Muscle Morphology and the Insulin Resistance Syndrome
A population-based study of 70 year-old-men in Uppsala

by
Anu Hedman
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Abstract


Skeletal muscle accounts for the largest part of insulin-mediated glucose uptake. Insulin resistance (IR) is the main component of insulin resistance syndrome (IRS) and is an essential cause of a number of cardiovascular risk factors. This thesis investigates the relationships between muscle morphological characteristics and IRS because skeletal muscle is responsible for the majority of glucose uptake.

In this population-based sample of 70-year-old men, higher proportion of type I fibers as well as higher capillarization were related to higher insulin sensitivity and higher self-reported physical activity, which were related to a lower prevalence of type IIB fibers. Serum triglycerides, HDL cholesterol and plasminogen activator inhibitor-1 (PAI-1) activity were significantly related to fiber distribution and muscle capillarization and muscle morphology, in part, explained the association between these metabolic risk factors with physical activity level. BMI, glucose intolerance, PAI-1 activity, serum FFA concentration, proportion of type IIB fibers, HDL cholesterol level, drug treatment, physical activity level, and W/H ratio together explained 55% of the variation in the insulin sensitivity index. In addition, almost a twofold improvement of the correlations was seen after correcting for intraindividual variation. Glucose tolerant hypertensive subjects showed a lower capillary supply when compared to controls. Capillary density was negatively correlated to the increase in mean arterial pressure over two decades as well as to supine heart rate 20 years before. Interestingly, supine heart rate showed an independent inverse association to the percentage of type I fibers and a positive correlation to the percentage of type IIB muscle fibers. Capillary density and elevated serum free fatty (FFA) acid values were inversely associated with insulin-mediated blood flow and thus to endothelial dysfunction, which has been linked to IR. In fact, capillary density and serum FFA level together explained 71% of the variation in insulin-mediated leg blood flow changes.

In conclusion, these population-based findings support the observations that muscle morphological features and insulin sensitivity are related to each other. Muscle morphology might explain some of the beneficial impact of physical activity on the components of IRS. Accordingly, we suggest that alterations in muscle morphology should be considered as an essential part of the IRS.

Keywords: muscle morphology, fiber distribution, capillary density, insulin resistance syndrome, physical activity, hypertension, insulin-mediated blood flow.

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To my family

This thesis is based on the following investigations, which will be referred to by their Roman numerals:


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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<td>IRS</td>
<td>insulin resistance syndrome</td>
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<td>HDL</td>
<td>high density lipoproteins</td>
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<td>LDL</td>
<td>low density lipoproteins</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<td>IGT</td>
<td>impaired glucose tolerance</td>
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<td>IR</td>
<td>insulin resistance</td>
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<td>Myosin ATPase</td>
<td>myosin adenosine triphosphatase</td>
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<td>MHC</td>
<td>myosin heavy chain</td>
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<td>GLUT4</td>
<td>glucose transporter protein 4</td>
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<td>TG</td>
<td>triglycerides</td>
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<td>PAS</td>
<td>amylase-periodic acid-Schiff</td>
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<td>CV</td>
<td>coefficient of variation</td>
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<td>HR</td>
<td>heart rate</td>
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<td>SBP</td>
<td>systolic blood pressure</td>
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<td>DBP</td>
<td>diastolic blood pressure</td>
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<td>FFA</td>
<td>free fatty acid</td>
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<td>LPL</td>
<td>lipoprotein lipase</td>
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<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
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<td>SNS</td>
<td>sympathetic nervous activity</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>LBF</td>
<td>leg blood flow</td>
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<td>ULSAM</td>
<td>Uppsala Longitudinal Study of Adult Men</td>
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<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
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<td>BP</td>
<td>blood pressure</td>
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<td>M/I</td>
<td>insulin sensitivity index</td>
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<td>M</td>
<td>insulin-stimulated glucose uptake</td>
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<td>AUC</td>
<td>area under the curve</td>
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<td>PA</td>
<td>physical activity</td>
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<td>W/H ratio</td>
<td>waist-to-hip ratio</td>
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<td>VTI</td>
<td>velocity time integral</td>
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<td>SV</td>
<td>stroke volume</td>
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<td>MV</td>
<td>minute volume</td>
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<tr>
<td>ICC</td>
<td>intra-class correlation coefficient</td>
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<td>CF</td>
<td>correction factor</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>CI</td>
<td>confidence interval</td>
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<td>ANCOVA</td>
<td>analysis of covariance</td>
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<td>SD</td>
<td>standard deviation</td>
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<td>ACE</td>
<td>angiotensin converting enzyme</td>
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Introduction

In 1923, a Swedish physician Eskil Kylin described "das Hypertonie-Hyperglykämie-Hyperurikämiesyndrom" in Zentralblatt für innere Medizin 1. However, the modern concept and pathophysiological mechanisms behind this syndrome were elegantly introduced by Gerald Reaven, who called it syndrome X 2. His initiative resulted in a profound research and a public interest about the syndrome, which is crucial for development of type 2 diabetes – a disease predicted to affect about 220 million people worldwide by 2010 3.

Interest in this syndrome has been both intense and complex. This is probably the reason why various researchers have their own favorite expression to label the syndrome. For example; the metabolic syndrome 4, syndrome X 2, the deadly quartet 5 and insulin resistance syndrome (IRS) 6.

IRS is a constellation of associated clinical and laboratory findings, consisting of glucose intolerance, central obesity, dyslipidemia (increased triglycerides, decreased HDL, increased small dense LDL), hypertension, increased prothrombotic factors, and a predisposition for atherosclerotic vascular disease 7. Sixty years ago according to Himsworth and Kerr 8, the underlying state essential for development of this syndrome is the insulin resistance defined as an impaired biological response to insulin. This biological action of insulin does not apply only to the glucose uptake by target organs, but also includes its effects on lipid and protein metabolism and on vascular endothelial function.

IRS is a very prevalent condition, but due to a lack of widely accepted criteria, the prevalence of this syndrome shows large differences between studies 9-11. In 1998, the WHO proposed a unifying definition for the syndrome. The WHO chose to call it the metabolic syndrome rather than the insulin resistance syndrome. This was primarily because it was not agreed that insulin resistance (IR) was the cause of all components of the syndrome 12. In spite of that, measures of IR were still included in the definition. The prevalence of IRS in the present cohort is 12.5% at age 50 years and 18.8% at age 70 years 13. In another Swedish population of more than 4000 men and women from Stockholm County at 60 years of age the prevalence of IRS, defined by the WHO definition, was 29% among men and 15% among women (Maj-Lis Hellenius 2001, personal communication). In the population of Framingham, USA, the prevalence of IRS ranged from 22% to 27%14. One recent population-based Botnia study with 4483 subjects from Sweden and Finland showed an overall prevalence of 15% for IRS among men with normal glucose tolerance 10. In several population-based studies, among men with impaired glucose tolerance (IGT), 64% to 66% showed IR, which was present in 84% to 88% of men with type 2 diabetes 9, 10.

Insulin resistance (IR) to insulin action on glucose uptake can be measured by several methods, but hyperinsulinemic euglycemic clamp has been considered to be the “golden standard” 15. One of the crucial findings about the fait of the circulating glucose was made by Ralph DeFronzo et al. 16. They calculated that
during insulin-stimulated condition the majority, i.e. up to 85%, of circulating glucose was taken up by skeletal muscle. Prior to that time, skeletal muscle and its structure had almost exclusively been the interest of researchers involved in exercise physiology. Thus, it was reasonable to expect that skeletal muscle characteristics may have important health-related implications such as playing an important role in whole-body glucose metabolism. Studies described in the present thesis were therefore aimed at investigating the associations between skeletal muscle characteristics and insulin sensitivity as well as different components of insulin resistance syndrome in a population-based sample.

