to atherosclerotic plaques (19). The result is in contrast to findings of KA being associated with mortality after coronary artery disease when being analysed without adjusting for inflammation (39). Although exploratory, our results indicate that downregulation of KA might be one of the molecular pathways mediating the hazards of sedentary behaviour in disease progression. This should be further investigated.

Stroke subtype

We found that patients with cardioembolic strokes at baseline had higher levels of CRP, IL-6, and neopterin at three months, indicating a difference in the inflammatory profile between

these subgroups. The link between stroke and inflammation has primarily been investigated in all the subtypes combined (14, 46) or in stroke caused by the progression of atherosclerosis (47) or CSVD (48). The role of inflammation in cardioembolic strokes, in particular, is less studied. However, increasing evidence exists of the importance of inflammation for the presence of atrial fibrillation and the associated cardioembolic risk (7, 8, 49). It can be argued that concurrent vascular disease might mediate the increased risk of stroke associated with inflammation found in patients with atrial fibrillation. However, Packer argued in a review for the contribution of inflammation as a risk factor independent of vascular disease, as the risk exceeded that predicted by cardiovascular risk factors (50). Inflammatory biomarkers might be important for identifying those in need of prolonged ECG monitoring to identify paroxysmal atrial fibrillation, and when evaluating stroke risk in patients with atrial fibrillation (7, 38, 50-52). Altogether, these observations indicate that the stroke subtype is relevant when studying inflammation in stroke and underlines the importance that such studies are powered for stratified analyses.

Strength and weaknesses

The information about stroke recurrence was based on the Norwegian Stroke Registry, which identifies patients with stroke admitted to hospital. The registry reports coverage of 84-87% in this period (26) and has been shown to be reliable(53). The registry includes only patients admitted to hospital and where the recurrent stroke has been clinically recognised. Not all patients are admitted to hospital, and not all strokes are clinically overt. Zeestraten et al. found a recurrence rate of 3% based on clinical judgement, while 27% had new lacunas on imaging at the five-year follow-up (54). These strokes might be more clinically subtle but are still of clinical importance. The use of imaging, such as MRI, could increase the sensitivity of the

stroke diagnosis, and this has been included in the updated stroke definition (55). Hence, we might have underestimated the incidence of ischemic stroke recurrence.

The mean follow-up time of the patients was 2.5 years, and for the patients included at the end of the study, the follow-up was limited to 16 months after the three-month follow-up. Khanevski et al. studied patients with ischemic stroke and transient ischemic attack and found that 5.4% had stroke recurrence after 1 year, 10.7% after 5 years, and 14.2% at the end of the study (mean 5.6 years) (30). In addition, we had to exclude half of the stroke recurrences because they occurred before the three-month follow-up. The short follow-up and the low number of stroke recurrences in our study increase the risk of a type II error, and in particular negative findings should be evaluated with caution.

To avoid the acute inflammatory response associated with the acute stroke event, we chose the three-month follow-up after the acute stroke as the baseline for this study, assuming the inflammatory biomarkers were not affected by the acute event at that point in time-point(16). The correlation between the inflammatory biomarkers before and three months after is not known. Still, it characterises these subgroups of patients and could be valuable for prospective studies. Stroke subtype is based on clinical judgement of the most likely cause of the stroke in question and may be challenging to determine (6). Also, several causes of stroke in a single patient are common, i.e. a combination of large artery atherosclerosis, CSVD, and atrial fibrillation. These patients are often included in the category “unknown”.

Conclusion

Neopterin seems to be a useful prognostic biomarker in stroke populations. The novel finding of a potential protective effect of KA in response to inflammation in disease progression should be investigated further. The results also underline the importance of stroke subtypes

when investigating the impact of inflammation in ischemic stroke. The results highlights the importance of inflammation in cerebrovascular disease. Measures should be made to reduce inflammation as a secondary preventive strategy, i.e. by reducing sedentary behaviour. In addition, patients with undetected atrial fibrillation are receiving suboptimal drug treatment, and investigating the usefulness of inflammatory biomarkers to identify these patients could have significant therapeutic consequences for these individuals.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the institutional guidelines and approved by the Regional Committee of Medical and Health Research Ethics (REK) (Nor-COAST study, application number: REK no: 2017/2060. This sub-study, application number: REK no: 2015/171, Application ID: 12253). The study was registered at Clinicaltrials.gov (NCT02650531). To participate, the patients had to be able to give written informed consent for themselves. Patients who were unable to express consent for themselves were also included if their next of kin did not decline. This is in line with the Norwegian consent procedures for patients not able to consent for themselves.

