

Effects of exercise in the postprandial and fasted state on glycemia and markers of inflammation in persons prone to or with diabetes

Doctoral thesis by Håvard Nygaard

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If you take 100 steps after each meal, you'll live to be 99 years old

Chinese folk saying

True?

Preface

The thesis presents research conducted at Department of Sport Science at Inland Norway University of Applied Sciences (former “Lillehammer University College”) and Institute of Health and Society at the University of Oslo.

There are numerous persons to acknowledge, from my parents and siblings, to all colleagues in the “scientific community” that I have asked for advice. There is however some that have been of special importance for this PhD project.

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Dear Tove, your sacrifice and love have been absolute crucial for me. You are my rock.

Olava and Vilma, you are my heart.

Lillehammer, March 2017

Håvard Nygaard

Abstract

The prevalence of hyperglycemia and diabetes is increasing worldwide. It is therefore crucial to develop simple and effective strategies for diabetes prevention and to clarify related physiological mechanisms. The purpose of this thesis was to test if one bout of exercise performed in either the postprandial or the fasted state acutely affects glucose values and markers of inflammation, and to test whether regular postprandial exercise can improve glycemia in persons prone to or with diabetes.

To answer the research questions, we performed two studies. In Study 1, we assessed the acute response on interstitial glucose values and markers of inflammation of exercise performed either before (fasted) or after breakfast (postprandial), utilizing a randomized crossover design. Interstitial glucose was measured continuously until the next morning, while the markers of inflammation; sVCAM-1 and CRP were assessed during the first 3.5 hours after breakfast and once next morning. Study 2 was a randomized controlled study, encompassing an intervention group undertaking regular postprandial exercise during 12 weeks, and measures of HbA1c, fasted blood glucose, and glucose tolerance.

The results from Study 1 showed that postprandial exercise decreased peak glucose values and glycemic variability, while this was not the case for fasted exercise. Merged data from both exercise interventions showed that exercise per se increased sVCAM-1, with no substantial difference between the two types of exercise. CRP remained unchanged. In Study 2, none of the measured glycemic variables seemed to change as a result of regular postprandial exercise. Thus, postprandial exercise improves glycemia acutely, but apparently not in the long term.

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List of papers

- I. Nygaard H, Rønnestad BR, Hammarström D, Holmboe-Ottesen G, Høstmark AT. Effects of exercise in the fasted and postprandial state on interstitial glucose in hyperglycemic individuals. Accepted for publication in Journal of Sports Science and Medicine.
- II. Nygaard H, Falch GS, Whist JE, Hollan I, Ellefsen S, Holmboe-Ottesen G, Rønnestad BR, Høstmark AT. Acute effects of post-absorptive and postprandial moderate exercise on markers of inflammation in hyperglycemic individuals. European Journal of Applied Physiology. 2017. DOI: 10.1007/s00421-017-3576-2
- III. Nygaard H, Grindaker E, Rønnestad BR, Holmboe-Ottesen G, Høstmark AT. Long Term Effects of Daily Postprandial Physical Activity on Blood Glucose: A Randomized Controlled Trial. Applied Physiology, Nutrition, and Metabolism. 2017. DOI: 10.1139/apnm-2016-0467

Abbreviations

BrEx	Test day with exercise after breakfast
CON	Control day (paper I and II) and Control group (paper III)
CONGA2	Standard deviation of the differences between each glucose reading and the glucose reading 2 hours later
CONGA4	Standard deviation of the differences between each glucose reading and the glucose reading 4 hours later
CRP	C-reactive protein
ExBr	Test day with exercise before breakfast
HbA1c	Glycosylated hemoglobin
HDL	High density lipoprotein
INT	Intervention group
IQR	Interquartile range
LDL	Low density lipoprotein
RPE	Rating of perceived exertion
SD	Standard deviation
sVCAM-1	Soluble vascular cell adhesion molecule 1

Definitions

Fasted	After an overnight fast
Glycemia	The level of blood glucose. In the present thesis, glycemia refers to measures of the level of HbA1c, fasted glucose, 2-hour glucose, or to measures of interstitial glucose assessed by continuous glucose monitoring (that mirrors blood glucose).
Postprandial	Refers to the time after a meal when nutrients is absorbed from the intestine to the blood. We aimed to perform the postprandial exercise bouts in the early phase of the postprandial period, before peak blood glucose concentration.
Prone to diabetes	Persons with high risk of type 2 diabetes. Refers to both persons with hyperglycemia below the defined threshold for diabetes (paper I and II) and persons without known hyperglycemia defined as high risk individuals for type 2 diabetes due to Ramachandran's risk score for Asian Indians (91) (paper III)

Background

Glucose regulation

Human glucose regulation is a complex interplay between organs, tissue, energy intake, and energy consumption. Maintenance of normal blood glucose levels is crucial; on one hand, hypoglycemia can lead to neural tissue malfunction and even death (1), on the other hand, hyperglycemia can lead to serious health problems over time (2-5). Blood glucose is therefore a small but important part of the human carbohydrate stores.

The largest carbohydrate stores are in the liver and in the muscles. While muscle glycogen is metabolized in muscle only, glycogen stores in the liver serves as a ready supply of glucose for tissue via the blood stream (6). In the postprandial phase, after carbohydrate intake, glucose is absorbed from the intestine to the blood, leading to an increase of blood glucose. While glucose absorption from the intestine is undertaken by glucose transporters, the passage of glucose between the capillaries and the interstitial fluid mainly occurs by simple diffusion in most tissues (7). Consequently, there is equilibrium between plasma and interstitial glucose. However, changes in the level of plasma or interstitial glucose will cause a shift in this balance. The length of time before equilibrium is re-established will depend on a number of factors, such as insulin level, types of metabolic condition causing the fluctuations, and types of tissue. That being said, most studies put the lag time in the range of 5 – 15 minutes (8).

Some of the glucose is taken up from the blood by non-insulin-dependent tissue (or at least “less-insulin-dependent” tissue) like the brain (9, 10), while some is taken up in an insulin dependent manner by the liver and by muscle and fat. The beta-cells in the pancreas are sensitive to glucose and increases insulin secretion in accordance with the rise in blood glucose level. The postprandial increase in blood glucose is therefore accompanied with a concomitant increase in insulin level, which in turn decreases blood glucose level (11). In a fasting condition, the blood glucose level is maintained by glycogenolysis and gluconeogenesis, which mainly takes place in the liver, but also in the kidneys (11, 12). The endogenous glucose production during fasting is promoted by glucagon secretion and low insulin levels (6, 11). Catecholamines, cortisol, growth hormone, thyroid hormones,

angiotensin II and the sympathetic nerve system can also increase hepatic glucose output (6, 11).

The cellular uptake of glucose depends on specific glucose transporters, of which several isoforms have been identified. GLUT-4 is the main glucose transporter in skeletal muscle. Glucose transport is probably the rate-limiting step for muscle glucose uptake during rest. During exercise however, GLUT-4 is translocated independent of insulin to the sarcolemma as well as to the T-tubular system, leading to an effective increase of the transport capacity (13). Exercise intensity and duration is the primary determinants of GLUT-4 translocation in working muscles (13). The molecular mechanisms behind contraction induced translocation of GLUT-4 involves altered energy substrate balance and calcium release in the myocytes (14), and these mechanisms are maintained in insulin resistance (14). In addition to translocation of GLUT-4 during exercise, the expression of GLUT-4 increases both acutely after exercise and chronically as an adaptation to training (13). The delivery of glucose to contracting muscles is also increased by enlarged blood flow. Furthermore, an increased hepatic glucose output during exercise can compensate for the muscle uptake of glucose, thus maintaining the diffusion gradient of glucose between the blood and the tissue (13).

Insulin resistance is a hallmark of type 2 diabetes mellitus. Insulin resistance can be detected in persons who will develop type 2 diabetes before blood glucose levels exceeds the normal range. In this early phase of diabetes development, the insulin secretion from pancreas is augmented to compensate for the loss of insulin sensitivity (15). In persons that develop hyperglycemia, the beta cells begin to fail and glucose level increases over time. The severity and progression of hyperglycemia depend on degree of deterioration in the beta cells' ability to compensate for the insulin resistance (15). Loss of beta cell function can be caused by hyperglycemia per se, however, the mechanisms behind insulin resistance and loss of beta cell function is complex and often involve several organ systems (12).

Hyperglycemia and its comorbid conditions

According to the International Diabetes Federation (16), the global prevalence of diabetes in 2015 was about 8.8%. This number is expected to increase to 10.4% by 2040. In addition, many people have hyperglycemia below the definition of diabetes. The prevalence of impaired glucose tolerance was 6.7% in 2015 and is expected to increase to about 7.8% by

2040. The majority of those cases is caused by insulin resistance, which can be prevented by a healthy lifestyle, including exercise (16). It is therefore of importance to find lifestyle strategies for prevention and treatment of hyperglycemia that can be implemented in daily life of most people. Moreover, it is in general important to explore the effects of exercise on blood glucose and other health related variables.

Several studies have demonstrated a strong relationship between the magnitude of glycemia and the risk of cardiovascular diseases (2-4). Especially the magnitude of postprandial glycemia, which is the blood glucose level in the absorptive phase, shows a strong relation to these diseases and ensuing deaths (3, 4, 17-26). The largest risk for cardiovascular disease is observed in persons with postprandial blood glucose values characterized as diabetes or impaired glucose tolerance. However, this association shows no threshold level and starts well below the area of hyperglycemia (3, 17, 27). An elevated risk is therefore present in the upper areas of "normal blood glucose". Postprandial hyperglycemia is also associated with microvascular complications, cancer and impaired cognitive function in elderly people (5). In addition to the glucose levels per se, the variability of the glucose levels is associated with risk of cardiovascular disease (28-30). There are for example indications that large fluctuations in blood glucose are more deleterious to endothelial cells than a constant high glucose level (31).

Several studies have pointed out oxidative stress as a plausible mechanism linking elevated blood glucose to cardiovascular disease. Acute hyperglycemia entails an overproduction of reactive oxygen (32-37) and nitrogen species (38). These radicals in turn, mediate atherosclerosis by several mechanisms, including an activation of the innate immune system (39). For example, glucose mediated oxidative stress may increase the levels of pro-inflammatory cytokines (32, 33) and adhesion molecules (33, 34). Oxidative stress during hyperglycemia also increases the production of advanced glycated end products (38), and have the potential of decreasing endothelial function and vasodilation (38, 40). Experimental studies have shown that intermittent high glucose levels may induce more oxidative stress than constant high glucose levels (31, 35, 37).

It is generally accepted that hyperglycemia induced systemic inflammation is involved in both micro- and macrovascular complications (41), and atherosclerosis can be characterized as a chronic low-grade inflammatory condition (39). Systemic inflammation may also be involved in the progression of mild hyperglycemia into type 2 diabetes (12, 41). Therefore, systemic

low-grade inflammation appears to be a key factor in the vicious circle of hyperglycemia, cardiovascular disease and other comorbid conditions of diabetes. Numerous markers of inflammation have been extensively studied, including C-reactive protein (CRP) and soluble vascular cell adhesion molecule (sVCAM-1) (39, 41). CRP is an acute phase protein synthesized in response to homeostatic disturbances (42). Besides being a crucial part of the innate immune system, it predicts disease and death. The concentration of CRP measured with a high sensitivity assay exhibits a continuous association with the risk of cardiovascular disease and death from several cancers and lung diseases (43). CRP may provide an overall readout of inflammatory status (44) and has been proposed as a marker of cardiovascular risk for clinical use (41). VCAM-1 is crucial for leucocyte migration into tissue during inflammation and atherosclerosis, by adhering the leucocytes firmly to the endothelial cell membranes (45). A proportion of the membrane expressed VCAM-1 is cleaved from the endothelial cells after cytokine activation and can be measured in plasma as sVCAM-1 (46). The plasma concentration of sVCAM-1 have predictive value for cardiovascular disease in hyperglycemic persons (41).

The effect of exercise on hyperglycemia

Exercise is known as a cornerstone in prevention and treatment of hyperglycemia. The lowering effect of endurance and strength training exercise on blood glucose is explored in numerous studies (47-49). Basically, the effect of exercise on blood glucose is a result of increased energy expenditure, and the oxidation of glucose is multiplied several times even during low intensity exercise (50). The improvement in glycemia over time due to regular exercise are related to several physiological mechanisms, like increased muscular uptake of glucose via non-insulin-dependent pathways (51), improved insulin receptor function (52), increased concentration of GLUT-4, increased capillary density, changed fiber type composition (53), decreased systemic inflammation (54), gain in muscle mass, increased synthesis of proteins in the insulin signaling pathways, mobilization of abdominal fat, increased synthesis of glycolytic enzymes (55), increase in mitochondrial volume, increase in the amount of aerobic enzymes (56), and a decrease of intramyocellular lipids (57, 58).