**Skeletal muscle characteristics: fiber type distribution and capillary density**

Human skeletal muscle is composed of two major fiber types which differ in their metabolic and physiologic characteristics. The histochemical staining patterns of these fiber types became a research focus subsequent to the description of the needle biopsy technique in 1962 by Bergström. Currently, fiber types are most often distinguished on the basis of their susceptibility to loss of myosin ATPase activity in response to changes in preincubation pH. In human skeletal muscle there are two main categories of fiber types – type I and type II. Type II fibers are further sub-classified as type IIA and IIB based on their resistance to loss of histochemically detectable myofibrillar ATPase at low pH values (Figure 1). Type IIA fibers are intermediate in most respects between type I and type IIB fibers. Myosin from type II fibers is alkaline stable but acid labile. The opposite is true for myosin from type I muscle fibers. The subgroup of IIB fibers may more properly be named type IIX. Recent studies have revealed IIB fibers most closely correspond to the type IIX fiber in rat, when the mRNA of myosin heavy chain (MHC) isoforms were analyzed immunohistochemically and electrophoretically. Determining the distribution of different fiber types by their content of MHC isoforms is now considered an acceptable alternative to staining for myosin ATPase activity and is currently often used in studies on muscle morphology and function.

Type I fibers have slow contraction velocities and are referred to as slow-twitch; whereas type II fibers have fast contraction velocities and are designated as fast-twitch.

The most extensively studied human muscle is the lateral portion of the quadriceps femoris muscle (vastus lateralis). The mean proportion of type I fibers in the vastus lateralis muscle is approximately 50% and type IIA fibers are typically twice as common as type IIB fibers.
Figure 1. Human beings, in contrast to other species, have a more homogeneous mixture of fibers in nearly all muscles. This figure shows the myosin ATPase stain (preincubation pH=4.58) of the muscle fibers of one study subject. Type I fibers are stained black, type IIA fibers are stained white, and type IIB are stained gray.

Histochemical staining of human skeletal muscle has shown the existence of major differences in the metabolic profiles of type I and type II fibers. In general, type I fibers have higher aerobic capabilities by virtue of their increased oxidative enzyme activities. The activities of such oxidative enzymes like 3-hydroxyacyl-CoA dehydrogenase, succinate dehydrogenase and citrate synthase are 30% to 50% higher in type I fibers as compared to type II fibers. Type I fibers also exhibit a higher concentration of mitochondria than type II fibers. Based on rodent research, it has been found that type I fibers have a higher insulin sensitivity and insulin responsiveness than type IIB fibers. These differences in insulin action have been attributed to higher insulin binding capacity, insulin receptor kinase activity and phosphorylation in muscles containing type I fibers. In rats, key protein functions and expressions of the insulin-signaling cascade are greater in oxidative versus glycolytic muscle. Additionally, there is direct evidence that glucose transporter GLUT4 in humans is more abundant in type I muscle fibers than type II fibers. There is a twofold-higher mean value for the activity of glycolytic enzymes in type II than in type I fibers, whereas those activities are 20%-30% higher in type IIB than type IIA fibers. Lipid content of individual fibers shows a distinct difference with type I fibers containing three to five times higher levels of triglycerides (TG) than type II fibers. This might partially be explained by the finding that higher level of fatty acid binding protein has been related to higher proportion of type I fibers. Fatty acid composition in the
skeletal muscle membrane phospholipids is also related to the different types of skeletal muscle fibers. An increased content of polyunsaturated fatty acids within cell membranes has been shown to be associated with an increase of membrane fluidity, insulin receptor density and insulin binding. Opposite effects have been noted when the content of saturated fatty acids increases in membranes. In humans, lower proportions of saturated palmitic acid and higher proportions of long-chain polyunsaturated fatty acids in muscle membrane were positively correlated to the percentage of type I fibers.

Abundant evidence is available demonstrating differences in capillary density for type I and type II fibers. The number of capillaries surrounding a specific fiber type in mixed muscles is largest for type I fibers. Capillaries range between 4-11 and 2.5-3 for type IIA and 2-3 for type IIB fibers, respectively. The size of various fiber types should also be taken into account, since type I fibers are usually smaller than type II fibers. In untrained muscle the average area supplied by each capillary is 10%-30% more for type II fibers than for type I fibers. There are approximately 300-400 capillaries in a square millimeter of muscle tissue. From among variables used to quantitate capillarization, the question of which variable serves as the best indicator of diffusing conditions is still unsettled. Some authors prefer the number of capillaries per fiber, whereas others favor Krogh’s concept of a capillary and its “diffusing” cylinder.

The most widely used method for staining capillaries is the amylase-periodic acid-Schiff (PAS) staining reaction, which stains the basement membrane of the capillaries of the human muscle. This method is able to detect all capillaries present in a muscle sample compared to electron microscopy. Recently, a new immunohistochemical method has been developed in order to better visualize capillaries with the help of antibodies against endothelial cells. This new method has been claimed to be superior to the more commonly used PAS method.

Reproducibility of skeletal muscle morphology

An important question is whether small muscle samples from a human muscle, such as those obtained with a biopsy technique, will give acceptable information about the fiber composition, fiber size, capillary supply and chemical composition of the entire muscle. The reproducibility of determination of muscle fiber composition by the needle biopsy technique has been reported in a number of studies with duplicate biopsies taken from the same site of the muscle, where the coefficient of variation (CV) of fiber composition ranges from 5% to 44%. Biopsies taken from different depths of the lateral vastus and tibialis anterior muscles have demonstrated predominance of type II fibers at the surface and type I fibers in deeper regions. Differences in fiber type distribution between dominant and non-dominant leg have been reported to be significant in one study, but not in others. Reproducibility of capillary density has been determined only in a few studies. It has been analyzed in different sections of the muscle...
same biopsy with the CV ranging from 4% to 9% 54-56. A level of 200 fibers per biopsy has been suggested as a limit above which adequate muscle morphology data are obtained 48. However, large samples have not been shown to reduce this variation substantially nor does an open versus needle biopsy produce a significant difference 24, 57. Due to this quite high biological and individual variation, the relationships including muscle morphological characteristics might be attenuated and this could imply that we might fail to see some relationships that in fact do exist. A method where the correlation coefficients are corrected relying on reproducibility data of muscle morphology has not been used previously, although it has been applied to correct for variation in insulin sensitivity 58. Therefore, we have undertaken a study aimed at correcting for the variations in muscle morphological features and calculating the true correlations by adjusting for that bias.

**Skeletal muscle morphology and physical activity**

The role of skeletal muscle characteristics in athletic performance has been broadly investigated over the last 25 years. The reintroduction of the muscle needle biopsy for exercise research by Bergström and Hultman 17 has allowed direct measurements not only in muscle chemistry, but also in muscle morphology. Buller et al. provided the first important contribution about exercise-induced changes in muscle fiber type in 1960, when they reported the occurrence of fiber transformations during the cross-innervation of slow muscles by fast nerves and vice versa 59. The fact that muscle fibers are convertible was further developed in experimental studies on rodents with cross-innervation and electrical stimulation of different types of muscles 60-62. In these experiments, when a slow muscle was stimulated at 40 Hz, it showed more rapid contractions 60 and a decreased capillary supply 63. In addition, a transformation of fast- to slow-twitch muscles has been observed after low-frequency pacing 61, 62. It is known, that the skeletal muscles of world-class sprinters contain a high percentage of fast-twitch fibers (type II), whereas the skeletal muscles of elite endurance athletes have a high percentage of slow-twitch type I fibers 64, however these alterations in fiber pattern have mostly been ascribed to genetic factors 65. The current data suggest it may be possible to induce some shift within type II fibers from type IIB to type IIA 66, 67. Trained muscles have 3-4 times higher oxidative enzyme levels 68, whereas the content of glycolytic enzymes is only marginally affected 64. Skeletal muscle capillarization in man is enhanced by 50% in response to endurance training 37 and the difference in capillarization between endurance athletes and untrained individuals has been found to be two-to threefold in favor of athletes 19. In fact, all variables of capillary supply (capillaries per fiber, capillary density per square millimeter, and number of capillaries found around fiber) increased. Capillaries per fiber, which is less influenced by fiber size, is the most frequently used measure in training studies because this indicator is most closely related to whole-body maximal oxygen uptake of a subject 19. The increase in capillaries is larger than the increase in fiber
size resulting in a definite reduction in the fiber-type area each capillary needs to supply 19.