Consent for publication

Not applicable. The publication does not contain individual data.

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due to Norwegian regulations and conditions for informed consent but are available from the corresponding author on reasonable request.

Competing interest

ABK has been/is the principal site investigator in three clinical trials (Boehringer-Ingelheim 1346.0023, Roche BN29553, and Novo Nordisk NN6535-4730), IS has been an investigator in the clinical trial Boehringer-Ingelheim 1346.0023 and part of the advisory board for Biogen.

The rest of the authors do not have any competing interests to declare.

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Authors´ contribution

KNA has planned this study. She has participated in the processing of the registry data, planned and performed the statistical analyses and interpreted the results. She has written the article. AU has contributed to the statistical analyses and the interpretation of the results. He has contributed with detailed knowledge of the biomarkers from Bevital and to the process of writing the manuscript.

TA has contributed to the design of this study and was responsible for the physical activity data from the main study. She has contributed to the revision of the manuscript.

JA has supervised the process of developing and describing the statistical method and contributed to the interpretation and presentation of the data.

TEM has analysed the cytokines used in this study. He has contributed in the post-analytical work and defined the method of handling the data and interpreting the results. He has also contributed in the revision of the manuscript.

MN has contributed to the development of the study and revised the manuscript.

HN has contributed to the development of the study and revised the manuscript. He has also contributed to the development of the statistical method and the interpretation of the results.

IS is the principal investigator of the main project and has contributed to the revision of the manuscript.

PMU has been in charge of analysing the biomarkers from Bevital. He has also contributed in the preparation phase of designing the study and selecting the analytic platform, and in interpreting the results and the revision of the manuscript.

ABK has contributed to the development of the study and to the selection of biomarkers and interpretation of the results. She has also contributed to the writing process.

All authors have read and agreed to the published version of the manuscript and are accountable for the content.

References

1. Emerging Risk Factors C, Kaptoge S, Di Angelantonio E, Lowe G, Pepys MB, Thompson SG, et al. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: An individual participant meta-analysis. Lancet. 2010;375(9709):132-40.
2. Parikh NS, Merkler AE, Iadecola C. Inflammation, autoimmunity, infection, and stroke: Epidemiology and lessons from therapeutic intervention. Stroke. 2020;51(3):711-8.
3. Dempsey PC, Matthews CE, Dashti SG, Doherty AR, Bergouignan A, van Roekel EH, et al. Sedentary behavior and chronic disease: Mechanisms and future directions. J Phys Act Health. 2020;17(1):52-61.
4. Liberale L, Montecucco F, Schwarz L, Luscher TF, Camici GG. Inflammation and cardiovascular diseases: Lessons from seminal clinical trials. Cardiovasc Res. 2021;117(2):411-22.
5. Adams HP, Jr., Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. Stroke. 1993;24(1):35-41.
6. Rucker V, Heuschmann PU, O'Flaherty M, Weingartner M, Hess M, Sedlak C, et al. Twenty-year time trends in long-term case-fatality and recurrence rates after ischemic stroke stratified by etiology. Stroke. 2020;51(9):2778-85.
7. Berg DD, Ruff CT, Morrow DA. Biomarkers for risk assessment in atrial fibrillation. Clin Chem. 2021;67(1):87-95.
8. Wallentin L, Hijazi Z, Andersson U, Alexander JH, De Caterina R, Hanna M, et al. Growth differentiation factor 15, a marker of oxidative stress and inflammation, for risk assessment in patients with atrial fibrillation: Insights from the Apixaban for Reduction In STroke and Other THromboemboLic Events in Atrial Fibrillation (ARISTOTLE) trial. Circulation. 2014;130(21):1847-58.
9. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: A dynamic balance. Nat Rev Immunol. 2013;13(10):709-21.
10. Poggesi A, Pasi M, Pescini F, Pantoni L, Inzitari D. Circulating biologic markers of endothelial dysfunction in cerebral small vessel disease: A review. J Cereb Blood Flow Metab. 2016;36(1):72-94.
11. Nasjonal faglig retningslinje - hjerneslag (National guidelines - stroke): Norwegian Directorate of Health; 2017 [updated 2020-04-27. Available from: https://www.helsedirektoratet.no/retningslinjer/hjerneslag.
12. Noz MP, Hartman YAW, Hopman MTE, Willems P, Tack CJ, Joosten LAB, et al. Sixteen-week physical activity intervention in subjects with increased cardiometabolic risk shifts innate immune function towards a less proinflammatory state. J Am Heart Assoc. 2019;8(21):e013764.
13. Alme KNA, T.; Assmus, J.; Mollnes, T.E.; Naik, M.; Næss, H.; Saltvedt, I.; Ueland, P.M.; Ulvik, A.; Knapskog, A.B. Investigating novel biomarkers of immune activation and modulation in the context of sedentary behaviour: A multicentre prospective ischemic stroke cohort study. . BMC Neurology. 2021;In press. .
14. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, et al. Antiinflammatory therapy with Canakinumab for atherosclerotic disease. N Engl J Med. 2017;377(12):1119-31.
15. Castillo J, Alvarez-Sabin J, Martinez-Vila E, Montaner J, Sobrino T, Vivancos J, et al. Inflammation markers and prediction of post-stroke vascular disease recurrence: The MITICO study. J Neurol. 2009;256(2):217-24.
16. Pedersen ER, Midttun O, Ueland PM, Schartum-Hansen H, Seifert R, Igland J, et al. Systemic markers of interferon-gamma-mediated immune activation and long-term prognosis in patients with stable coronary artery disease. Arterioscler Thromb Vasc Biol. 2011;31(3):698-704.
17. Sulo G, Vollset SE, Nygard O, Midttun O, Ueland PM, Eussen SJ, et al. Neopterin and kynurenine-tryptophan ratio as predictors of coronary events in older adults, the Hordaland Health Study. Int J Cardiol. 2013;168(2):1435-40.