The timing between exercise and food intake

A number of studies have aimed to reduce postprandial glycemia acutely by exercise. Both the effect of one bout with strength exercise (59) as well as endurance exercise (60-66) prior to a meal have been investigated. However, the largest acute decreases in postprandial glycemia is predominantly found when exercise is performed after carbohydrate intake, when glucose is increased above fasting values (64, 67-79). This effect is also apparent after very light or small amounts of postprandial exercise (73-76, 78-80). An example of typical blood glucose responses to postprandial exercise is given in figure 1. Some studies have compared the effect of exercise both in the fasted and the postprandial state in persons with metabolic syndrome and diabetes. The results indicates that postprandial exercise decreases postprandial glycemia, while exercise in the fasted state does not (64, 81, 82). An example from a study comparing exercise in the fasted and postprandial state is given in figure 2. These studies focused on the acute glycemic effect of exercise in the first postprandial period after a meal (64) or within the first 10 hours thereafter (81, 82).

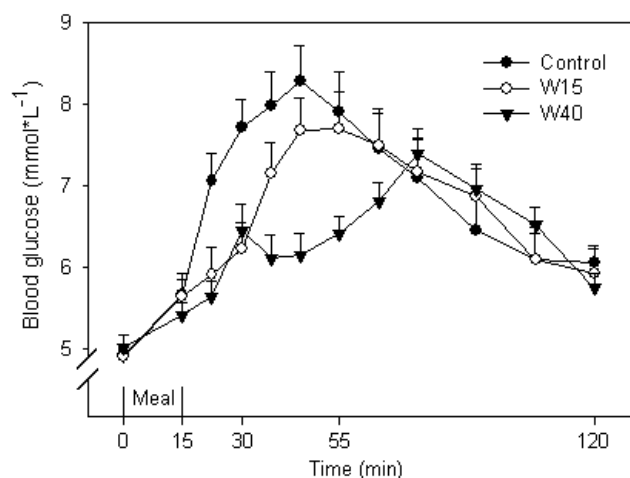


Figure 1. Blood glucose responses to postprandial light exercise in normoglycemic subjects. The figure shows blood glucose responses to a carbohydrate rich meal during a control condition without exercise (Control), 15 minutes (W15), and 40 minutes (W40) of slow walking immediately after the meal. The incremental area under the blood glucose curves differed significantly between W40 and control ($p=0.014$). Participants were healthy postmenopausal women. Reprinted from Nygaard, H., Tomten, S. E., & Hostmark, A. T. (2009). Slow postmeal walking reduces postprandial glycemia in middle-aged women. *Appl Physiol Nutr Metab*, 34(6), 1087-1092, with permission from NRC research press.

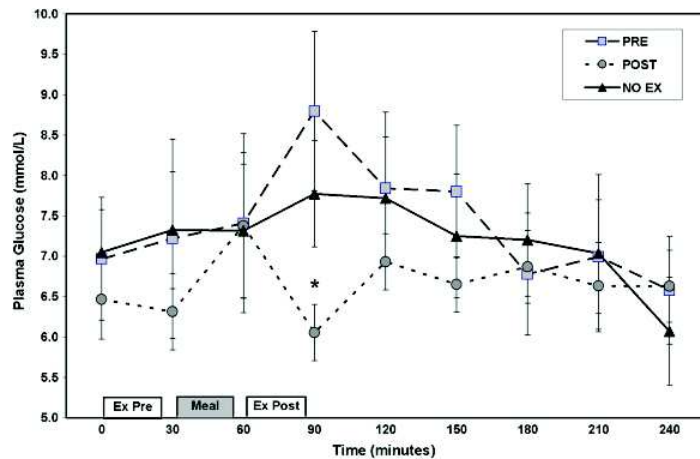


Figure 2. Blood glucose responses to exercise in the fasted versus the postprandial state in type 2 diabetic persons. The figure shows blood glucose responses during a condition with 20 minutes of walking before (PRE) or after a dinner meal (POST) and a control condition (NO EX) without exercise. Blood glucose values differed significantly ($p < 0.05$, indicated by *) between PRE and POST and between NO EX and POST 90 min into the experiment. Reprinted from Colberg, S. R., Grieco, C. R., & Somma, C. T. (2014). Exercise effects on postprandial glycemia, mood, and sympathovagal balance in type 2 diabetes. *J Am Med Dir Assoc*, 15(4), 261-266, with permission from Elsevier.

The effect of exercise on markers of inflammation

Exercise affects inflammatory status. Whereas long-term regular exercise reduces CRP levels in diabetic persons (83), one bout of strenuous exercise, may entail the opposite acute effect (42). Less is known about the effect of moderate exercise on markers of inflammation, and exercise may affect markers of inflammation in an intensity-dependent manner, since metabolic activity in general is linked to the level of oxidative stress (84).

Also the nutritional status might affect the inflammatory response of exercise. Possibly, the inflammatory response may be altered via the effect of exercise on postprandial glycemia, providing a link between high glycemic excursions and inflammation (32-34). For example, hyperglycemia after a carbohydrate rich meal has the ability to increase sVCAM-1 acutely in diabetic individuals (33), but this glycemic excursion may be blunted if exercise is performed after the meal (64, 67-79). In addition, during exercise the nutritional status might influence cortisol secretion that is known to depress several parts of the immune system. Glycogen depleting exercise activates the hypothalamic-pituitary-adrenal axis and secretion of ACTH and cortisol into the circulation for maintenance of blood glucose levels. Carbohydrate ingestion in connection with exercise therefore inhibit the secretion of cortisol (85).

Aims of the thesis

A priori, physical activity, whether in the postprandial or the fasted state, may not have any effect on blood glucose concentrations, or it could reduce or even augment glycemia. Based on above-mentioned studies it seems like one bout of light or moderate postprandial, but not fasted exercise entails an intermittent blunting effect on postprandial glycemia. Notably, the sampling of blood glucose levels was limited to the first 4 – 10 hours after exercise and glycemic variability was not measured in the studies directly comparing fasted and postprandial exercise (64, 81, 82). Lately, devices for continuous interstitial glucose monitoring have become commercially available, making it feasible to monitor glucose for longer durations and to calculate glycemic variability. Systemic inflammation is related to glycemia, food intake and exercise and may thus also be affected by exercise in the postprandial or the fasted state. However, practical guidelines for the timing between exercise and food intake cannot be based on acute studies only. Consequently, long-term studies with clinical outcomes in this field are needed.

The aim of this thesis was to examine acute and long-term effects of exercise according to the timing of food intake. Specifically, we examined the acute effects of moderate exercise, performed in either the postprandial or the fasted state on interstitial glucose (paper I) and on markers of inflammation (paper II). Finally, we aimed to examine the long-term effect of regular postprandial exercise on glycemia (paper III).

We point out that the present work does not aim at exploring detailed physiological mechanisms, but will rather focus on whether the present exercise interventions influences the selected measurements.

Hypotheses:

In persons prone to or with type 2 diabetes;

1. Moderate exercise performed in the postprandial or the fasted state influences the level, and / or the variability of interstitial glucose over 22 hours compared to no exercise.
2. Moderate exercise performed in the postprandial state influences the level, and / or variability of interstitial glucose over 22 hours differently from exercise performed in the fasted state.
3. Moderate exercise has an acute effect on CRP and sVCAM-1 levels.
4. The acute effects of moderate exercise on CRP and sVCAM-1 levels differ depending on whether the exercise is performed in the postprandial or the fasted state.
5. Performing light postprandial exercise on a regular basis over the course of 12 weeks affects glycemia.

Methods

Overview of the designs

To test the hypotheses of the present thesis, we performed two studies. One study had a randomized crossover design, assessing the acute response on interstitial glucose values and markers of inflammation. The other study had a randomized controlled design, and the participants in the intervention group performed regular postprandial exercise during 12 weeks.

Study 1

Acute effects of exercise in the postprandial and fasted state on glucose and markers of inflammation (paper I and II)

Participants

The inclusion were restricted to individuals diagnosed with hyperglycemia, i.e. previously measured fasting venous plasma glucose ≥ 6.1 mmol/L and/or 2 hour glucose tolerance ≥ 7.8 mmol/L, and who did not use glucose-lowering medications. Autoimmune disease, cancer, or other diseases directly and / or significantly affecting inflammatory status or metabolism, except for diabetes, were set as exclusion criteria. Sample size calculations were performed for the markers of inflammation, showing that 8 participants would be enough to detect a significant difference between test days with standard deviation (SD) equal to the mean difference, $\alpha=0.05$ and power 80%. We also did calculations for expected change in glucose concentrations during the first two hours after breakfast, and concluded that a lower number of participants would be sufficient. Taking into account the uncertainty in the calculation for inflammatory markers we included 13 participants, of which one was excluded from the data set because further examination of her patient journal after enrollment showed that she did not meet the inclusion criteria. Inclusion was based on self-reported hyperglycemia. Four women and eight men completed the study and are included in the results. Four of the participants were diagnosed with type 2 diabetes, and all were of European descent. Their characteristics

are summarized in Table 1. Some of the participants used lipid-lowering therapy (n=4), antithrombotic agents (n=4), angiotensin II receptor antagonists (n=2) and ACE inhibitors (n=1). The dosage and timing of intake of these drugs were kept stable during the study period. Median time from diagnosis of hyperglycemia to participation in the study was $8.5 \pm$ interquartile range (IQR) 31 months. Seven of the participants had at least one parent or one sibling with diabetes. All women were postmenopausal. The participants reported that they had performed 219 ± 237 minutes of endurance or strength training, 177 ± 143 minutes of walking and 122 ± 49 minutes of lighter activity (like gardening and housework) per week for the last three months prior to study enrollment. To obtain a general impression of the dietary habits of the participants prior to the study, they answered a questionnaire. When asked about which type of bread they ate, eleven answered wholegrain bread, none answered semi-wholegrain or non-wholegrain bread and one did not eat bread. When asked about how many times per week they ate a selection of specified types of food, answers were as follows: sugar-containing jam or chocolate spread, 0 ± 0 ; sugar-rich beverages, 0.04 ± 0.01 ; candy / chocolate / snacks, 1.4 ± 1.2 ; boiled food, 3.5 ± 1.6 ; pan fried food, 2.7 ± 1.4 ; deep-fried food, 0.1 ± 0.3 ; fruit and vegetables, 16.6 ± 9.0 .

Table 1. Characteristics of the participants. Mean \pm SD.

n	12
Age, yr	65 \pm 8
Body weight, kg	73.3 \pm 9.7
Height, m	1.73 \pm 0.08
Body mass index, kg/m ²	24.5 \pm 1.9
HbA1c, %	6.1 \pm 0.6
Diagnosed with diabetes, n	4
Total cholesterol, mmol/L	5.1 \pm 1.3
HDL cholesterol, mmol/L	1.4 \pm 0.6
LDL cholesterol, mmol/L	3.0 \pm 1.0
Systolic blood pressure, mmHg	133 \pm 18
Diastolic blood pressure, mmHg	74 \pm 6

Ethics statement

The Regional Ethics Committee (REK Sør-Øst, Norway) approved the study, and all participants gave their written informed consent prior to enrollment.

General design

The study was performed using a randomized crossover design. Each subject undertook three test days (Figure 3) in a balanced order, separated by at least six days and no more than 21 days: one test day with a bout of exercise performed in the fasted state before breakfast (ExBr), one test day with identical exercise in the postprandial state after breakfast (BrEx), and one test day without physical exercise (CON). All experimental days were identical, except for the different exercise regimes or the lack thereof. Participants were sedentary on test days except for the exercise sessions, and all meals were standardized. All test days started in the morning, and the breakfast was approximately at the same time for each participant (within one hour). Continuous glucose measurement was performed until at least 22 hours after breakfast, and blood samples were taken at standardized time points in the postprandial period after breakfast (until 3.5 hours after start of breakfast) and once after 24 hours (Figure 3).