Physical activity and features of insulin resistance syndrome

Exercise training results in multiple physical and metabolic changes which have important implications especially for individuals with IGT destined to develop type 2 diabetes. Prospective cohort studies demonstrate a reduced risk of developing type 2 diabetes with increased physical activity 69-71. In addition feasibility studies in Sweden and China showed an ~50% reduction in the number of subjects who developed diabetes over a five-to six-year follow-up in the group that exercised compared with the group that did not 72, 73.

In 1972, Björntorp et al. suggested that exercise training might increase tissue sensitivity to insulin 74. Soon after, studies using the hyperinsulinemic-euglycemic clamp demonstrated that exercise-trained people have a higher rate of insulin-stimulated glucose disposal than do their sedentary counterparts 75-78. This impact on insulin sensitivity has been explained by substantial evidence about the parallel increase in GLUT4 expression by 60%-80% during exercise training 79-81. Interestingly, exercise training increases GLUT4 even without the presence of insulin implying the utilization of a different pathway for activation of glucose transport 82. It appears it is the summed effects of regular, individual bouts of exercise that accounts for much of the improvement in insulin sensitivity and glucose disposal during training regimens 83.

The evidence of the efficacy of regularly performed exercise in lowering blood pressure is rather extensive. Three meta-analyses, with the earliest including 29 studies 84 and the two latest including 44 studies 85, 86, concluded that low-to-moderate intensity exercise [30% to 74% VO2max or maximum heart rate (HR)] might be more effective in lowering blood pressure than higher intensity exercise 84. In this analysis, the decrement of blood pressure evoked by exercise was not sufficient to produce normotension in many studies. A more pronounced decrease in systolic blood pressure (SBP) by 7-10 mmHg and diastolic blood pressure (DBP) by 6-8 mmHg with exercise appears to be limited only to hypertensive patients, especially with concomitant insulin-resistance 85-88.

Endurance training increases the capacity for clearance of free fatty acids (FFA) and TG by skeletal muscle, which is one of the main target tissues for FFA disposal together with the liver 89. Skeletal muscle might be responsible for approximately 50% of the TG removal 90. It is known that trained individuals rely more on fat as an energy substrate than untrained ones, in spite of the fact that FFA levels are often lower in endurance-trained subjects 19. The most consistent effect of increased physical activity on the plasma levels of lipids has been observed to be a decrease in plasma levels of TG. It has been shown that physical training decreases plasma TG concentration by 15%-30% 91, 92. An increase in levels of HDL has been demonstrated only if exercise is intense and prolonged 93. Elevation of HDL cholesterol levels is usually seen due to enhanced lipoprotein
lipase activity (LPL) in skeletal muscle. Training increases LPL activity in skeletal muscle causing muscle TG storage to be replenished from serum TG-rich lipoproteins after exercise. Muscle morphological features might play a role in these processes. In one study, a higher proportion of type I fibers was associated with lower serum TG. In fact, lipoprotein lipase activity in skeletal muscle was shown to correlate to capillary density, which together with the percentage of type I fibers explained 75% of the variation in serum triglyceride level. Besides, poor oxidation of FFA has been suggested to be associated with a low percentage of type I fibers.

In patients with IRS, impaired fibrinolytic activity is largely the result of increased activity of plasminogen activator inhibitor-1 (PAI-1), which in turn is related to elevated levels of insulin and TG, but can be lowered by regular physical exercise.

Obesity, especially when situated abdominally is one of the most prominent findings in patients with IRS. Regular exercise is rarely associated with substantial weight loss in the absence of concomitant caloric restriction. Importantly, in one follow-up study aiming to assess the impact of cardiorespiratory fitness on all-cause and cardiovascular mortality by Lee et al., in a cohort of 22,000 subjects, they observed that obese and fit men had a lower risk of all cause and cardiovascular mortality than did the unfit and lean men. Thus it seems, that increased physical activity protects against the health hazards induced by obesity.

Elevated heart rate is an established independent cardiovascular risk factor and is also an essential component of IRS. One characteristic change associated with endurance training is a decrease in resting heart rate. The underlying mechanism of this bradycardia may be a combination of three factors: a reduction in the intrinsic heart rate, increased parasympathetic tone, and a somewhat decreased sympathetic nervous activity (SNS) as reviewed recently. Thus, the impact of increased physical activity on autonomic function might be one possible explanation for its cardioprotective effect and other health benefits.

Questionnaire assessment is the most widely used method for exploring habitual physical activity in large-scale epidemiological studies. Various questionnaire assessments, involving self-rating of habitual physical activity, have been extensively validated. To date, however, no validation study concerning physical activity has included the determination of skeletal muscle morphology characteristics such as fiber type distribution and capillary density. In the present study, we used a large sample of elderly men from the general population to study if muscle morphological features are associated with self-reported physical activity and the features of IRS. Secondly, we investigated the importance of muscle morphology when the association between physical activity and insulin sensitivity is evaluated.
Muscle morphology and insulin resistant states

In support of a role for muscle morphology in IRS, a predominance of type II and particularly type IIB muscle fibers together with a low capillary density has been linked to insulin resistance 116-121, 122. However, in a large number of studies a correlation of insulin action to different types of muscle fibers or capillary supply has not been confirmed 35, 123-126. Large variation either in measurement of insulin action or in muscle morphology or in both, and small subject numbers in study groups might explain this lack of confirmation. Individuals with type 2 diabetes have also been found to have a low percentage of type I fibers with high proportion of type IIB fibers 119, 120. It has been observed that obesity-related insulin resistance is associated with a greater percentage of type IIB fibers and capillary rarefaction 116, 117, 127, 128. Furthermore, the amount of body fat is inversely related to the proportion of type I fibers 129 and a higher proportion of type IIB fibers has been observed in the skeletal muscle of obese subjects 98. In fact, obesity might explain up to 40% of the variations in muscle morphology 98.

Hypertension also has its origin in insulin resistance 2, 130, 131. A more intriguing and less studied aspect is to what degree muscle structure, i.e. fiber composition and capillary density, is related to hypertension. Hypertension is characterized by elevated peripheral vascular resistance as a result of microvascular constriction 132. Hutchins and Darnell were the first to suggest that microvascular rarefaction represents an important mechanism in primary hypertension at least in experimental settings 133. It has been proposed that in hypertension a microvascular constriction can reach the point of non-perfusion of the vessel leading to its disappearance 134. This is because in hypertension, which is a state with heightened sympathetic nervous system activity, small arterioles have increased sensitivity 135 and reactivity 136, 137 to norepinephrine and other vasoconstrictive agents. Vascular hypertrophy, a measure of vascular resistance 138, is significantly related to plasma norepinephrine levels 139. These observations led to human studies, where a reduced capillarization was observed in the muscle of hypertensive subjects 140, as well as in other tissues 141-145. Additionally, some capillary rarefaction was observed in the skeletal muscle in hypertension-prone men 146. In one previous study, the percentage of oxidative type I fibers was found to be lower in hypertensives than in normotensive subjects 147. In another study a fairly higher proportion of fast-twitch, type II fibers was found in hypertensives than in normotensives (P=0.1) 148. Blood pressure has been related to the percentages of type I as well as type IIB muscle fibers 147-151. In women with a wide range of obesity, 20-30% of the variation in systolic and diastolic blood pressure could be explained by the variables of muscle morphology 149. In contrast, in a recent Norwegian study, neither the fiber distribution nor the capillarization of muscle differed between non-diabetic, hypertensive subjects and normotensive controls matched for body mass index (BMI) 152. These authors, therefore, questioned the claims of earlier studies suggesting that deteriorated
glucose metabolism, obesity and abdominal obesity had confounded the results. As alterations in muscle morphology are associated with insulin resistance and obesity, we aimed at comparing the muscle morphology in glucose tolerant, untreated hypertensives to that of healthy subjects in a population-based sample.