18. Zuo H, Tell GS, Ueland PM, Nygard O, Vollset SE, Midttun O, et al. The PAr index, an indicator reflecting altered vitamin B-6 homeostasis, is associated with long-term risk of stroke in the general population: The Hordaland Health Study (HUSK). Am J Clin Nutr. 2018;107(1):105-12.
19. Baumgartner R, Berg M, Matic L, Polyzos KP, Forteza MJ, Hjorth SA, et al. Evidence that a deviation in the kynurenine pathway aggravates atherosclerotic disease in humans. J Intern Med. 2021;289(1):53-68.
20. Joisten N, Walzik D, Metcalfe AJ, Bloch W, Zimmer P. Physical exercise as kynurenine pathway modulator in chronic diseases: Implications for immune and energy homeostasis. Int J Tryptophan Res. 2020;13:1178646920938688.
21. Thingstad P, Askim T, Beyer MK, Brathen G, Ellekjaer H, Ihle-Hansen H, et al. The Norwegian COgnitive impairment After STroke (Nor-COAST) study: Study protocol of a multicentre, prospective cohort study. BMC Neurol. 2018;18(1):193.
22. Kuvas KR, Saltvedt I, Aam S, Thingstad P, Ellekjaer H, Askim T. The risk of selection bias in a clinical multi-center cohort study. Results from the Norwegian COgnitive impairment After STroke (Nor-COAST) Study. Clin Epidemiol. 2020;12:1327-36.
23. Pedersen ED, Waje-Andreassen U, Vedeler CA, Aamodt G, Mollnes TE. Systemic complement activation following human acute ischaemic stroke. Clin Exp Immunol. 2004;137(1):117-22.
24. Ulvik A, Midttun O, Pedersen ER, Eussen SJ, Nygard O, Ueland PM. Evidence for increased catabolism of vitamin B-6 during systemic inflammation. Am J Clin Nutr. 2014;100(1):250-5.
25. Midttun O, Hustad S, Ueland PM. Quantitative profiling of biomarkers related to B-vitamin status, tryptophan metabolism and inflammation in human plasma by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom. 2009;23(9):1371-9.
26. Norwegian Stroke Registry (Norsk Hjerneslagregister) - annual reports Norwegian Stroke Registry (Norsk hjerneslagregister); 2021 [Available from: https://stolav.no/fag-og-forskning/medisinske-kvalitetsregistre/norsk-hjerneslagregister/rapporter.
27. Pedersen AG, Ellingsen CL. Data quality in the Causes of Death Registry. Tidsskr Nor Laegeforen. 2015;135(8):768-70.
28. Fine JPG, R. J. A proportional hazards model for the subdistribution of a competing risk. Journal of the American Statistical Association 1999;94(443):14.
29. Austin PC, Fine JP. Practical recommendations for reporting Fine-Gray model analyses for competing risk data. Stat Med. 2017;36(27):4391-400.
30. Khanevski AN, Bjerkreim AT, Novotny V, Naess H, Thomassen L, Logallo N, et al. Recurrent ischemic stroke: Incidence, predictors, and impact on mortality. Acta Neurol Scand. 2019;140(1):3-8.
31. Mosarrezaii A, Amiri-Nikpour MR, Mehryar HR, Choobi Anzali B, Nourooz-Zadeh S, Babaei S, et al. Investigating the relationship between interleukin-6 serum levels and outcome in acute ischemic CVA. Brain Behav. 2020;10(8):e01668.
32. Gu Y, Gutierrez J, Meier IB, Guzman VA, Manly JJ, Schupf N, et al. Circulating inflammatory biomarkers are related to cerebrovascular disease in older adults. Neurol Neuroimmunol Neuroinflamm. 2019;6(1):e521.
33. Everett BM, Glynn RJ, MacFadyen JG, Ridker PM. Rosuvastatin in the prevention of stroke among men and women with elevated levels of C-reactive protein: Justification for the Use of statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER). Circulation. 2010;121(1):143-50.
34. Aulin J, Hijazi Z, Siegbahn A, Andersson U, Alexander JH, Connolly SJ, et al. Serial measurement of interleukin-6 and risk of mortality in anticoagulated patients with atrial fibrillation: Insights from ARISTOTLE and RE-LY trials. J Thromb Haemost. 2020;18(9):2287-95.
35. Ganz P, Amarenco P, Goldstein LB, Sillesen H, Bao W, Preston GM, et al. Association of osteopontin, neopterin, and myeloperoxidase with stroke risk in patients with prior stroke or transient ischemic attacks: Results of an analysis of 13 biomarkers from the stroke prevention by aggressive reduction in cholesterol levels trial. Stroke. 2017;48(12):3223-31.