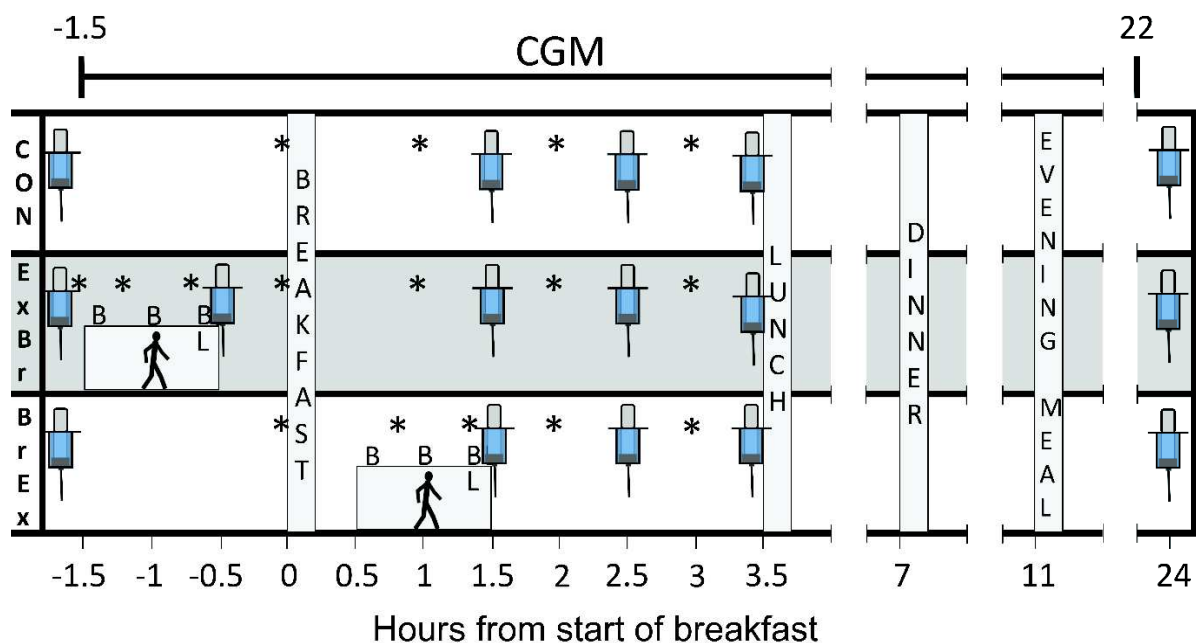


Figure 3. Overview of the test protocol. A control day (CON) in the upper row, a day with exercise in the fasted state (ExBr) in the middle row and a day with exercise in the postprandial state (BrEx) in the bottom row. CGM= continuous glucose monitoring, was performed until 22 hours after breakfast in each of the three test days. Frame with walking man indicates when the exercise bouts (60 min moderate treadmill walking) were performed. Syringe = blood sample. * = Measure of heart rate, oxygen consumption and respiratory exchange ratio (RER) values. B = Measure of perceived exertion (Borg scale). L = measure of blood lactate.

Exercise and food intake

All participants remained in the laboratory facility until the lunch meal was ingested, whereupon the participants were transported home, and stayed home for the remaining time of the 22 hours glucose-monitoring period. They had a checklist with all details concerning the standardization of the experiments, including instructions about timing and amount of food intake. Prior to the first test day, each participant was given a detailed lesson about the importance of experimental standardization, and all procedures necessary for exact standardization. At the end of each test day, we asked the participants to comment on the standardization, and no divergences were reported.

The participants were instructed to avoid exercise during the last three days prior to each test day. Any light activity performed on these days prior to the first test day, were recorded and repeated in the same manner before the second and third test day. The exercise sessions in both ExBr and BrEx consisted of 60 minutes of treadmill walking at an individually standardized speed at 8% inclination. The individual speed was decided during a familiarization session >6 days before the first test, and defined as the speed corresponding to 12 at the Borg 6-20 RPE scale (86) after 30 minutes of walking at 8% inclination. At the end of exercise at test days the rate of perceived exertion for ExBr and BrEx, respectively, was 12.0 ± 0.2 and 12.4 ± 0.2 ($p=0.021$) on Borg scale, and corresponding blood lactate levels were 1.4 ± 0.2 and 1.7 ± 0.2 mmol/L ($p=0.038$). All other physical activity was limited to what was absolute necessary, like walking to the car and moving between living room, toilet, kitchen and bedroom.

Use of antioxidants or anti-inflammatory agents was not allowed during the last month leading up to study participation. We instructed the participants to standardize their diet three days prior to each test by writing down food intake in the days leading up to the first test day and repeating this regimen before the second and third test day. An absolute dietary standardization was performed from the evening 10 hours before breakfast on each test day until 22 hours after the breakfast. Macronutrient intakes from this period are given in Table 2. Standardization was achieved by repeating the diet eaten on the first test day on the second and third test day. A digital kitchen scale and a diary were used to carefully register each meal the first test day and repeat it on the second and third test day. The participants were instructed to eat and register a self-chosen meal containing >30 g carbohydrate in the evening 10 hours preceding breakfast on test days. For breakfast on test days, the participants ingested 250 mL semi-skimmed milk and cornflakes corresponding to 1g carbohydrate per kg body

weight. Lunch (3.5 hours after start of breakfast) contained a yogurt and self-chosen amounts of wholegrain crispbread, butter, cheese and water. For dinner (7 hours after start of breakfast), the participants could choose between several boil-in-bag dinner packages (salmon with rice and vegetables, chicken casserole, meatballs potatoes and creamed peas or minced steak with stewed cabbage and potatoes; Fjordland AS, Norway). Leftovers were registered on test day one, and the corresponding amount of food was removed on the second and the third test day. The evening meal (11 hours after start of breakfast) consisted of a self-chosen amount of whole meal bread, butter, cheese and skimmed milk that also was carefully registered and repeated. The research team provided the subjects with all food on test days (not including the evening meal prior to test day).

Table 2. Macronutrient intake before and during test days. Mean \pm SD

	KJ	Protein, g	Fat, g	Carbohydrate, g
Evening meal, prior to test day	1371 \pm 966	12 \pm 6	10 \pm 4	43 \pm 27
Breakfast	1589 \pm 200	13 \pm 2	3 \pm 1	74 \pm 9
Lunch	2101 \pm 823	28 \pm 14	19 \pm 10	52 \pm 19
Dinner	2535 \pm 813	37 \pm 17	27 \pm 10	51 \pm 17
Evening meal	2634 \pm 978	30 \pm 8	29 \pm 12	59 \pm 33
Total test day (from breakfast)	8859 \pm 1884	108 \pm 33	77 \pm 22	235 \pm 47

Data sampling

Continuous glucose monitoring

Interstitial glucose was monitored by a continuous glucose monitoring system consisting of a sensor which measures interstitial glucose via a subcutaneous microdialysis fiber (Enlite Glucose Sensor MMT-7008A, Medtronic MiniMed, Northridge, USA) and a recorder (iPro2 MMT-7745WW, Medtronic MiniMed, Northridge, USA) attached to the sensor. The recorder stores mean glucose values every 5th minute. We attached the sensor and the recorder, which together weighs about 10 g, to the skin at the anterior abdominal area lateral to the periumbilical region. The participants were given a glucose meter (Contour XT, Bayer Consumer Care AG, Basel, Switzerland) and were instructed in capillary blood sampling, for determination of capillary blood glucose at baseline, and before the meals at 3.5, 11 and 22 hours after breakfast. Determination of capillary blood glucose was done in triplicate at each

time point. We downloaded data from the continuous glucose recorder to the accompanying software and calibrated the data by the capillary blood glucose recordings, before exporting data into Microsoft Excel.

In order to test the validity of continuous glucose monitoring system as an indicator of blood glucose we used venous blood sampled 2.5 hours after breakfast. Mean plasma venous blood glucose for all 2.5 hours samples was 7.3 ± 2.3 mmol/L, while corresponding continuous glucose monitoring mean value was 7.2 ± 2.0 mmol/L. Intraclass correlation was $r=0.95$, mean difference 0.7 mmol/L and coefficient of variation between continuous glucose monitoring and venous blood samples was 7.3%. However, fluctuations in glucose entails a delay between blood glucose and interstitial glucose (8) that may add inaccuracy to this validation.

Venous blood samples

Blood samples were drawn from an antecubital vein at baseline and thereafter 1.5, 2.5, 3.5 and 24 hours after breakfast. In addition, blood was sampled at the end of the ExBr exercise bout (Figure 3). Blood was drawn into EDTA tubes and centrifuged immediately at 2600 g for 12 minutes before freezing. The plasma samples were thawed and frozen again prior to analysis. However, the results from the plasma samples that had undergone this procedure were found to be reliable, see Appendix 1. Glucose and triglycerides were analyzed at Furst Medical Laboratories, Oslo, (Advia 2400 Chemistry System, Siemens Healthcare Diagnostics Inc). High-sensitivity CRP was determined by a solid-phase, chemiluminescent immunometric assay (Immulite 2000, Diagnostic Products Corporation, USA). In 35 out of 192 samples, CRP levels were below the minimum range of the assay, i.e. 0.2 mg/L. These samples were taken from 4 different persons (6, 5, 16 and 8 samples from each person, respectively), and were set at the minimum range of the assay; 0.2 mg/L. We analyzed sVCAM-1 with commercially available ELISA kits (Human sVCAM-1/CD106 immunoassay Quantikine ELISA, R&D systems Inc, Minneapolis, USA). All analyses of CRP and sVCAM-1 from any particular subject were analyzed intra-assay. Intra-assay coefficients of variation were 7% for CRP and 1% for sVCAM-1.

Heart rate, metabolism and perceived exertion

Data on oxygen consumption, respiratory exchange ratio (Oxycon Pro, Erich Jaeger, Hoechberg, Germany) and heart rate were retrieved for 10 minutes at several standardized time points until 3 hours after breakfast (Figure 3). Mean values from the last 2 min of

sampling were used in the analyses. Blood lactate concentration (Biosen C-line, EKF-diagnostic GmbH, Germany) was measured 55 minutes into each bout of exercise and Borg 6-20 RPE (86) was reported 5, 30 and 55 minutes into the exercise bouts.

Calculations and statistical analysis

Expenditure of energy and carbohydrate and fat metabolism were calculated from VO_2 and RER values using a table given in McArdle, Katch and Katch textbook of exercise physiology (page 188) (87). We did the statistical analysis with IBM SPSS statistics, version 22.0, using a linear mixed model with participant number as the repeated “subjects” variable. Random intercept was included in the models. We checked the residuals for normality and homogeneity. The α -level was set at 0.05 and a p value <0.1 was considered as a tendency towards statistical significance.

Test day was used as a fixed factor in the analysis of the data from the continuous glucose monitoring, and due to Bonferroni correction, the α -level was set at ≤ 0.017 , and $p < 0.034$ was considered a tendency towards statistical significance in pairwise comparisons between test days. Because measures were calibrated against blood glucose values from finger sticks, taken four times per day, the numerous continuous values are not independent of each other. We were not able to control for this dependency in the model. Therefore, we utilized mean values instead of all values in the analysis. As an indicator of the severity of the glycemic excursions we calculated mean of the 10 highest glucose values on each test day, independent of when those values were observed, and we assessed glucose variability by calculation of continuous overall net glycemic action (CONGA) (88). CONGA is the SD of the differences between each glucose reading and the glucose reading n hours later. We used $n=2$ hours (CONGA2) and $n=4$ hours (CONGA4).

Test day and time (baseline vs. subsequent measure) were used as fixed factors in the analysis of data from the venous blood samples, and effect of intervention was defined as an interaction between test-day and time. Absolute values were utilized in the analysis. To assess the effect of exercise on the markers of inflammation, independent of timing between exercise and food intake, we used merged data from both exercise interventions.

We calculated effect sizes (ES) for the effect of intervention and between interventions by using Cohen's d_z (89). ES were interpreted according to Hopkins et al. (90): $d \geq 0.2$ =small effect, $d \geq 0.6$ =moderate effect, $d \geq 1.2$ =large effect. Data are presented as means \pm SD.