Muscle fiber type distribution and heart rate were not related in pooled analyses of normo-and hypertensive subjects 147, 148, 153. Although, in one study, young healthy males with higher proportions of fast-twitch fibers showed a higher resting heart rate 154. Therefore, one of the aims of our study was to test the hypothesis of a relationship between muscle fiber pattern, capillary density and heart rate in a normo-and hypertensive population.

**Endothelial function and capillary density**

It is claimed that endothelium is the largest organ, which covers an area of approximately 700 m² and weighs about 1-1.5 kg in a person with a body weight of 70 kg 155. Endothelial function is important for the maintenance of blood flow. A hallmark of endothelial dysfunction is a reduced availability of nitric oxide (NO). Along with other functions, insulin has also been found to increase the blood flow in skeletal muscle beds through vasodilation, which is endothelium-dependent 156, 157. The vasoactive effect of insulin implies a balance between its local vasodilatory capacity and its vasoconstrictive central sympatho-excitatory effect with the purpose of maintaining blood flow 158-160. Many disease states with increased sympathetic tone and insulin resistance, like hypertension (though not yet fully clear) 161-164, obesity 156 and type 2 diabetes 165 are also characterized by endothelial dysfunction. According to a suggestion by Baron and Laakso, insulin-mediated augmentation in blood flow and muscle perfusion are accompanied by capillary recruitment in skeletal muscle 156, 166. In one recent study in young subjects, the number of capillaries per fiber in the leg and the insulin-induced blood flow in the forearm showed a close association 121. This has also been also seen in skin 167. In studies investigating insulin-stimulated blood flow in healthy individuals, elderly subjects are often excluded. This exclusion is partly based on observations indicating a deleterious impact of aging on endothelial function 161, 168-173. Therefore, in a subsample of 15 healthy elderly men from a population-based cohort, we investigated to what degree differences in insulin-induced changes in leg blood flow (LBF) are explained by capillarization in the femoral muscle bed.
Aims of the study

I To investigate the relationships between insulin sensitivity and muscle morphology Additionally, to evaluate the influence of intra-individual variability on correlations between muscle morphology and measures of insulin sensitivity in a population-based sample of 515 elderly men and to calculate the unconfounded correlations by adjusting for that variation.

II To investigate whether muscle morphological characteristics are related to self-reported physical activity and to the components of insulin resistance syndrome. An additional aim was to study to what degree muscle morphology contributes to the association between physical activity and features of the insulin resistance syndrome.

III To compare the muscle morphology in untreated, newly diagnosed hypertensive subjects with that in controls and to test the hypothesis of an association between muscle morphology, heart rate, and development of hypertension during 20 years in a state of normal glucose tolerance.

IV To determine to what degree differences in insulin-induced changes in LBF are explained by capillarization in the femoral muscle bed in 15 healthy elderly subjects with a known metabolic profile.
Methods

A population-based cohort

The subjects in this study participated in the Uppsala Longitudinal Study of Adult Men (ULSAM) aimed at identifying risk factors for cardiovascular disease as previously described. All men in Uppsala born between 1920 and 1924 (n=2841) were invited for a health investigation in 1970, with 2322 (82%) participating. Participants still alive and living in Uppsala were traced by their ten-digit social security number and invited for re-examination 20 years later, in 1991, with 1221 men (73%) participating. Investigations included an oral glucose tolerance test (OGTT) as well as euglycemic, hyperinsulinaemic clamp. Muscle biopsy specimens obtained for muscle morphology assessment were available from a random sample of 515 70-year-old men (Figure 2).

Figure 2. The ULSAM study

Study subjects

In study 1, all 515 men with muscle morphology determinations were included. The men included in the study showed no difference from the remainder of the cohort men regarding the prevalence of IGT (24%) and type 2 diabetes (14%) according to WHO criteria from 1985. Around half of the men in the study (57%) were on drug treatment. Of these, 65% (n=177) received cardiovascular drug treatment and 8% (n=21) received antidiabetic drugs. The prevalence of smoking was 20% (101 men). A subgroup of subjects (n=23) volunteered for the reproducibility study where muscle biopsy, OGTT and clamp procedure (n=21) were repeated within 4–6 weeks. These repeated procedures were used to determine the combined effects of biological variation and measurement error on measured variables. Among the subjects who underwent a repeated muscle biopsy procedure, five showed impaired glucose tolerance, three were diabetics and seven had hypertension. Fifteen
subjects received drug treatment. None of the men were involved in intense physical training as investigated by a questionnaire about their leisure time physical activity. **In study II**, all 515 men were included in a correlation analysis of morphology and the features of IRS. In forty men, data about their physical activity was missing. Thus the relationship between self-reported physical activity and muscle morphology was performed for 475 subjects.

**The population in study III**, which was selected from among 211 glucose tolerant subjects, consisted of untreated, and thus, newly diagnosed hypertensive men and healthy controls. Subjects were selected for this study as untreated hypertensives (n=43) when they were not receiving any drug treatment and had a supine systolic blood pressure of $\geq 160$ mmHg and/or a diastolic blood pressure of $\geq 90$ mmHg on two separate occasions in accordance with Swedish guidelines for diagnosing hypertension in 1990 and 1993178. The control group (n=113) was composed entirely of men who declared themselves to be healthy, with a normal blood pressure and who were not receiving any drug treatment. Subjects with coronary heart disease (data from Official Swedish In-patient Registry) were excluded from the present study.

**Study IV subjects** were drawn from a subgroup of 46 men, who had data on femoral artery blood flow measurement. Fifteen men with normal glucose tolerance and blood pressure were selected. None of them had a coronary heart disease (data from Official Swedish In-patient Registry) or received pharmacological treatment. Four of the study subjects were cigarette smokers.

The Ethics Committee of the Medical Faculty, Uppsala University, approved this study and each subject gave consent prior to participation.

**Investigations at age 50**

Investigations performed at age 50 years that were used in study II have been described in depth previously174. Blood pressure (BP) was measured on the right arm after 10 minutes' rest in the recumbent position using mercury manometers. The BP cuff had a rubber bladder, 12.5 cm wide and 35 cm long. SBP and DBP were read to the nearest 5 mmHg. DBP was recorded at the disappearance of the Korotkoff sounds (phase V). The BP was taken either by a registered nurse or a physician. The mean supine BP obtained by the physician in 231 subjects was 131.5/83.0 mmHg. The corresponding mean pressure obtained by the nurse in 216 men was 131.8/83.7 mmHg. Thus, there was no systematic error in the pressures obtained by the observers. The radial pulse rate was counted after 10 minutes rest before the BP measurement was taken. The mean pulse rate obtained by the physician in the same subjects as mentioned above was 69.4 and that obtained by the nurse was 69.0.

**Investigations at age 70**

All investigations were performed after an overnight fast.
Determination of muscle morphology

A biopsy of the right musculus vastus lateralis was obtained via an incision through the skin and fascia from the midlateral part of the muscle using a Bergström needle under local anesthesia. The biopsy was divided into three pieces; one for histochemical studies and the others for biochemical analyses described elsewhere. Biopsy samples were mounted in an embedding matrix (TISSUE-TEK®, O.C.T. compound 4583, Miles Laboratory, USA). The samples were quickly frozen in isopentane cooled liquid nitrogen and stored at −70°C until the histochemical assay was done.

Serial transverse sections (10 µm and 16 µm for analyzing fibers and capillaries, respectively) were cut in a cryostat at −20°C and mounted on cover slips. To identify fibers as type I, IIA and IIB, sections were stained for myofibrillar ATPase in a glycine buffer at pH 9.4 (ATP used was SIGMA A-5395) after acid preincubation (in acetate buffer at pH 4.58 for 60 seconds at a temperature of 21°C) 18, 180.

Sections to be stained to visualize capillaries were kept at −20°C and brought to room temperature just before staining by the Amylase-PAS method 36. After fixing the sections with Carnoy’s fixative (10 min) they were digested in 0.35% α-amylase (SIGMA A – 6880; 30 min, 37°C), oxidized in 2% periodic acid (H5IO6; MERCK 1.00524.; 10 min) and stained with Schiff’s reagent (Apteksbolaget, Stockholm; containing 0.45% fuchsine). This method has been proven to detect all capillaries present in a muscle sample 41. Dry sections were mounted with Kaiser’s glycerogelatin on cover glass. Sections from four different biopsies were stained and prepared at the same time.