36. Sugioka K, Naruko T, Hozumi T, Nakagawa M, Kitabayashi C, Ikura Y, et al. Elevated levels of neopterin are associated with carotid plaques with complex morphology in patients with stable angina pectoris. Atherosclerosis. 2010;208(2):524-30.
37. Rouhl RP, Damoiseaux JG, Lodder J, Theunissen RO, Knottnerus IL, Staals J, et al. Vascular inflammation in cerebral small vessel disease. Neurobiol Aging. 2012;33(8):1800-6.
38. Lewicka E, Dudzinska-Gehrmann J, Dabrowska-Kugacka A, Zagozdzon P, Lizewska A, Danilowicz-Szymanowicz L, et al. Neopterin and interleukin-6 as predictors of recurrent atrial fibrillation. Anatol J Cardiol. 2016;16(8):563-71.
39. Yu E, Ruiz-Canela M, Guasch-Ferre M, Zheng Y, Toledo E, Clish CB, et al. Increases in plasma tryptophan are inversely associated with incident cardiovascular disease in the prevencion con dieta mediterranea (PREDIMED) study. J Nutr. 2017;147(3):314-22.
40. Pedersen ER, Tuseth N, Eussen SJ, Ueland PM, Strand E, Svingen GF, et al. Associations of plasma kynurenines with risk of acute myocardial infarction in patients with stable angina pectoris. Arterioscler Thromb Vasc Biol. 2015;35(2):455-62.
41. Zapolski T, Kaminska A, Kocki T, Wysokinski A, Urbanska EM. Aortic stiffness: Is kynurenic acid a novel marker? Cross-sectional study in patients with persistent atrial fibrillation. PLoS One. 2020;15(7):e0236413.
42. Hijazi Z, Aulin J, Andersson U, Alexander JH, Gersh B, Granger CB, et al. Biomarkers of inflammation and risk of cardiovascular events in anticoagulated patients with atrial fibrillation. Heart. 2016;102(7):508-17.
43. Ulvik A, Pedersen ER, Svingen GF, McCann A, Midttun O, Nygard O, et al. Vitamin B-6 catabolism and long-term mortality risk in patients with coronary artery disease. Am J Clin Nutr. 2016;103(6):1417-25.
44. Badawy AA. Hypothesis kynurenic and quinolinic acids: The main players of the kynurenine pathway and opponents in inflammatory disease. Med Hypotheses. 2018;118:129-38.
45. Pingle SK, Tumane RG, Jawade AA. Neopterin: Biomarker of cell-mediated immunity and potent usage as biomarker in silicosis and other occupational diseases. Indian J Occup Environ Med. 2008;12(3):107-11.
46. Tardif JC, Kouz S, Waters DD, Bertrand OF, Diaz R, Maggioni AP, et al. Efficacy and safety of low-dose Colchicine after myocardial infarction. N Engl J Med. 2019;381(26):2497-505.
47. Arenillas JF, Alvarez-Sabin J, Molina CA, Chacon P, Fernandez-Cadenas I, Ribo M, et al. Progression of symptomatic intracranial large artery atherosclerosis is associated with a proinflammatory state and impaired fibrinolysis. Stroke. 2008;39(5):1456-63.
48. Noz MP, Ter Telgte A, Wiegertjes K, Joosten LAB, Netea MG, de Leeuw FE, et al. Trained immunity characteristics are associated with progressive cerebral small vessel disease. Stroke. 2018;49(12):2910-7.
49. Li Y, Tan W, Ye F, Wen S, Hu R, Cai X, et al. Inflammation as a risk factor for stroke in atrial fibrillation: Data from a microarray data analysis. J Int Med Res. 2020;48(5):300060520921671.
50. Packer M. Characterization, pathogenesis, and clinical implications of inflammation-related atrial myopathy as an important cause of atrial fibrillation. J Am Heart Assoc. 2020;9(7):e015343.
51. Ma Y, Pan Z, Fan D, Xu S, Pan F. The increased risk of atrial fibrillation in inflammatory arthritis: A systematic review and meta-analysis of cohort studies. Immunol Invest. 2021:1-13.
52. Tancin Lambert A, Kong XY, Ratajczak-Tretel B, Atar D, Russell D, Skjelland M, et al. Biomarkers associated with atrial fibrillation in patients with ischemic stroke: A pilot study from the NOR-FIB Study. Cerebrovasc Dis Extra. 2020;10(1):11-20.
53. Varmdal T, Ellekjaer H, Fjaertoft H, Indredavik B, Lydersen S, Bonaa KH. Inter-rater reliability of a national acute stroke register. BMC Res Notes. 2015;8:584.
54. Zeestraten EA, Lawrence AJ, Lambert C, Benjamin P, Brookes RL, Mackinnon AD, et al. Change in multimodal MRI markers predicts dementia risk in cerebral small vessel disease. Neurology. 2017;89(18):1869-76.