Study 2

Long-term effects of regular postprandial exercise on glycemia (paper III)

Participants and recruitment process

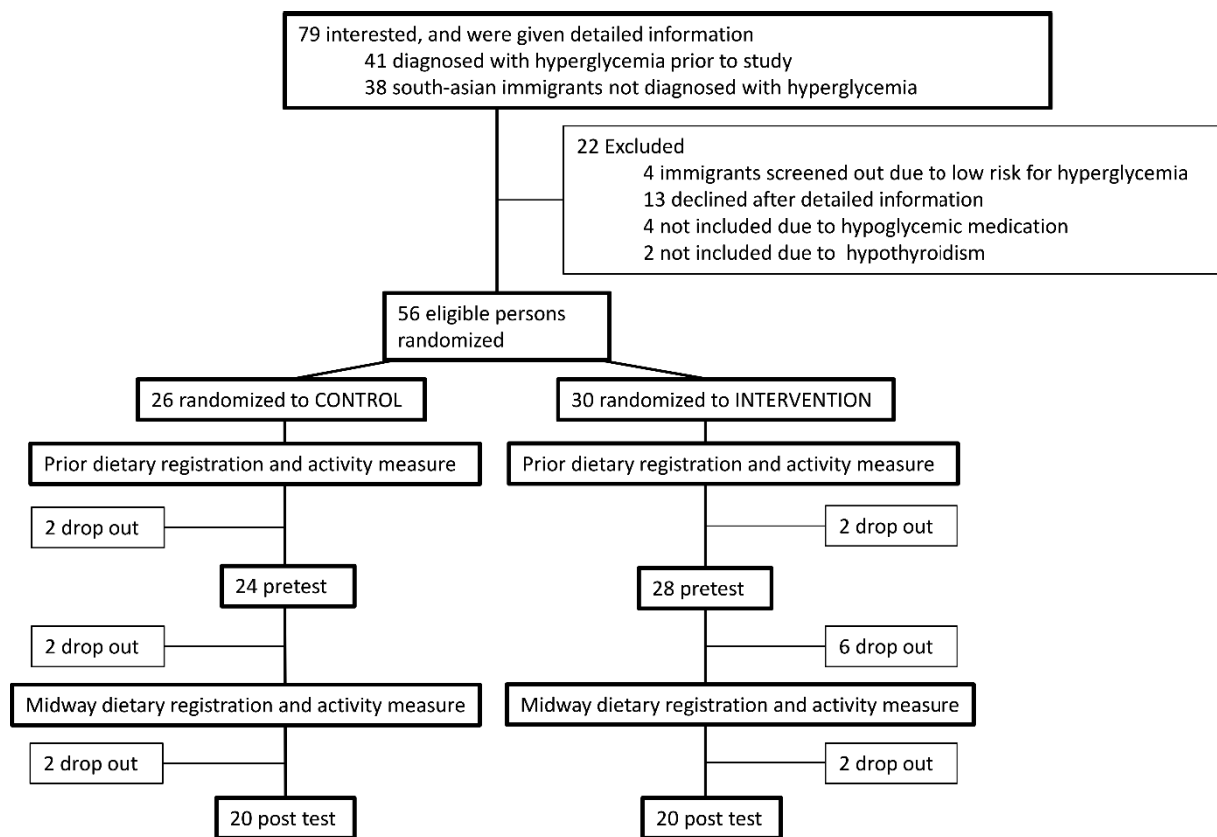


Figure 4. Participant flow chart

We recruited the participants from Lillehammer, Oslo and its surroundings, in Norway by advertising in media, by posters, and by presentations in local diabetes associations, mosques,

temples and community events. Recruitment started in May 2010 and data collection was completed in June 2014. We included hyperglycemic persons (independent of origin) treated with lifestyle change only, and south Asian immigrants with high risk of type 2 diabetes according to Ramachandran's risk score for Asian Indians (91). Hyperglycemia was defined as previously measured fasting venous plasma glucose ≥ 6.1 mmol/L and / or 2 hour glucose tolerance ≥ 7.8 mmol/L, and a cut off >21 was used for the risk-score according to Ramachandran's recommendations (91). Originally, only hyperglycemic persons were included, but due to a low number of participants recruited (in 2010 and 2011), we changed eligibility to also include Asian immigrants with high risk of type 2 diabetes (from 2012). We considered medication or illnesses directly affecting glycemia (other than hyperglycemia per se) as exclusion criteria. We chose HbA1c, fasting glucose and 2 hour glucose as primary outcome measures. In sample size calculations we considered a 5% improvement in the primary outcomes; HbA1c or glucose as clinical relevant, and we expected a SD of 5% on the change. Accordingly, 20 participants in each group would be enough to detect a clinical relevant improvement with a two-sided test, $\alpha=0.05$ and power=80%. Actual SD on change from pre- to post-test was 5, 18 and 27% for HbA1c, fasted and 2 hour glucose respectively. Interested persons were given detailed written information. A total of 56 persons were randomized to a control group (CON) or an intervention group (INT), of which 16 dropped out after inclusion (Figure 4). Randomization was done in accordance with the random allocation rule (92). To ensure equal group sizes of $n=20$, the lot from a participant that dropped-out were replaced in the lottery. To limit interchange of information from INT to CON, family members or close friends that enrolled at the same time were randomized to the same group. Enrollment and randomization was done by HN. Four persons dropped out between randomization and pre-test (Figure 4), 12 persons dropped out during the intervention period, whereas 40 persons completed the post-tests with 20 persons in each group. Table 3 shows the baseline characteristics of the participants that completed the study.

Table 3. Baseline characteristics of the participants that completed the long-term study

	CON	INT
Participants, n	20	20
Previously diagnosed with hyperglycemia, n	10	13
of which diagnosed with diabetes, n	5	5
Time since diagnosis of hyperglycemia, months	26 ± 27	35 ± 39
Included by Ramachandran's risk score, n	10	7
Asian origin, n	11	12
European origin, n	9	8
Female, n	7	6
Age, years	46 ± 8	53 ± 9
Postmenopausal women, n	5	5
Height, cm	172 ± 10	168 ± 10
Body weight, kg	80.7 ± 17.8	74.4 ± 10.7
Body mass index, kg·m ⁻²	27.4 ± 4.5	26.3 ± 2.4

Hyperglycemia was defined as previously measured fasting venous plasma glucose ≥ 6.1 mmol/L and/or 2 hour glucose tolerance ≥ 7.8 mmol/L. Diabetes was defined as previously measured fasting venous plasma glucose ≥ 7 mmol/L and/or 2 hour glucose tolerance ≥ 11.1 mmol/L. A cut off >21 was used for the risk-score according to Ramachandran's recommendations (91).

Four of the Norwegian participants in the intervention group and two in the control group reported to be on a low carbohydrate diet. Results from dietary recordings performed for four days prior to, and during study, are shown in Table 4.

Table 4. Dietary intake per day.

	CON (n=12)		INT (n=17)	
	Pre	Midway	Pre	Midway
Energy intake, KJ	8279 ± 2150	8043 ± 1819	8231 ± 2216	7364 ± 1602 \$
Protein, g	86 ± 18	83 ± 14	88 ± 24	83 ± 26
Fat, g	78 ± 25	84 ± 28	82 ± 39	66 ± 32 * #
Carbohydrate, g	212 ± 87	193 ± 68	199 ± 97	194 ± 88
Mono + disaccharide, g	91 ± 62	85 ± 46	72 ± 39	61 ± 29 \$
Starch, g	117 ± 45	105 ± 36	126 ± 77	131 ± 72
Added sugar, g	36 ± 46	35 ± 26	26 ± 24	21 ± 18
Dietary fiber, g	30 ± 12	22 ± 9 *	23 ± 8	23 ± 8 #

Mean ± SD calculated from four days dietary recordings performed before and during the study period (midway). \$=p<0.1 within group. *=p<0.05 within group. #=p<0.05 between groups, change from pre to during study.

Ethics Statement

The Regional Ethics Committee (REK Sør-Øst, Norway) approved the study, and all subjects gave their written informed consent. The trial is registered at clinicaltrials.gov. ID:

NCT02536066, URL:

<https://clinicaltrials.gov/ct2/show/NCT02536066?term=h%C3%A5vard+nygaard&rank=2>.

The trial was not registered prior to enrollment because we were not aware of this requirement.

Intervention

Participants in INT underwent an individual motivation session. Everyone were instructed to add >30 minutes of physical activity each day, starting <30 minutes after a meal, during the 12 week intervention. However, they were free to do more than 30 minutes, and everyone developed individual targets for the level and pattern of postprandial physical activity during intervention. Individual targets were based on each participant's motivation, wishes and possibilities for performing activity after meals. Our intention was to increase the level of postprandial physical activity as much as possible for each individual in INT. The intervention was "home-based", and they were free to do whatever type of activity they wanted, as long as it involved the legs. Prior to target-setting they were given information about the acute effects of postprandial physical activity on blood glucose (64, 67-79, 81, 82). They were free to choose which of the daily meals that should be followed by physical activity, but they were informed that the effect was anticipated to be largest after the meals with largest carbohydrate intake. Furthermore, all participants were told to maintain their usual diet and exercise habits, and live as normal. We contacted the participants in INT by telephone every 2 – 3 week during the study period to help them maintain motivation for the intervention.

Measurements

The participants were instructed not to do intense or exhausting exercise during the last three days leading up to pre or post-test. Light activity (the intervention included) was allowed.

Time from the most recent activity bout to post-test was 2 ± 3 days for CON and 1 ± 0 days for INT (median \pm IQR).

Venous blood samples were analyzed commercially by Furst Medical Laboratories, Oslo (HbA1c by HPLC- G8, Tosoh Bioscience. Glucose, triglycerides and all cholesterol levels by Advia 2400 Chemistry system, Siemens Healthcare Diagnostics Inc, and insulin and c-peptide by immunoassays, Advia Centaur XP, Siemens Healthcare Diagnostics Inc.).

The original protocol included measures of HbA1c and fasted values of glucose, insulin, triglycerides, HDL cholesterol and LDL cholesterol, as well as 2 hour glucose (75 g glucose challenge), systolic blood pressure, diastolic blood pressure, body weight, waist circumference, dietary recordings, accelerometer recordings and questionnaires. After the 8th participant we added fasting and 2 hour c-peptide, 2 hour insulin and finger sticks with capillary glucose measurements every 15 minute during the 2 hour oral glucose tolerance test. All data were collected by HN.

In three subjects the 2 hour insulin value exceeded the upper measurement range of the analysis instrument (all three at the pre-test and two of them at post-test), which were 2080 pmol/L. Those values were set to 2080 pmol/L in the analysis. Blood pressure is presented as mean of two measures; one in the fasting state after 5 minutes rest and the other 1 hour after start of glucose intake.

To measure the level of physical activity we used questionnaires, activity diaries and accelerometer recordings. In the questionnaire at the pre-test, the participants were asked about the level of physical activity, defined as walking, bicycling or more intense activity during the 3 months prior to the study. In the questionnaire at the post-test, they were asked about the magnitude of change in such activity from before the study to within the study period. During the entire study period they kept an activity diary which included type of activity, duration, perceived exertion (86), and time from end of last meal to start of activity. In the diary, physical activity was defined as all activity involving the legs and lasting >10 minutes. Accelerometers were used to estimate the level of physical activity pre study and during study. Before the study, the participants used the accelerometers (ActiGraph GT3X, ActiGraph. LLC, Pensacola, FL, US) for four consecutive days at home; 3 weekdays and 1 day during the weekend. The accelerometers were carried at the right hip, secured with an elastic belt, during waking hours (93). This procedure was repeated on the same weekdays in

the middle of the study period. The accelerometers registered vertical acceleration 30 times per second in units called counts. Mean count values were stored in 10-second intervals. We downloaded the data to the ActiLife software provided by the manufacturer (ActiGraph, LLC). To control for the influence of wear-time on the total amount of counts we used wear-time computed by the software. This was defined as total registration time minus all time with at least 60 consecutive minutes with zero counts, with allowance for up to 1 minute with counts greater than zero.

To assess dietary habits, the participants also recorded their food intake. They did this registration at the same days as the accelerometer recordings. Every component of every meal was carefully registered by a digital kitchen scale and noted in the diary during this four-day period. Dietary assessment data were analyzed using a nutrient analysis program (Mat på Data 5.0, The Norwegian Food Safety Authority, Oslo, Norway).

Statistics

We did the statistical analyses with IBM SPSS statistics, version 22.0. Analysis of within group changes and comparisons between groups of within group changes were done with a linear mixed model. Participant number was used as the “repeated subject variable” with random intercept. Group (CON, INT) and time (pre, post) were used as fixed factors. The effect of intervention was defined as the group-time interaction. Residuals were checked for normality and homogeneity. Between group comparisons of data sampled only on one time point were done with independent t-tests. Correlations were done with Pearson’s *r*. However, there were some exceptions since the residuals for the comparison of the pre and post values for 2 hour insulin (CON and INT) and LDL cholesterol (INT) were indistinctly skewed. We performed both parametric and nonparametric tests (Wilcoxon) for the within group comparisons, and chose to report the highest *p* value for each variable. However, choice of method did not affect whether any difference was significant or not. The level of physical activity for three months prior to study and during study were markedly skewed (both CON and INT) and *p* values were obtained with Mann Whitney U. The α -level was set at 0.05 and a *p* value <0.1 was considered as a tendency towards statistical significance. The data are presented as means \pm SD except from physical activity for the three months prior to and during study, which are presented as median \pm IQR.