A single investigator, who was blinded regarding the clinical data of the subjects, determined all morphological characteristics of the studied skeletal muscle morphology. Characteristics of skeletal muscle morphology were determined with the use of a computerized image analysis system designed for the analysis of skeletal muscle (Bio-Rad Scan Beam, Hadsund, Denmark), linked to an optical microscope (Leitz, Germany) by video camera (DAGE–MTI, Inc., CCD–72, USA). Determination of fiber type distribution and fiber areas, calculated automatically by the system, was performed from myofibrillar ATPase stained sections and capillaries from Amylase-PAS stained sections which were magnified x150. The diffusion distance, derived automatically by the system, represents the muscle area supplied by one capillary and was calculated by dividing the mean fiber type area by the mean number of capillaries surrounding this fiber type.

Capillary supply was determined both as capillary density per mm² and as the number of capillaries per fiber. Capillary counts were also calculated as the number found around each specific fiber type. In those cases where a capillary was cut longitudinally, it was counted as one at each cell junction as suggested earlier 37. On average 232±89 capillaries were counted per biopsy. The size of analyzed specimen areas in duplicate samples ranged from 0.40 to 1.18 mm² and was
dependent on the size of the biopsy; as large an area as possible was examined. Samples for muscle morphology determination in our cohort study were relatively small due to the fact that after obtaining the muscle biopsy it was dissected into several parts for different assays.

Reproducibility of the method was established by performing the morphology analyses for duplicate muscle biopsies (n=23) and duplicate measurements of the same biopsy by one or two investigators to determine intra- and inter-observer variation (n=10). A duplicate biopsy, performed 30 - 47 days later, was obtained from the same thigh, but 2 - 3 cm upward from the former biopsy site. The average amount (and range) of muscle fibers in specimens estimating reproducibility was 165 fibers (73-336).

**Determination of insulin sensitivity and glucose uptake**

Whole-body sensitivity to insulin and glucose uptake was measured by the euglycaemic hyperinsulinaemic clamp procedure according to the method of DeFronzo 15 with minor modifications. Insulin (Actrapid Human®, Novo, Copenhagen, Denmark) was infused in a priming dose for 10 minutes and then as a continuous infusion at a rate of 56 mU/m$^2$/min (instead of 40 mU/m$^2$/min) for 110 minutes, resulting in a steady-state plasma insulin concentration of 105 to 107 mU/l. This concentration of insulin has been shown to inhibit hepatic glucose output by 88-95% also in diabetics 181. The target level of plasma glucose during the clamp study was 5.1 mmol/l. Insulin sensitivity index (M/I) was calculated as the amount of glucose (M) infused per minute divided by steady-state plasma insulin (I), multiplied by 100 (mg/min/kg per mU/l). The CV for M/I was 14%.

**OGGT**

After an overnight fast the subjects were given 75 g of D-glucose in 300 ml of water. Blood samples were taken for plasma glucose and serum insulin determination 0, 30, 60, 90 and 120 min after the ingestion of glucose. Plasma glucose was measured by the glucose dehydrogenase method (Gluc-DH®, Merck, Darmstadt, Germany). Serum insulin was determined by an enzymatic-immunological assay (Enzymmun®, Boehringer Mannheim, Germany) performed in an ES300 automatic analyzer (Boehringer Mannheim, Germany). The incremental area under the curve (AUC) for glucose and insulin was calculated according to the trapezoidal rule estimate182.

**Physical activity level**

Leisure-time physical activity (PA) was assessed using four questions included in the medical questionnaire: 1) Do you spend most of your time reading, watching TV, going to the cinema or engaging in other, mostly sedentary, activities? 2) Do you often go walking or cycling for pleasure? 3) Do you engage in any active sport or
heavy gardening for at least 3 hours every week? 4) Do you regularly engage in hard physical training or competitive sport? Based on these questions, PA categories were constructed: Sedentary (I), Moderate (II), Regular (III), and Athletic (IV). The PA categories used in this study have been used and validated by others 110-115.

**Blood pressure, heart rate and anthropometrical measurements**

Blood pressure at age 70 years was measured in the right arm with the subject in the supine position after resting for 10 minutes. Measurements were made twice and the values were recorded to the nearest even figure. The mean of the two values was used for each blood pressure. The cuff size was 12x35 cm or 15x45 cm depending on the arm circumference. SBP and DBP were defined as Korotkoff’s sounds I and V, respectively. The mean arterial blood pressure (MAP) was calculated according to the formula MAP=DBP+[(SBP-DBP)/3]. The coefficient of variation (CV) for blood pressure variables ranged from 5.1% to 6.8%. Heart rate was measured in the supine position as radial pulse rate during one minute. BMI was calculated as the ratio of weight (in kg) to height (in meters) squared (kg/m²). The waist and hip circumferences were measured in the supine position. The waist was measured midway between the lowest rib and the iliac crest, and the hip at its widest girth. The waist/hip (W/H) ratio was calculated.

**Lipid and PAI-1 measurements**

Triglyceride and cholesterol concentrations in serum and in the isolated lipoprotein fractions were determined enzymatically (Boehringer Mannheim, Mannheim, Germany) in a Monarch instrument (Instrumentation Laboratories, Lexington, USA). HDL particles were separated by precipitation with magnesium chloride/phosphotungstate. The free fatty acid (FFA) concentration in serum was measured by an enzymatic method (Wako Chemicals GmbH, Neuss, Germany). The CV was 5.7% for serum total cholesterol, 11.1% for HDL cholesterol, 6.6% for LDL cholesterol and 14.8% and 24.2% for serum TG and FFA, respectively, in our laboratory. Plasminogen activator inhibitor-1 (PAI-1) activity was analyzed with a commercial two-step indirect enzymatic assay (Spectrolyse/pL PAI kits, Biopool AB, Umeå, Sweden). The CV was 7.2%.

**Assessment of insulin-mediated LBF**

LBF was measured in connection with euglycaemic hyperinsulinaemic clamp using the Doppler ultrasound technique. This method for blood flow measurements was considered preferable to venous occlusion plethysmography because it measures whole LBF. The technique for measuring and calculating LBF has been described in detail previously 183. Blood flow variables used in this study were velocity time integral (VTI), femoral artery stroke volume (SV) and femoral artery minute volume (MV). VTI, expressed in meters, representing the area under the systolic portion of the mean velocity trace, reflects the mean forward distance traveled by
the erythrocytes during a given systole, the stroke distance. Femoral artery SV (ml) was obtained by multiplying VTI by the cross-sectional area of the artery (cm²), with adjustment for the angle between the sample volume and the vessel wall [SV=VTI x 100 x (diameter/2)² x π x 2]. The corresponding femoral artery MV (ml/min) was then calculated by multiplying SV by the simultaneously measured heart rate. This femoral artery MV is hereafter referred to as LBF. The coefficient of variation (CV) for VTI, SV and LBF were 11%, 15% and 18%, respectively. Our study presents the measurement of LBF that takes the artery cross-sectional area into account and thereby represents an accurate volume estimation when measuring blood flow as recently suggested. Measurement of LBF with the Doppler ultrasound technique has previously been validated by the venous thermodilution technique and by venous strain-gauge plethysmography. Furthermore, this method of determining insulin-induced blood flow changes, measured in a large conduit artery, is also suggested to reflect the endothelial function in resistance vessels during hyperinsulinemia.

**Statistical analyses**

The SAS analysis system program (version 6.0.8 for Windows) and JMP 3.0.2 for Apple Macintosh computers (both SAS Institute Inc., Cary, NC, USA) and Stata 6.0 statistical software (Stata Corporation, College Station, Texas, USA) were used for calculating results. Spearman’s Rank Correlation was used to test linear regressions for significance, ANOVA was applied to compare group means and Student’s t-test (or Wilcoxon Signed Rank Sum test for non-normally distributed data) was used for post-hoc analyses. The Chi square or Fisher's exact test were used to calculate proportional differences between the groups. \( P < 0.05 \) was considered significant.