55. Sacco RL, Kasner SE, Broderick JP, Caplan LR, Connors JJ, Culebras A, et al. An updated definition of stroke for the 21st century: A statement for healthcare professionals from the American Heart Association/American Stroke Association. Stroke. 2013;44(7):2064-89.

Abbreviations

ATC: anatomical therapeutic chemical classification
ADL: Activities of daily living
BI: Barthel Index
CI=confidence interval
CRP: C-reactive protein
CSVD: cerebral small vessel disease
eGFR: estimated glomerular filtration rate
ECG: electro cardiogram
HR=hazard ratio
IL-6: interleukin-6
IL-10: interleukin-10
IQR=interquartile range
KA: kynurenic acid
LDL: Low density lipoprotein
mRS: Modified Rankin Scale
MRI: magnetic resonance imaging
NIHSS: National Institute of Stroke Scale
Nor-COAST: Norwegian Cognitive Impairment After Stroke Study
PAr-index: pyridoxal acid ratio index
PLP: pyridoxal-5-phosphate
PL: pyridoxal
PA: pyridoxal acid
REK: regional ethics committee
SD=standard deviation
TOAST= Trial of Org 10172 in Acute Stroke Treatment
WHO: World Health Organization

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Supplementary table 1: Adjusted competing event (death) regression of the associations between biomarkers and the outcomes ischemic stroke recurrence.

	Model 1			Model 2		
	SHR	CI	p	SHR	CI	p
CRP	0.83	(0.40, 1.71)	0.613	0.89	(0.43, 1.84)	0.745
IL-6	1.17	(0.67, 2.04)	0.578	1.09	(0.60, 2.00)	0.771
Neopterin	1.42	(0.82, 2.47)	0.215	1.36	(0.81, 2.29)	0.245
PAr-index	1.11	(0.59, 2.10)	0.748	1.20	(0.64, 2.23)	0.587
Kynurenic acid	0.81	(0.42, 1.58)	0.544	0.88	(0.41, 1.89)	0.737

Model 1: age, sex, prior cerebrovascular disease, modified Rankin scale at three months, creatinine.
Model 2: As model 1 + TOAST-classification at baseline (TOAST=Trial of Org 10172 in Acute Stroke Treatment)
For CRP n was 212, 8 and 13 for all, recurrent IS and death, respectively.
SHR=subdistribution hazard ratio, CI=confidence interval, CRP= C-reactive protein, IL-6=Interleukin 6, IL-10=Interleukin 10, PAr-index=4-pyridoxic acid:(pyridoxal+pyridoxal-5`-phosphate), KA=kynurenic acid.

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