Summary of results

Paper I

There were no significant effect of type of test day on mean glucose values for the entire 22 hours period ($p=0.111$). Glucose curves for the 22 hours period are shown in Figure 5. None of the exercise interventions did affect mean postprandial glycemia after breakfast, lunch and dinner significantly. However, BrEx bout tended to decrease glucose values after the evening meal compared to the ExBr (6.9 ± 0.5 vs. 7.9 ± 0.5 mmol/L, $p=0.032$). Furthermore, BrEx decreased the mean of the 10 highest glucose values measured in each individual (8.6 ± 0.5 mmol/L) compared to both CON (9.3 ± 0.6 mmol/L) and ExBr (9.6 ± 0.5 mmol/L, $p=0.012$ and 0.009 respectively). BrEx also decreased the glycemic variability compared to CON, measured as CONGA4 (1.22 ± 0.14 vs. 1.58 ± 0.15 mmol/L, $p=0.015$).

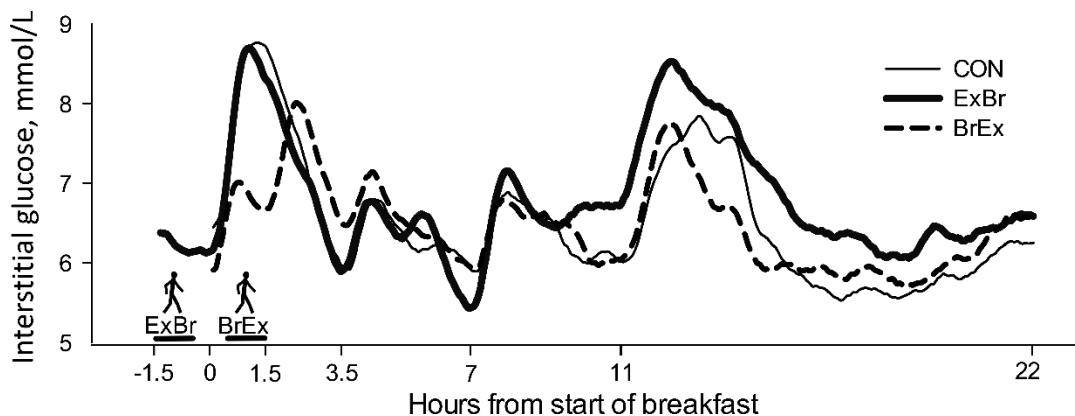


Figure 5. Results from the continuous glucose monitoring. Glucose curves for the entire test period of the three test days; The control day (CON), the day with exercise in the fasted state (ExBr) and the day with exercise in the postprandial state (BrEx).

Paper II

Merged data from the two exercise days showed that sVCAM-1 increased from baseline (4 ± 16 ng/mL) compared to CON (-28 ± 47 ng/mL) causing a difference of 32 ± 47 ng/ml ($p=0.024$). The difference in sVCAM-1 between ExBr (-5 ± 19 ng/mL) and BrEx (16 ± 32 ng/mL) did not reach statistical significance ($p=0.193$ for the difference of 21 ± 40 ng/ml). Exercise did not affect CRP values. Triglyceride values increased from baseline to end of exercise within both ExBr ($p=0.005$) and BrEx ($p=0.001$). Results are shown in Figure 6.

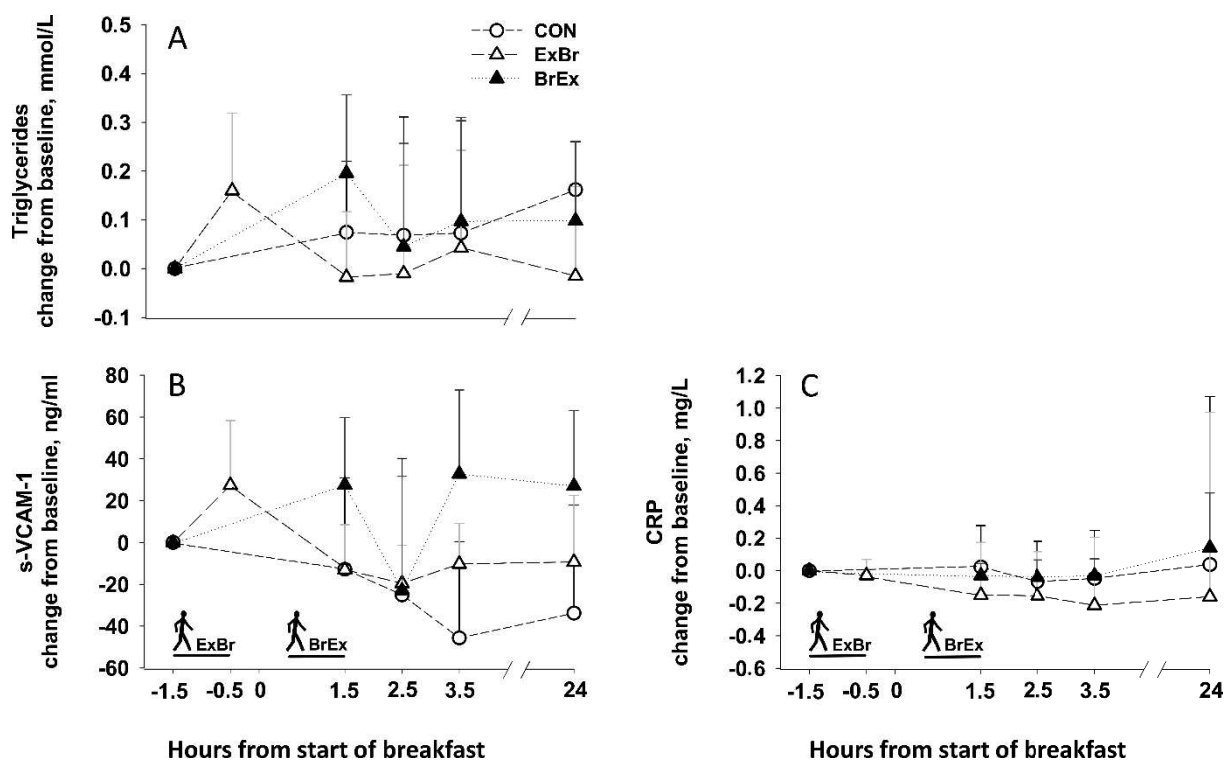


Figure 6. Blood values of triglycerides and markers of inflammation. Triglycerides (A), sVCAM-1 (B) and CRP (C) during the three test days; the control day (CON, open circles), the day with exercise in the fasted state (ExBr, open triangles) and the day with postprandial exercise (BrEx, black triangles).

Paper III

40 participants completed the study and are included in the results. The participants in CON reported 19 minutes more physical activity per day for the last three months prior to study than INT (defined as walking, bicycling or more intense activity, $p=0.001$). Baseline accelerometer counts were 14% higher for CON vs. INT, however the difference was not significant. INT reported a higher increase in activity level from before intervention to within intervention (41 ± 25 minutes / day) than CON (2 ± 16 minutes / day, $p<0.001$). There was also a tendency that INT had a higher increase in accelerometer counts from before to midway in study ($44 \pm 70\%$) than CON ($5 \pm 56\%$, $p=0.061$). Results from the activity diaries showed that the actual level of physical activity tended to be higher in INT (58 ± 72 minutes / day) compared to CON (38 ± 48 minutes/day, $p=0.085$) during study (defined as all activity involving the legs and lasting >10 minutes). Mean start of activity was earlier after meals in INT (30 ± 13 minutes) than CON (100 ± 57 minutes, $p=<0.001$). Perceived exertion during physical activity was about the same in INT and CON (11 ± 1 and 11 ± 2 on Borg's 6-20 scale). The predominant activity during intervention in INT was walking, but also some bicycling, gardening and swimming were registered. One subject in INT reported swimming as main activity during INT.

There were no within or between group differences in blood glucose or HbA1c from pre- to post-test (Table 5). The change in level of physical activity reported by questionnaire or measured by accelerometer did not correlate significantly with change in any glycemic variable in INT. Correlation coefficients to change in HbA1c, fasting and 2 hour glucose were 0.302, -0.071 and 0.262 for change in physical activity based on questionnaire results, and 0.236, 0.005 and -0.003 for the change in physical activity based on accelerometer counts respectively. There was no significant correlation between the baseline values and change in any glycemic variable in INT. There was no significant interaction between the effect of intervention and carbohydrate intake or ethnicity.

Table 5. Results of the long-term study.

	CON		INT	
	Pre	Post	Pre	Post
HbA1c, %	6.2 ± 0.6	6.1 ± 0.8	6.2 ± 0.5	6.1 ± 0.8
Glucose fasted, mmol/L	5.9 ± 1.1	6.0 ± 1.5	6.0 ± 1.3	6.2 ± 1.8
Glucose 2hour, mmol/L	7.7 ± 3.5	8.6 ± 5.1	8.8 ± 4.0	8.9 ± 4.8
Waist circumference, cm	97.8 ± 13.8	99.0 ± 12.1	96.2 ± 6.0	94.8 ± 6.0 \$ #
Body weight, kg	80.7 ± 17.8	81.3 ± 17.1	74.4 ± 10.7	73.7 ± 10.4 * #

Mean values ± SD. \$=p<0.1 within group. *=p<0.05 within group. #=p<0.05 between groups for change from pre to post. n=20 in both CON and INT.

Discussion

The main findings in these projects demonstrate that postprandial exercise may decrease glucose excursions during the subsequent day and night in hyperglycemic participants, whereas fasted exercise seems not to entail such decreases (paper I). Furthermore, exercise appears to increase levels of sVCAM-1 acutely, but not CRP levels, although without any significant difference between the two types of exercise (paper II). When hyperglycemic participants and normoglycemic participants with high risk of type 2 diabetes performed postprandial exercise on a regular basis for 12 weeks, glycemia or HbA1c was not improved compared to a control group (paper III). Accordingly, it might be questioned whether the observed favorable acute effects of postprandial exercise on blood glucose is insufficient to change the measured variables over time. We cannot rule out that the characteristics of the experimental conditions, for example regarding intensity and duration of the exercise, as well as the dietary habits of the participants might have changed the outcome in the long-term intervention. It is also crucial that the participants actually performed the post meal exercise. These issues and other considerations on sample size estimations, selection bias, measurement methods, calculations and analysis, in both studies, are discussed below. We also discuss a physiological mechanism indicating that high carbohydrate availability during exercise might blunt some of the favorable adaptations to exercise in long-term.

Considerations about the samples and randomization

The sample size in study 1 (paper I and II) might have been too low to detect significant effects on mean glucose values for the entire test period (paper I) and on the difference in sVCAM-1 between the two exercise interventions (paper II). This is indicated by the sample size calculations and the numerical values that show non-significant differences. The limitations of the sample size calculations are obvious since calculations were not performed for glucose for the entire test period, and the actual variability of change for sVCAM-1 was larger than the expected value used in the calculations. Thus, although we included more participants than determined by the sample size calculations and the observed non-significant differences were small, the calculations were inappropriate and a type 2 error might have occurred.

The sample size calculation in the long-term study (paper III) was also imprecise. The calculation for HbA1c seems to have been accurate, but the actual variability of change was larger than expected for fasted glucose and 2 hour glucose. This may have entailed a type 2 error. However, the observed mean changes from pre to post do not indicate any positive effect of intervention on fasted and 2 hour glucose. Therefore, there are no obvious indications of a type 2 error, other than a faulty sample size calculation, and the conclusion might not be affected by the low number of participants.

Use of volunteers as study participants often entails a selection bias. Volunteers are usually more motivated for the intervention than the general population and are often not representative for the entire population that the results are generalized to (94). The present studies are likely no exception from this. The degree of external validity is difficult to establish, but the dietary habits reported by the participants in the acute study (paper I and II), e.g. the low intake of sugar, can be interpreted as indications of selection bias. The reported activity level also appears to be higher than what is reported by newly diagnosed diabetics (95), and the generalizability of the results to hyperglycemic persons in general can therefore be questioned. The Norwegian participants in the long-term study (paper III), were recruited the same way and from the same locations as the ones in the acute study, and four persons participated in both studies. It therefore seems likely that some degree of selection bias has occurred also in this study. The possible effect of selection bias on the results is further discussed below, in *“the influence of carbohydrate load”*.

Since we included both hyperglycemic persons and persons with high risk of hyperglycemia in the long-term study (paper III), the sample obtained was rather heterogenous. This might increase the variability of the results and weaken the generalizability of the study to one specific group within the spectra included. However, no significant relation between the severity of hyperglycemia and effect of the intervention was present in this study.