**Study I.** All the estimates of reproducibility concerning duplicate biopsies were calculated by ANOVA with factors for subject and time period. The time factor was used to correct for possible systematic differences that could arise from biopsy sampling on different locations of the thigh. Intra-class correlation coefficients (ICC) and corrected correlation coefficients (\( r_{true} \)) for muscle morphology variables were estimated to remove the bias caused by within-subject variation in multiple correlations according to the method provided by Rosner and Willett (1988): \( ICC=[1+(\overline{\delta^2_w/\delta^2_B})^{-1}] \). where \( \overline{\delta^2_w} \) is the within-subject variance and \( \overline{\delta^2_B} \) is the between-subject variance. When ICC is \( \geq 0.50 \), the design efficiency is usually optimal (i.e. standard error is minimal), if the number of replicates is no more than two. \( R_{true} \) for the correlation was calculated by multiplying the observed correlation coefficient (\( r_{obs} \)) with the correction factor (CF) \( [\sqrt{1+(\overline{\delta^2_w/\delta^2_B})}] \) for the variables involved. Thus, the CF reflects the underestimation of a true correlation due to intra-individual variation. For example, in a case the CF equals 1.5, the underestimation is 33% \( [100-(100/CF)] \).
Intra-individual differences between mean values were tested for significance using Student’s paired t-test. When normality was not achieved by logarithmic transformation of data, a non-parametric test (Spearman’s Rank or Spearman’s Rank Partial Correlations) was used.

**Study II.** Spearman’s Partial Rank Correlation was used to correct for possible effects of known confounders (BMI, W/H ratio, glucose intolerance, drug treatment, blood pressure, smoking and/or physical activity level). All correlation coefficients (except those in multiple regression analysis) were adjusted for the intra-individual variation with the help of the correction factor of the respective variable. A multivariate stepwise regression analysis with forward selection was performed to investigate determinants for insulin sensitivity. Finally a standard multiple regression, based on the last step in the stepwise regression, and calculation of partial correlations were performed. Candidate independent variables for the model were variables with a significant correlation with the insulin sensitivity index in the bivariate analysis. Results are presented as cumulative $r^2$ for each step and partial correlation and P value for the final model. Confidence intervals (CI) were two-sided.

**Study III.** Changes over time were tested for significance with the paired t-test. When normality was not achieved by logarithmic transformation of data, a non-parametric test (Spearman’s Rank Correlations) was used. ANCOVA (Pearson’s Partial or Spearman’s Partial Rank Correlation) was used to correct for possible effects of the three known confounders of muscle morphology namely BMI, W/H ratio and the level of physical activity. All correlation coefficients were adjusted for intra-individual variations of the respective variables.

**Study IV.** ANCOVA (Pearson’s Partial or Spearman’s Partial Rank Correlation) was used to correct for possible effects of known confounders, namely BMI, W/H ratio, level of physical activity and smoking status. The dependent variable in multiple regression analysis was the change in LBF during hyperinsulinaemia. The independent variables were the capillary density in muscle and the serum level of FFA. Calculation of a 95% confidence interval (CI) of the correlation coefficient was performed according to Steiger and Fouladi 195.
Results

Reproducibility of muscle morphology variables (I)

The CV for different characteristics of muscle morphology was between 11% and 42% in duplicate biopsies. The CV for markers of insulin sensitivity ranged between 12% and 39%. The variability reflected by intra-class correlation ranged from 0.23 to 0.60 for muscle morphology and from 0.68 to 0.96 for estimates of insulin sensitivity and glucose tolerance. There was no significant difference when the assessment of muscle morphology determination was compared between two investigators, but the variability did tend to be somewhat smaller, when only one investigator performed the analysis.

Relationships between muscle morphology and insulin sensitivity (I)

The proportions of type I and type IIB fibers as well as capillary density in mm$^2$ were significantly related to measurements of insulin sensitivity and insulin (Table 1). These correlations stayed significant after adjustment for obesity and physical activity. Most pronounced was the positive relationship between capillary density in mm$^2$ and the insulin sensitivity index. Insulin sensitivity correlated negatively and insulin variables correlated positively to all fiber areas, most strongly with the type IIB fiber area. After correcting these relationships for within-subject variation (ICC), up to a twofold improvement of the correlation coefficient ($r_{true}$ compared to $r$) was seen due to removal of the bias caused by variation within the muscle (Table 1).

Table 1. Correlations between muscle morphology, insulin sensitivity and insulin ($r_{true}$) and without ($r$) adjusting for within-subject variation in a population-based sample of 515 men.

<table>
<thead>
<tr>
<th></th>
<th>Type I fibers (%)</th>
<th>Type IIB fibers (%)</th>
<th>Capillaries in mm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r$</td>
<td>$r_{true}$</td>
<td>$P$</td>
</tr>
<tr>
<td>M/I</td>
<td>0.21</td>
<td>0.33</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>f-insulin</td>
<td>-0.15</td>
<td>-0.23</td>
<td>0.0005</td>
</tr>
<tr>
<td>2-insulin</td>
<td>-0.12</td>
<td>-0.21</td>
<td>0.0063</td>
</tr>
<tr>
<td>AUC insulin</td>
<td>-0.08</td>
<td>-0.14</td>
<td>0.0911</td>
</tr>
</tbody>
</table>

AUC - area under the curve during the OGTT
Relationships between muscle morphology and self-reported physical activity (II)

The prevalence of men at each level of physical activity agreed with the figures from the whole cohort confirming the sub-sample of study II was representative of the population (Table 2).

Table 2. Prevalence of men at each level of physical activity (PA) in the whole cohort and in Study II.

<table>
<thead>
<tr>
<th>PA category</th>
<th>Whole cohort (n=1098)</th>
<th>Population in study II (n=475)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>I</td>
<td>43</td>
<td>4</td>
</tr>
<tr>
<td>II</td>
<td>370</td>
<td>34</td>
</tr>
<tr>
<td>III</td>
<td>619</td>
<td>56</td>
</tr>
<tr>
<td>IV</td>
<td>66</td>
<td>6</td>
</tr>
</tbody>
</table>

PA categories: I, sedentary; II, moderate; III, regular; IV, athletic

The percentages of type I and type IIB fibers showed significant associations with the level of physical activity and these relationships were independent of obesity (BMI, W/H ratio), smoking habits, diabetes, hypertension, drug treatment and heart rate (Figure 3). The number of capillaries around the different fiber types (except that of type IIB fibers) also showed significant positive and independent relationships to physical activity level. This resulted in a 30% higher number of capillaries per fiber in the group with the highest PA, compared with the lowest PA group ($P<0.001$). Capillary density expressed as number of capillaries per mm$^2$ is highly dependent on the average fiber area. Mean fiber area showed a significant (200-300µm$^2$) increase with one level of increase in PA. This was attributed primarily to a significant positive trend for type I fiber area with increasing level of PA. The 10% difference in number of capillaries per mm$^2$ between the first and the fourth physical activity group was therefore not statistically significant.

Figure 3. Muscle fiber composition at different levels of self-reported physical activity (n=475). P, P for trend; $P_{adj}$, P for trend adjusted for obesity (BMI, W/H ratio), smoking habits, diabetes, hypertension, drug treatment and heart rate.
Relationships between self-reported physical activity and features of IRS (II)

The prevalence of type 2 diabetes decreased significantly with increasing level of PA (P<0.05), whereas the decrease in the prevalence of IGT did not reach significance (P=0.09). Insulin sensitivity and glucose uptake were positively and independently related to physical activity level (P<0.001 for both). Lower levels of serum triglycerides (P<0.01), PAI-1 activity (P<0.05) and heart rate (P<0.05) were associated with a higher physical activity level. However, these relationships were explained by muscle morphology variables as they lost significance after adjusting for fiber type distribution and capillary density in mm². The measures of obesity (BMI, W/H ratio), blood pressure, serum total or LDL cholesterol were not significantly related to PA level. Furthermore, HDL cholesterol showed a positive and serum FFA concentration a negative, though insignificant, trend with increasing PA level.

Relationships between muscle morphology and features of IRS (II)

As shown in Table 3, there were significant relationships between muscle morphological variables and serum TG levels, HDL cholesterol and PAI-1 levels. The covariance analysis showed that the relationship between the percentage of type I fibers and serum TG was most influenced by PA level. HDL cholesterol was significantly related to the percentage of type I and IIB fibers as well as to capillary density, which appeared to be independent of diabetes, smoking, drug treatment and physical activity. The association lost its significance when the measures of obesity were added to the equation (P=0.06).

The proportion of type I fibers was significantly related to the PAI-1 level and remained marginally significant after adjustment for all possible confounders including serum TG, which only had a slight effect on this relationship (r=-0.18; P=0.05). The inverse association between capillary density and PAI-1 activity was not influenced by BMI, smoking, lipid levels, PA level, diabetes or drug treatment, but was attenuated when further adjusted for W/H ratio (r=-0.16, P=0.1).

Table 3. Significant associations of fiber type distribution and capillary density to serum lipids and plasminogen activator inhibitor-1 (PAI-1) activity in 515 men.

<table>
<thead>
<tr>
<th></th>
<th>Type I (%)</th>
<th>Type IIB (%)</th>
<th>Capillary density in mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum TG (mmol/l)</td>
<td>-0.19</td>
<td>0.0035</td>
<td>0.12</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>0.21</td>
<td>0.0012</td>
<td>-0.19</td>
</tr>
<tr>
<td>PAI-1 activity (U/ml)</td>
<td>-0.24</td>
<td>&lt;0.001</td>
<td>0.26</td>
</tr>
</tbody>
</table>

P, Spearman’s Rank Correlation; a confounded only by BMI and waist-to-hip ratio; b Confounded only by waist-to-hip ratio.
Relative proportions of type I and type IIB fibers were significantly correlated with supine heart rate when confounders (PA level, obesity, hypertension, treatment, diabetes and smoking) were taken into account ($r=-0.25$; $P<0.01$ and $r=0.18$; $P<0.05$, respectively). Capillary supply was not related to heart rate. All muscle morphology variables together, investigated in the present study, explained 16% of the variation in insulin sensitivity when forced into multiple regression model and half of this variation was attributed exclusively to the proportion of type IIB fibers. Regression analysis, including all relevant variables regarding insulin sensitivity, indicated the significant explanatory variables left in the equation were BMI, glucose intolerance, PAI-1 activity, serum FFA concentration, proportion of type IIB fibers, HDL cholesterol level, drug treatment, PA level, and W/H ratio, which together explained 55% of the variation in the insulin sensitivity index (Table 4).

**Table 4.** Multiple regression analysis with insulin sensitivity index (M/I) as a dependent variable

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Stepwise regression</th>
<th>Final model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Step</td>
<td>$r^2$</td>
</tr>
<tr>
<td>BMI</td>
<td>1</td>
<td>0.3119</td>
</tr>
<tr>
<td>Glucose intolerance (normal=0; IGT=1; type 2 diabetes=2)</td>
<td>2</td>
<td>0.4598</td>
</tr>
<tr>
<td>PAI-1 activity</td>
<td>3</td>
<td>0.4983</td>
</tr>
<tr>
<td>Serum FFA</td>
<td>4</td>
<td>0.5139</td>
</tr>
<tr>
<td>Proportion of type IIB fibers</td>
<td>5</td>
<td>0.5257</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>6</td>
<td>0.5345</td>
</tr>
<tr>
<td>Drug treatment (no=0; yes=1)</td>
<td>7</td>
<td>0.5410</td>
</tr>
<tr>
<td>Self-reported physical activity level</td>
<td>8</td>
<td>0.5472</td>
</tr>
<tr>
<td>W/H ratio</td>
<td>9</td>
<td>0.5518</td>
</tr>
</tbody>
</table>

$\beta$, standardized regression coefficient; IGT, impaired glucose tolerance; PAI-1, plasminogen activator inhibitor; FFA, free fatty acid; HDL, high density lipoprotein; W/H ratio, waist-to-hip ratio

Smokers compared to non-smokers showed a significantly lower proportion of type I fibers (44% vs. 50%, $P<0.01$) and capillary density in mm$^2$ (307 vs. 310, $P<0.05$) even after adjusting for possible confounders.

**Comparison of haemodynamic, metabolic and muscle morphological characteristics between untreated glucose tolerant hypertensives and healthy controls (III)**

Blood pressure and resting heart rate were significantly higher in the hypertensive group than in the controls at age 70 years. Blood pressure in hypertensive subjects
was already higher at age 50 years and changes over 20 years were highly significant for all hemodynamic parameters for the later group (Table 5).

**Table 5.** Office blood pressure and supine heart rate at ages 50 and 70 years in the study groups.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=113)</th>
<th>Hypertensives (n=43)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at age 50</td>
<td>at age 70</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>123 ± 11</td>
<td>134 ± 12**</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>77 ± 7</td>
<td>78 ± 6</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>93 ± 7</td>
<td>97 ± 7**</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>65 ± 10</td>
<td>63 ± 8*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, resting heart rate; *P<0.05 and ***P<0.001 compared to controls, †P<0.01 and ‡P<0.001 compared to age 50 years.

The groups did not differ regarding the measures of obesity (BMI, W/H ratio), physical activity level, plasma insulin and insulin sensitivity. However, the hypertensive group compared to the controls showed a significantly higher fasting glucose level (5.4 vs. 5.2 mmol/l, P<0.05) and 2-h glucose concentrations (6.2 vs. 5.7 mmol/l, P<0.05).

After correcting for BMI, W/H ratio and physical activity level the groups did not differ significantly with regard to fiber type distribution or fiber areas, but the hypertensive subjects had a significantly lower capillary supply than the controls, when analyzed as number of capillaries around different fiber types (Table 6).

**Table 6.** Characteristics of muscle morphology in normo- and hypertensive subjects adjusted for body mass index, waist-to-hip ratio and physical activity.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=113)</th>
<th>Hypertensives (n=43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution of fiber type (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>53.8 ± 17.0</td>
<td>50.6 ± 15.3</td>
</tr>
<tr>
<td>II</td>
<td>46.2 ± 17.0</td>
<td>49.4 ± 15.3</td>
</tr>
<tr>
<td>IIA</td>
<td>30.4 ± 13.5</td>
<td>32.3 ± 13.1</td>
</tr>
<tr>
<td>IIB</td>
<td>15.8 ± 11.3</td>
<td>17.1 ± 12.5</td>
</tr>
<tr>
<td>Area of fiber types (µm²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5190 ± 1240</td>
<td>5310 ± 1830</td>
</tr>
<tr>
<td>IIA</td>
<td>5080 ± 1410</td>
<td>4950 ± 1830</td>
</tr>
<tr>
<td>IIB</td>
<td>4420 ± 1280</td>
<td>4330 ± 1710</td>
</tr>
<tr>
<td>Mean area</td>
<td>5090 ± 1100</td>
<td>5110 ± 1750</td>
</tr>
<tr>
<td>No. of capillaries around different fiber types</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>4.4 ± 0.7</td>
<td>4.2 ± 0.7*</td>
</tr>
<tr>
<td>Type IIA</td>
<td>3.9 ± 0.9</td>
<td>3.6 ± 0.9*</td>
</tr>
<tr>
<td>Type IIB</td>
<td>3.5 ± 0.9</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td>Capillaries in mm²</td>
<td>326 ± 61</td>
<td>315 ± 73</td>
</tr>
<tr>
<td>Capillaries per fiber</td>
<td>1.64 ± 0.32</td>
<td>1.53 ± 0.34*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD; *P < 0.05 compared to normotensive controls.
Relationships between muscle morphology, blood pressure and heart rate (III)

There was no correlation between blood pressure and fiber type distribution in the whole group (n=156), but in the hypertensive group alone the proportions of type I and type II fibers were significantly related to MAP even when correction was made for obesity and physical activity (r= –0.56 and r=0.52, respectively; P=0.03 for both).