To limit interchange of information and behavior from the intervention group to the control group in the long-term study (paper III), family members or close friends who enrolled at the same time (persons who wanted to “participate together”) were randomized to the same group. This is in conflict with the idea of true randomization. Indeed, there appeared to be some differences between the groups at baseline. The differences were most pronounced for the self-reported level of physical activity, with lower values for the intervention group compared to the control group. We cannot exclude that this difference is due to the

randomization method. However, the baseline levels in accelerometer counts did not differ substantially between groups, and the difference in self-reported baseline level of physical activity could therefore have been some kind of reporting bias. Participants often report their behavior biased toward what they consider to be expected (96). Regardless of this, the observation of no within group changes in glycemia reduces the concerns associated with baseline differences between groups.

Did they really change exercise patterns?

In the long-term study (paper III) we put emphasis on the motivation for the intervention and individual accommodations to increase the level of postprandial exercise as much as possible for each participant in the intervention group. Nevertheless, adherence to non-supervised exercise interventions can be lower than supervised interventions (97), and we had no absolute control over the activity patterns of the participants. Therefore, we assessed the participants' physical activity patterns with other methods than direct observation. Results from the activity diaries suggest that the intervention group increased their level of physical activity, since the reported activity level seemed to be higher than in the control group. However, the results from the activity diaries are difficult to interpret, since there is no baseline data, and as suggested by the results from the questionnaires, there may have been a higher baseline activity level in the control group than in the intervention group. The results from the questionnaires suggest considerable increases in the level of physical activity in the intervention group. The validity of such a subjective assessment of a behavioral intervention can be questioned since persons do have a tendency to report their behavior biased toward the expected performance (96). Objective methods are therefore considered as preferable for quantification of the amount of physical activity, and accelerometers is regarded as the objective method of choice in free-living studies (98). Actigraph accelerometers are validated several times and the correlation to other methods for assessing level of physical activity (e.g. indirect calorimetry) is in the range $r=0.45 - 0.90$ in former versions of the instrument (93). A more recent validation of the Actigraph accelerometer (99), utilizing a version of the instrument that is comparable to the one we used (100), showed a correlation to indirect calorimetric in the range $r=0.82 - 0.91$ at level and graded walking. In general, energy expenditure was somewhat overestimated during downhill walking and somewhat underestimated in uphill walking and the authors therefore argued that some of the inaccuracy

observed at graded surface would be evened out in a free-living setting (99). Tests of the inter-instrument reliability of Actigraph accelerometers, which can be interpreted as an indicator of the reliability of the “test-retest” regimen in the long-term study (paper III) have generally shown low coefficients of variation (typically <5%) (93). The 4 day recordings we used are in accordance with a recommendation that 3 to 5 days is enough to assess reliable results (93). The intervention group had a higher increase in accelerometer counts than the control group from before to midway into the study. The accelerometer recordings therefore reinforce the impression that the intervention group actually increased the level of physical activity, although the between-group difference had only a borderline significance. The accelerometer results may have underestimated the increases in activity level, since some of the participant in the intervention group practiced swimming (without the accelerometer) and bicycling as regular activities during intervention. Bicycling is poorly registered by accelerometer worn on the hip (99).

Based on those results from different methods for assessing activity level it seems reasonable to conclude that the intervention group really increased their level of physical activity. It is unclear whether the anthropometric measures support this conclusion or not. On one hand, there was a reduction in body weight and waist circumference in the intervention group. On the other hand, the body weight and waist circumference reductions were modest. Consequently, we cannot rule out that the reported changes in activity level are overestimated, and / or that a dietary compensation to the increased activity level have occurred. A dietary compensation is however not supported by the results from the dietary recordings. Alternatively, the intensity and duration of the added activity may have been too low to cause major reductions in fat mass.

Independent of the level of activity performed it seems convincing that the intervention group had changed their activity pattern with regard to timing to the preceding meal. A start of activity 30 minutes after the last meal was much earlier than the control group.

Were exercise stimuli large enough?

Regular exercise is in general expected to decrease hyperglycemia chronically (47-49), and it is reasonable to expect the same effect, or indeed, a larger effect, if the exercise is performed in the postprandial state (64, 81, 82), although, contrasting findings exists (101). The findings

in the acute study (paper I) supports previous findings of favorable effects of postprandial exercise although a significant decrease in mean glucose values was only present during the postprandial exercise session. Summarizing the studies in this field (64, 67-80), it seems convincing that the type of postprandial exercise performed in the long-term study (paper III) blunts postprandial glycemia acutely. At least HbA1c should have been affected in long-term, since the magnitude of HbA1c is a result of the magnitude of the blood glucose levels over time. Since this did not occur, it might be questioned whether the exercise stimuli was too negligible to change HbA1c substantially. However, substantial acute decreases in postprandial glycemia are observed after doses of postprandial exercise that might have been lower than the reported doses in the long-term study (paper III) (73-75, 78-80, 102). Furthermore, previous long-term interventions with comparable amounts of walking have decreased HbA1c in both diabetic (103) and healthy (104) participants. In addition, there was no significant correlation between the amount of postprandial physical activity added during intervention and the effect on glycemia (paper III). Indeed, if any relationship was present, it was in disfavor of the hypothesis. This observations indicates that the lack of effect was not a result of a too low exercise stimuli and it reinforces a conclusion that postprandial exercise might not decrease glycemia in long-term. However, we cannot exclude that the exercise stimuli was too low, or alternatively that the number of postprandial exercise bouts per day was too low to entail a substantial decrease in HbA1c.

Perspectives on intensity

The perceived exertion was about 12 on Borg's 6-20 scale in the acute study (paper I and II) and about 11 in the long-term study (paper III). We refer to the intensities as "moderate" and "light" respectively, according to the specifications in the scale (86). Previous studies have shown that light postprandial exercise is an effective way to reduce postprandial glycemia (73-76, 78, 80), but the results of the interventions might have been more pronounced with higher intensities. Exercise intensity is an interesting matter of debate, since the acute effect of different intensities on blood glucose seems to differ dependent on glycemia. One bout of moderate or high intensity exercise has the potential to increase blood glucose levels in healthy persons (105, 106) while it decreases blood glucose in diabetic persons, even if it is performed in the post-absorptive state (106). An increase can in general be explained by the catecholamine mediated increase in hepatic output, since catecholamines are secreted in

accordance with exercise intensity (107). The discrepancy between diabetic and healthy persons, might be a result of higher peripheral utilization of glucose in diabetics during high intensity exercise (106). In a healthy or mixed population, low exercise intensity might therefore be the safest choice targeting an acute decrease in glycemia. If the moderate or high intensity exercise is done in the postprandial phase however, when the blood glucose level is acutely increased, a decrease in blood glucose levels is likely to occur both in healthy (67) and diabetic persons (67, 68, 70, 71, 79). It seems plausible that the catecholamine effect on hepatic output is overruled by the high insulin-to-glucagon ratio in the postprandial phase (108). The acute effects on glucose observed in paper I might therefore have been larger if exercise intensity were higher. Many long-term physiological adaptations related to insulin sensitivity are probably also larger after intensive training compared to light training (56, 109-111). A very recent study in type 2 diabetic participants demonstrated reductions in HbA1c after a high-intensity training program, but not from work-matched exercise training at moderate intensity, (112). Intensive exercise might therefore entail favorable effects, and the results reported in paper III could have been more pronounced if higher intensities were used. However, high intensity exercise immediately after food intake might not be a good idea, considering gastrointestinal problems. The intensity can also affect the adherence to exercise and persons with hyperglycemia may be more motivated for light activity (113), but this is disputed as high intensity exercise represent a time-efficient strategy for promoting health (114).

Was the timing between meals and physical activity appropriate?

The postprandial physical exercise bout in the present acute study was initiated 30 minutes after the start of breakfast (paper I and II). This timing has been recommended for lowering postprandial glycemia in diabetic persons, since a high insulin-to-glucagon ratio is anticipated at this point of time (108). A high insulin-to-glucagon ratio will in turn inhibit the counter regulatory increase in hepatic glucose output that occurs when exercise is done in the post-absorptive phase (108). This notion is supported by the fact that the glucose level at onset of postprandial exercise has a strong relation to the subsequent decrease in glycemia (115). It is therefore reasonable to believe that an onset of exercise after peak glucose and / or insulin concentration will lead to lesser acute decrease in glucose than if the onset takes place before the peak. The exact timing of the onset of postprandial exercise might therefore be important,

at least for the glycemic changes in the postprandial period when exercise is performed. This also raises the question whether suboptimal timing between food intake and exercise might have contributed to the lack of effect in the long-term study (paper III). However, the reported mean interval between end of the last meal and start of exercise in the intervention group was 30 minutes (paper III), which is likely to be well within the area of high insulin-to-glucagon ratio (67, 116). The SD of the individual mean interval between food and exercise can also be characterized as low (± 13 minutes). However, we cannot exclude that variation in the timing of exercise influence the results. In any case, the timing between food intake and exercise could not have been ideal in every single activity session, since every individual had some variation, and there were certainly also variation in meal composition, time of peak postprandial glycemia and insulin-to-glucagon ratio.

Is favorable physiological adaptations to exercise attenuated when exercise is performed in the carbohydrate fed state?

The lack of effect on glycemia in the long-term study raises the question whether some of the anticipated adaptations to long-term exercise are attenuated as a result of the nutritional status during postprandial activity. One possible physiological explanation of the lack of positive results of the intervention is related to intramyocellular lipids in diabetes prone subjects. Accumulation of intramyocellular lipids is believed to be an important mechanism behind insulin resistance (117, 118). These lipids are used as an energy source during exercise. Glucose feeding during exercise has the ability to decrease the release of hormone sensitive lipase which is the rate-limiting enzyme in the breakdown of intramyocellular lipids (119). A few studies have demonstrated a larger breakdown of intramyocellular lipids after fasted exercise compared to exercise in the carbohydrate fed state / carbohydrate feeding during exercise in healthy participants (57, 58). Furthermore, fasted exercise, but not exercise accompanied with carbohydrate intake, had advantageous effects on fat metabolism capacity and insulin sensitivity in healthy participants fed with a fat-rich high-energy diet (120). Based on these studies, it seems likely that there is an interaction between excessive glucose availability during exercise and the related physical adaptations. We cannot exclude that breakdown of intramyocellular lipids is attenuated in the long-term study (paper III), possibly diminishing the positive effect on insulin sensitivity that is expected to occur after regular exercise. With regard to this, it would have been interesting if an intervention with fasted

exercise also had been included in the long-term study, but the study was limited to postprandial exercise only.

The influence of carbohydrate load

As discussed above (under “considerations about the samples and randomization”), a selection bias have likely occurred in the acute study (paper I and II), and the diet of those participants might differ from the diet of average hyperglycemic persons. Since the participants with known hyperglycemia in the long-term study (paper III) were recruited the same way as in the acute study (paper I and II) and some participated in both studies, a degree of selection bias might have occurred in this study as well. Indeed, some of the participants in the long-term study reported to be on a low carbohydrate diet (which was confirmed by the dietary recordings). The inclusion of participants on low carbohydrate diets may have been a major source of error, since a low carbohydrate intake entails smaller postprandial excursions in blood glucose than a large intake. It is therefore likely that a low carbohydrate intake reduces the potential for decreasing postprandial glycemia with exercise. We tested if there was an interaction between the registered amount of carbohydrate intake and the effect of intervention, but could not find such a relationship (paper III). However, the study was not powered for analysis of such interactions.

In addition to an eventually bias related to a low carbohydrate intake in general, the participants in the long-term study (paper III) were free to choose which meals to be followed by exercise. We did not assess any data on which meals that were followed by exercise or the carbohydrate amount of those meals. The participants were told that the effect of exercise on blood glucose is largest after the meals with the highest carbohydrate content, but we do not know to what degree this advice was complied with.

As in the long-term study (paper III) it is likely that the effect of exercise in the acute study (paper I) would have been more pronounced if the glycemic loads and the ensuing postprandial glycemic increases had been larger (74, 78, 115, 121). The effect of exercise did indeed appear to be largest after the meals with highest postprandial glucose excursions (paper I). Also the effect on markers of inflammation (paper II) might have been different if larger doses of carbohydrate were ingested. Apparently, there was no inflammatory response to the breakfast meal. If larger doses of carbohydrate were ingested, a hyperglycemia entailed

inflammatory response might have occurred, (30, 33, 122, 123), but potentially not after postprandial exercise, since such exercise decreased glucose excursions. The generalizability of studies utilizing exaggerated amounts of carbohydrate can however be questioned, especially since we observed a low carbohydrate intake in some participants. One might therefore say that the external validity of many acute studies in this field are biased by abnormally large carbohydrate intakes.

Validity of the results from the continuous glucose measurements

The use of a continuous glucose monitoring system make it easier to monitor glucose for longer durations compared to venous blood sampling. The measuring device used in paper I is small and light and the assessment can be done without pain and without affecting daily life substantially (124). Furthermore, it makes it easy to monitor glucose during sleep. The results may therefore be more generalizable to everyday life than if venous blood samples were used.

This system assesses interstitial glucose (paper I) and not blood glucose, but, since interstitial glucose mirrors blood glucose (7, 8) the interstitial glucose values may represent a valid estimate of blood glucose values. In a study comparing continuous glucose measures from the same device as utilized in paper I, with venous blood sampled for 24 hours, mean absolute relative difference between the measures was 17.6% (125). In another study using a former version of the device, mean absolute difference to venous values were ~ 0.55 mmol/L during exercise (126). In paper I, mean absolute difference to venous values (measured 2.5 hours after breakfast) were 0.7 mmol/L, while intraclass correlation was 0.95 and coefficient of variation was 7.3%. The continuous measures of interstitial glucose therefore seem to represent a valid estimate of blood glucose, although some differences exist. The differences can derive from the delay between blood glucose and interstitial glucose when glucose is fluctuating (8) as well as measurement error. However, one might question which of the extracellular fluids is the most relevant; blood plasma or interstitial fluid. It is likely to believe that plasma glucose is most relevant to glycosylation of plasma proteins and perhaps the endothelial glycemic stress, while interstitial glucose is most relevant to the glucose interaction with tissue cells. Test-retest reliability of the continuous interstitial measures might therefore be as interesting as the ability of the device to estimate blood glucose. Terada et al. (127) reported test-retest reliability from continuous measures assessed on two different

days with similar diet and behavior, utilizing the same device as we used in paper I. The coefficient of variation was 3.9% for mean glucose, 8.6% for CONGA2 and 15.8% for CONGA4, while intraclass correlations were 0.95, 0.95 and 0.86 respectively. The results indicate that our measures in paper I is reliable, although some random error are present, especially for CONGA4.

Intermittent elevations in glucose levels are related to cardiovascular damage (28-31, 37). It is therefore relevant to include estimates of the severity of such glucose excursions. It can be assessed in several ways, such as peak glucose concentrations and glycemic variability. In paper I we chose to utilize mean of the 10 highest values as a measure of peak values and CONGA scores as a measure of glycemic variability. One single peak value is typically used in studies evaluating the effect of exercise during one postprandial period (72-74, 77). The test days in the acute study (paper I) did however consist of several consecutive postprandial periods, and a mean score of the 10 highest values might therefore reflect total severity of the glycemic excursions better than a single value. In addition, a mean score is likely to be more reliable than a single value from the continuous measurement system. CONGA score is proven as a valid indicator of glycemic variability and is suggested to be the choice of preference for calculating intraday glycemic variability from continuous glucose measures (88).

Perspectives on the theoretical background

The theoretical foundation for this thesis is largely based on the assumption that there is a cause and effect relationship between postprandial glycemia and disease. A consequence of this assumption is the supposition that it is important to limit postprandial glycemic excursions. In reality however, a cause and effect relationship is difficult to prove. It is evident from longitudinal studies that there is a strong relationship between the magnitude of postprandial glycemia and subsequent worsening glycemia (5) as well as cardiovascular disease (3, 4, 17-20, 23-26) and other diseases (128-131), but causality is not proven in such study designs. A cause and effect relationship is in general best proven in experimental studies (94), but it is unrealistic to design and perform an experimental trial that generates definite answers about postprandial glycemia and its relation to diseases. Strong indicators of a causal relationship to cardiovascular disease are nevertheless achieved in experimental

studies using medication targeted at lowering postprandial glycemia (21, 22). However, other effects of the drug than reduced postprandial glycemia per se can bias the observed improved cardiovascular health. A relationship between glucose excursions and markers of cardiovascular risk is also observed in several acute studies (30), but such studies do not give any answers about the effect of repeating the stimuli regularly over long time on final outcomes. Overall, it is convincing that postprandial glycemia have a cause and effect relationship to its related diseases, but research in this field should be interpreted with reservations concerning the lack of a definite proof of causality. If such causality does not exist, there is no reason to believe that acute reductions in hyperglycemia per se can improve glycemia or prevent diseases in the long-term.

Specific comments to markers of inflammation

The main finding in paper II was an acute increase in sVCAM-1 but not CRP after moderate exercise in hyperglycemic participants. These findings support most of the previous research in the field (132-137). Oxidative stress induced by exercise per se, may be linked to the sVCAM-1 results since it increases after exercise. Like the concentration of sVCAM-1, the triglyceride levels also increased after exercise. Causality between triglyceride increases and observed sVCAM-1 increases cannot be ruled out since acute increases in triglyceride levels can entail oxidative stress and thereby increased levels of inflammatory markers, much the same way as hyperglycemic excursions are supposed to do (33). With regard to the self-reported activity level prior to the study, it seems plausible that the observed differences in sVCAM-1 levels also are a result of inactivity during the control condition. The control condition represented a decrease in activity level, and the test days may have reflected “real life” exercise more closely than the control condition for the participants.

The lack of effect on CRP of moderate uphill treadmill walking is not surprising considering the traditional view that vigorous eccentric muscle exercise and muscle damage is the mechanism behind post exercise CRP release (42).

Reliability of thawed blood samples

The blood samples used in paper II were thawed by accident during an electricity shutdown during the summer holiday that lasted for 13 days. We did an experiment to test the reliability

of the thawed blood samples. The report from this reliability test is attached as appendix 1. We concluded that the results from the thawed blood samples were reliable, and that the freezer accident did not affect the conclusions of paper I.

Perspectives on the possible practical implications

A discussion of whether or not practical health implications can be drawn from small studies, like the present ones, would be purely hypothetical. It is however tempting to consider some perspectives on the practical implications.

In paper I we showed that postprandial exercise in the morning has the ability to blunt the highest postprandial glucose excursions and decrease glycemic variability during the following day. Based on this, one might suggest that postprandial exercise should be a strategy to promote health in hyperglycemic persons not using antidiabetic drugs. However, this seems to be contradicted by the lack of glycemic changes in response to such activity in the long-term study (paper III). The paradoxical results are a reminder that behaviors that entails the largest acute effects are not necessarily effective habits to promote health in the long run.

Although there were no correlation between the amount of carbohydrate intake and the effect of intervention in the long-term study, it does not give any final answers about the effect of postprandial exercise in combination with high carbohydrate intake. Based on the acute study (paper I) and the anticipated relationship between the magnitude of postprandial glycemia and the effect of postprandial exercise (74, 78, 115, 121), we cannot rule out that postprandial exercise in the long term is a good strategy to decrease glycemia in persons with very high carbohydrate intake. Furthermore, intermittent high glucose concentrations, that is reduced by postprandial exercise in paper I, are associated with cardiovascular damage via several mechanisms (30), including endothelial dysfunction (28, 29, 31, 37). Postprandial exercise might therefore have advantageous effects on cardiovascular health despite a lack of a long-term glycemic affect per se. According to this, future studies of fasted and postprandial exercise should pay attention to markers of cardiovascular health in addition to glycemia. The acute results on the inflammatory markers (paper II) do not give any indications whether exercise should be done in the fasted or the postprandial state to obtain positive chronic effects. Such acute results on markers of inflammation are difficult to interpret, and it has also

previously been claimed that acute proinflammatory responses to exercise may be a mechanism behind an anti-inflammatory response over time (84). The finding that CRP is not affected by moderate exercise might be of relevance in clinical practice, e.g. if the patient is walking to the clinic.

Nevertheless, it is important not to interpret the present results as if light exercise does not matter to health. Walking and comparable activities, is simple, inexpensive, without adverse effects and may be a good strategy for implementing exercise in everyday life (138).

Summary of discussion

The main finding in paper I was that postprandial, but not fasted exercise can decrease glucose excursions- and variability during the subsequent day and night in hyperglycemic participants. There was no statistical significant effect on the mean glucose value, which could be a result of a type II error. Independent of whether exercise was performed in the fasted or postprandial state, it appear to increase levels of sVCAM-1 acutely, but not CRP levels (paper II). The lack of significant difference in sVCAM-1 response between the two types of exercise, might also be a type II error. The results in both paper I and II might have been different if the standardized diet had contained a larger dose of carbohydrate, and it seems likely that some degree of selection bias have occurred. When hyperglycemic participants and normoglycemic participants with high risk of type 2 diabetes performed postprandial exercise on a regular basis for 12 weeks, they did not improve glycemia compared to a control group (paper III). The lack of effect might be a result of a too low exercise stimulus, inappropriate timing between carbohydrate intake and exercise, low carbohydrate intake in some of the participants, or that some favorable adaptations to exercise training is blunted when exercise is performed consecutive to carbohydrate intake.

Conclusions

In persons prone to or with type 2 diabetes;

1. Moderate exercise performed in the postprandial state decreases the level of interstitial glucose excursions and variability over 22 hours compared to no exercise. In contrast, such exercise carried out in the fasted state appears to have no influence on these variables.
2. Moderate exercise performed in the postprandial state decreases the level of glucose excursions over 22 hours, compared to fasted exercise.
3. Moderate exercise entails an acute increase in sVCAM-1, whereas it does not affect CRP levels.
4. The acute effects of moderate exercise on CRP and sVCAM-1 levels appear not to differ substantially between exercise performed in the postprandial and the fasted state.
5. Performing light postprandial exercise on a regular basis over the course of 12 weeks appears not to affect glycemia, as measured by HbA1c, fasted- and 2-hour glucose.

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Appendix 1

Test of reliability of thawed blood samples

Summary

Because of an electricity shutdown that lasted for 13 days, all blood samples stored at -80°C thawed. In order to explore whether the thawed blood samples still could give us reliable results we performed a reconstruction of this accident. Duplicates of 24 plasma samples were either stored in -80°C until analysis or frozen in -80°C before a “13 days thaw and refreeze experiment”, which was an imitation of the original freezer accident.

The following intraclass correlations were present between frozen and thawed duplicates: sVCAM-1; 0.993, CRP; 0.997, Glucose; 0.996, Triglycerides; 0.997.

We conclude that the results from the thawed blood samples are reliable, and the freezer accident did not affect the conclusions of the main study.

Background

The electricity to the -80°C freezer was shut down by accident about 1:00 p.m. August 15th 2014. Electricity was turned on 13 days later, about 12:00 p.m. on August 28th. By that time, the temperature in the freezer had increased to room temperature (19°C) and all blood samples were thawed. The freezer reached -80°C within some hours after electricity was turned on again.

For six out of twelve participants, sVCAM-1 was analyzed before the electricity shut-down. The remaining blood samples including sVCAM-1 for six participants and CRP, glucose, triglycerides and marker of oxidative stress for all twelve participants were thawed and refreezed.

The purpose of this reconstruction was to explore whether the thawed blood samples still could give us reliable results.

Methods

To fully evaluate how much each blood variable was affected, we carefully reconstructed the accident with new blood samples drawn from 24 participants and treated identical to the samples in the main study. Except from one third of the participants that had a cold at the time of sampling the participants were healthy. Twenty-four blood samples were placed in the same freezer as in the accident (-80 °C, Herafreeze HFC 586 Basic, Thermo scientific) and a duplicate was kept as a positive control in a separate freezer at -80 °C. We did not believe that proper markers of oxidative stress could be stable in room temperature and decided not to include such variables in the experiment. The experiment was proven by the Regional Ethics Committee (REK Sør-Øst, Norway), and all participants gave their written informed consent.

After some days at -80 °C the electricity was disconnected from the same freezer as in the original freezer accident. The freezer contained the same volume of content as in the original accident. After 13 days the thawed samples were moved to the freezer containing the frozen duplicates and kept at -80 °C. Room temperature was stable at 19 °C during this 13 days period. Freezer temperature was logged during the reconstruction with a temperature logging system (Boomerang, ICU Scandinavia AB, Sweden).

The duplicates were analyzed simultaneously for comparison of sVCAM-1, hsCRP, glucose and triglycerides. Analyses were done similarly as in the main study. The number of analyzed duplicates were: 20, 24, 20 and 20 for sVCAM, CRP, glucose and triglycerides respectively. Results are presented as mean \pm SD, and coefficient of variation (CV) is mean of the CVs for each pair of duplicates. Intraclass correlations (ICC) were computed with IBM SPSS statistics, version 22.0, SPSS using a two way mixed model and the consistency approach.

Results

Freezer temperature from the reconstruction experiment is shown in Fig 1. In brief, temperature reached 0 °C <2 days after electricity was disconnected and 19 °C after about 7 days.

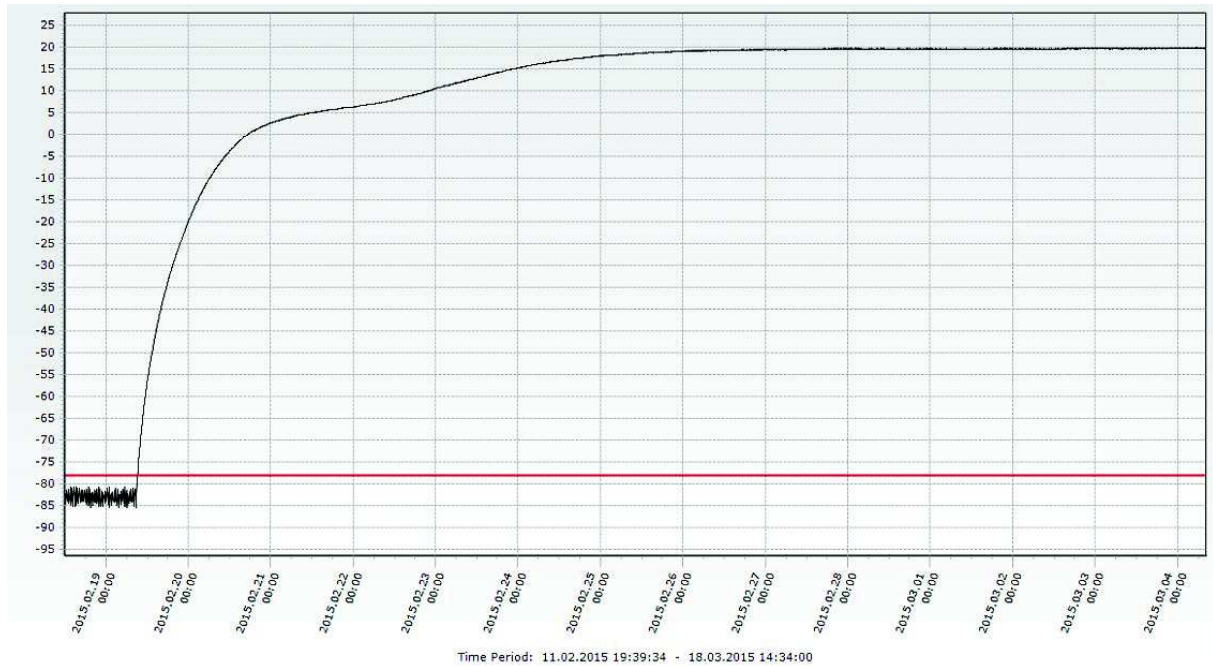


Figure 1. Freezer temperature during reconstruction. Temperature in °C on the y axis.

Mean sVCAM-1 value for the frozen duplicates was 681 ± 137 ng/mL while the corresponding value for the thawed duplicates was 668 ± 134 ng/mL (Fig 2). Mean decrease was 1.9% and CV 2.1%. Intraclass correlation (ICC) was 0.993.

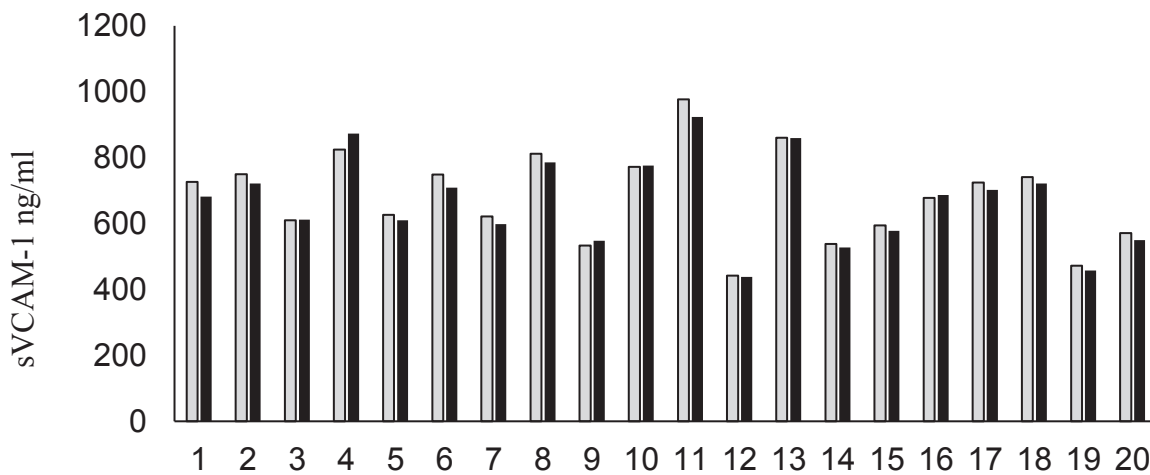


Figure 2. sVCAM-1 results. Twenty samples with the “Frozen” duplicate (grey) and the “thawed” duplicate (black).

Mean CRP value for the frozen duplicates was 1.84 ± 2.39 mg/L while the corresponding value for the thawed duplicates was 1.70 ± 2.25 mg/L. Mean decrease was 7.3% and CV 7.9% (Fig 3). ICC was 0.997.

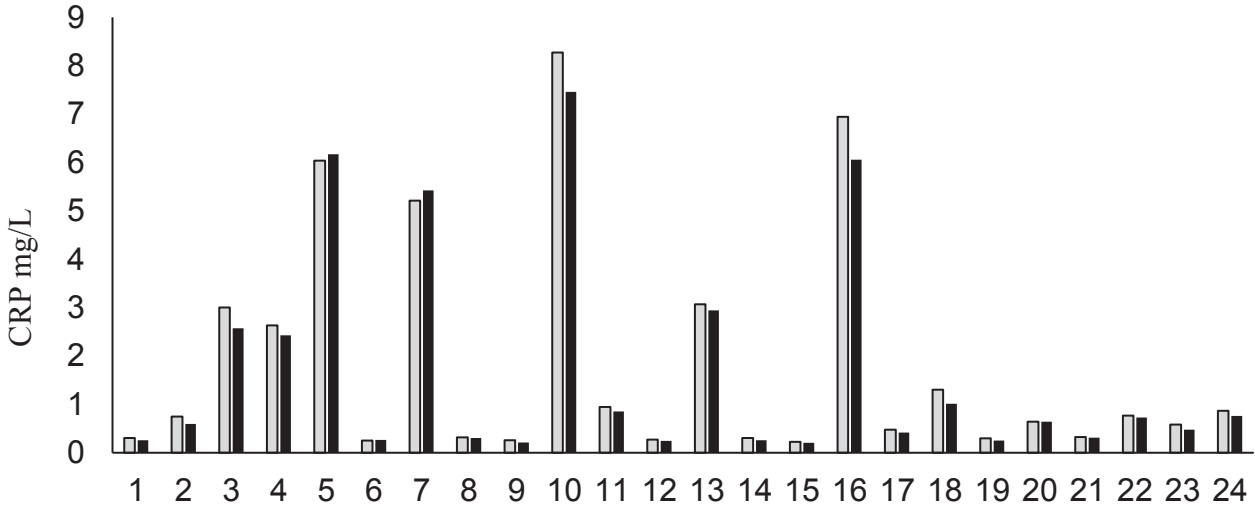


Figure 3. CRP results. Twentyfour samples with the “Frozen” duplicate (grey) and the “thawed” duplicate (black).

Mean glucose value for the frozen duplicates was 5.12 ± 0.57 mmol/L while the corresponding value for the thawed duplicates was 5.11 ± 0.58 mmol/L (Fig 4). Mean decrease was 0.2% and CV 0.6%. ICC was 0.996.

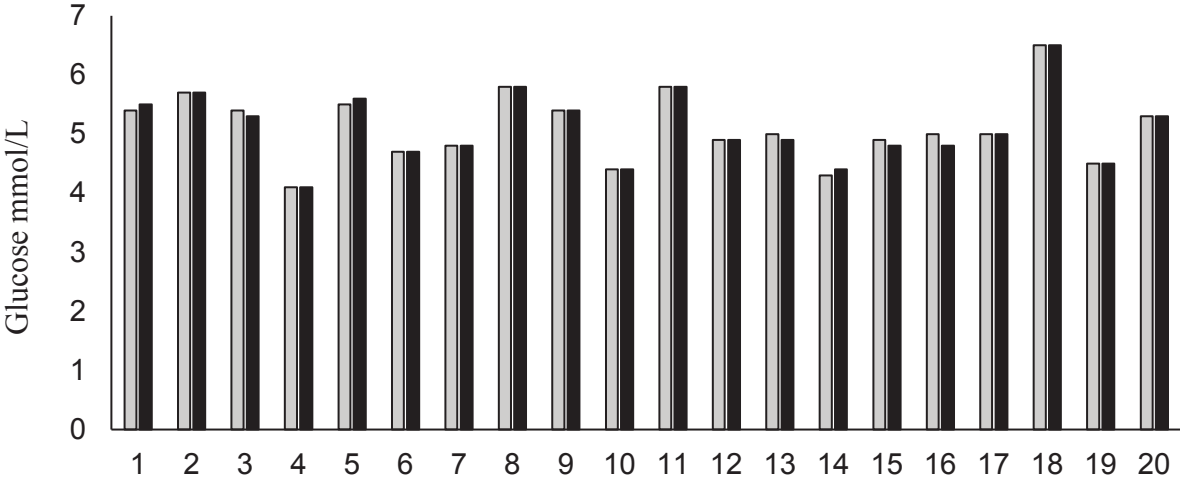


Figure 4. Blood glucose results. Twenty samples with the “Frozen” duplicate (grey) and the “thawed” duplicate (black).

Mean triglyceride value for the frozen duplicates was 1.42 ± 0.88 mmol/L while the corresponding value for the thawed duplicates was 1.41 ± 0.93 mmol/L (Fig 5). Mean decrease was 0.4% and CV 5.4%. ICC was 0.997.

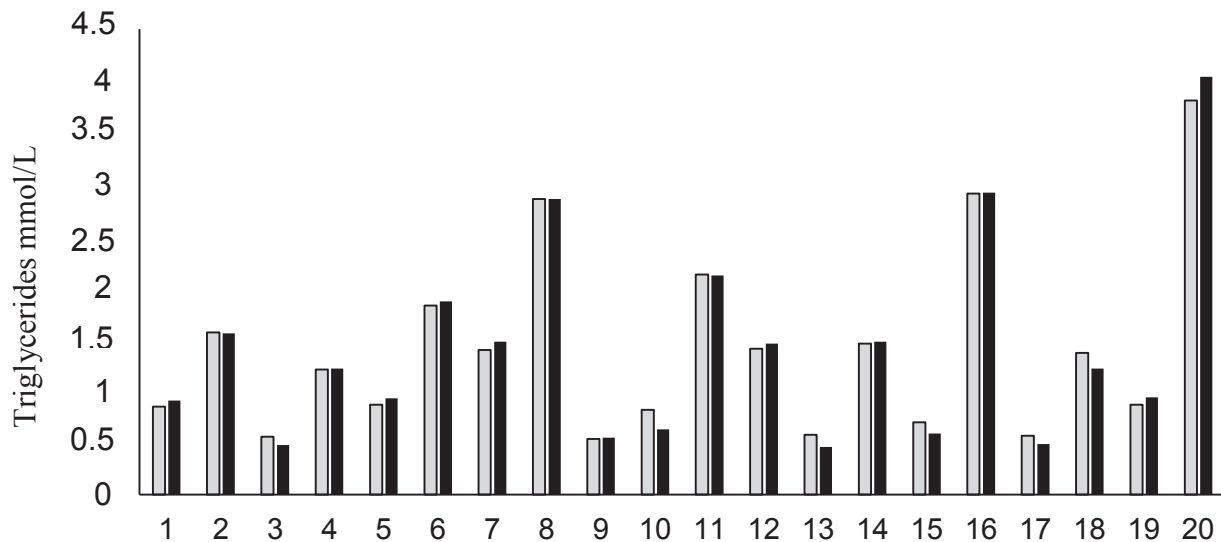


Figure 5. Triglyceride results. Twenty samples with the “Frozen” duplicate (grey) and the “thawed” duplicate (black).

Discussion

All variables showed a very high intraclass correlation between frozen and thawed duplicates. For each of the measured variables, we therefore assume that a significant group variation observed in the thawed samples is a true one, and not caused by the freezer accident. Mean values of the thawed samples was in general slightly lowered, but this is unlikely to affect the results of the main study, since all within subject samples for a variable is treated the same way and the intraclass correlation is very high. Our results are in accordance with previous studies showing that sVCAM-1 (1), CRP (1, 2), Glucose (2, 3) and Triglycerides (2, 3) are very stable in plasma.

Conclusion

The results from the thawed blood samples is reliable and the freezer accident did not affect the conclusion of the main study.

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