Compared to the controls, the hypertensives showed a significantly greater increase in blood pressure over the 20-year period, as shown in Table 1. The increase in MAP in the hypertensive subjects was strongly associated with capillary density in mm² (Figure 4).

![Figure 4. Relationship of capillary density in mm² to the change in mean arterial pressure (MAP) over a period of 20 years in untreated hypertensive subjects (n = 43).](image)

The total study population (n=156) was divided into tertiles according to heart rate at age 70 years. Trend analysis, with adjustments for BMI, W/H ratio and physical activity indicated significantly lower capillary density at age 70 years among those with the highest heart rate (Figure 5).

Capillary supply at age 70 years was even more strongly related to heart rate at age 50 years (Table 7), since the resting heart rate was higher at age 50 years than at age 70 years in both groups (Table 5).
Figure 5. Decrease in the mean number of capillaries per fiber over the tertiles of resting heart rate at age 70 years adjusted for body mass index, waist-to-hip ratio and physical activity (n=156).

Table 7. Relationship between capillary supply at age 70 years and resting heart rate at age 50 years adjusted for body mass index, waist-to-hip ratio and physical activity (n=156).

<table>
<thead>
<tr>
<th>Capillary supply</th>
<th>Resting heart rate at age 50 (beats/min)</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td></td>
<td>-0.35</td>
<td>0.01</td>
</tr>
<tr>
<td>Type IIA</td>
<td></td>
<td>-0.36</td>
<td>0.01</td>
</tr>
<tr>
<td>Type IIB</td>
<td></td>
<td>-0.25</td>
<td>0.08</td>
</tr>
<tr>
<td>Per fiber</td>
<td></td>
<td>-0.30</td>
<td>0.02</td>
</tr>
<tr>
<td>In mm²</td>
<td></td>
<td>-0.66</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

Insulin-mediated changes in leg blood flow and its relationship to capillary density in skeletal muscle (IV)

Baseline values of femoral artery blood flow showed no correlation to the skeletal muscle characteristics. A positive significant relationship was found between the insulin-induced change in LBF and the capillary density in mm² (Figure 6). Adjustment for BMI, W/H ratio, smoking status or physical activity level had no effect on this correlation. Change in LBF during hyperinsulinaemia was not related to the relative proportions of different fiber types. Additionally, the number of capillaries per fiber was not correlated to any of the estimates of femoral artery blood flow.

The change in LBF showed an inverse correlation to serum levels of FFA (r= -0.57; P<0.05) (Figure 6) that was confounded by W/H ratio. The change in LBF also showed a positive correlation to glucose uptake (r=0.51; P=0.05), but not after correction for BMI, W/H ratio, smoking and physical activity. This relationship
was independent of BMI, smoking status and physical activity. In multiple regression analysis 71\% of LBF variations (95\% CI: 23\% to 88\%) during hyperinsulinaemia were explained by capillary density and serum FFA level (28\% and 46\%, respectively; P< 0.001 for the model).

The moderate increase in LBF of 15 ± 11\% caused by physiological hyperinsulinaemia during the clamp test (serum insulin 107 mU/l) did not reach statistical significance (P=0.07). This was probably because of the large variation in LBF (95\% CI, –1.5 to 32.2 ml/min) and heart rate responses (95\% CI, -7 to 2 beats/min) to hyperinsulinaemia among the study subjects. In four individuals the LBF decreased during clamping 16 ± 8\%, ranging from - 36\% to - 3\%. In contrast, in 11 subjects it increased by 27 ± 8\% (P<0.01; range 1\% to 94\%).

**Figure 6.** Relationship between insulin-mediated change in leg blood flow (LBF) and a) the number of capillaries in mm$^2$; b) serum FFA concentration.
Summary of results

In this population-based sample of 70-year-old men, higher proportion of type I fibers as well as higher capillarization in skeletal muscle were related to higher insulin sensitivity and higher self-reported physical activity, which were related to a lower prevalence of type IIB fibers. Serum triglycerides, HDL cholesterol and plasminogen activator inhibitor-1 (PAI-1) activity were significantly related to fiber distribution and muscle capillarization and muscle morphology, in part, explained the association between these metabolic risk factors with physical activity level. BMI, glucose intolerance, PAI-1 activity, serum FFA concentration, proportion of type IIB fibers, HDL cholesterol level, drug treatment, physical activity level, and W/H ratio together explained 55% of the variation in the insulin sensitivity index. In addition, almost a twofold improvement of the correlations was seen after correcting for intra-individual variation. The skeletal muscle of glucose tolerant hypertensive subjects from this cohort showed a lower capillary supply when compared to controls. Capillary density in the muscle was negatively correlated to the change in mean arterial pressure over two decades as well as to supine heart rate 20 years before. Interestingly, elevated supine heart rate was independently associated with a lower percentage of type I muscle fibers and with a higher percentage of type IIB muscle fibers. Capillary rarefaction and elevated serum FFA values were associated with a decrease in insulin-mediated blood flow and therefore to endothelial dysfunction. In fact, capillary density and serum FFA level together explained 71% of the variation in insulin-mediated leg blood flow changes.
Discussion

Variability in muscle morphological characteristics and in measures of insulin sensitivity (I)

There was a large variation in the characteristics of muscle morphology within vastus lateralis muscle, as well as in the measurements of insulin sensitivity. Our results of reproducibility, reflected by the CV, for fiber distribution, areas and capillarization were in good agreement with previous studies \textsuperscript{24, 45-49, 53, 56, 127, 196, 197}. Variation in the insulin sensitivity index was also in agreement with earlier reports \textsuperscript{198-201}.

The ICC for fiber type distribution in the present study was $\geq 0.50$, while the ICC for area measurements varied from 0.30 to 0.60, resulting in an ICC of 0.50 for the mean area measurement. It was also observed that capillary supply per fiber showed a smaller variation within the muscle than capillary density per mm$^2$. This might be due to the fact, that capillary per fiber ratio is much less affected by tissue handling \textsuperscript{202}. The ICC was 0.30 for capillary density in mm$^2$ and for the areas of types I and IIB fibers. When ICC is $\geq 0.50$, the design efficiency is usually optimal (i.e. standard error is minimal), if the number of replicates is no more than two \textsuperscript{191-193}. If the ICC is less than 0.50, due to a large biological heterogeneity, then the use of a large number of replicates is recommended to obtain relatively stable correlations \textsuperscript{194}. This design is probably not suitable for large cohort studies, where the burden of obtaining replicate biopsies could eliminate many subjects. In the present study it was preferable to have a greater number of participants instead of a large number of replicates. This has been seen to have advantages concerning statistical efficiency and easier recruitment of a study population more representative of the general population. The development of statistical techniques, which can eliminate the influence of biological variation (within-person variability), substitutes for the need of replicate samples, once a reproducibility study in a small group is done.

Application of the measures of reproducibility to relationships between muscle morphology and insulin sensitivity (I)

Large biological variation in studied variables might attenuate the correlation coefficients, and thus, contribute to the underestimation of the relationships between them. Different from the CV, the intra-class correlation has the advantage not to be dependent on neither the mean nor the standard deviation. Moreover, with the help of the ICC, which takes within- and between-subject variability into account, one can calculate correction factors used in correcting the correlation coefficients \textsuperscript{194}. With the help of correction factors, a measure reflecting the underestimation of a correlation, we observed that correlations
could be underestimated by up to 50% due to the variations in muscle morphology (Table 1). This underestimation is avoided exclusively by using the ratio of within- and between-subject variation for calculating the true correlation.

In this cross-sectional investigation of a population-based sample of 70-year-old men, higher proportion of type I fibers, higher capillary density and lower prevalence of type IIB fibers were related to higher insulin sensitivity irrespective of obesity and physical activity level (Table 1). The data of reproducibility regarding muscle morphology and the measurements of insulin sensitivity were applied to estimate unconfounded (corrected) correlations, where the influence of the intra-individual variation had been eliminated. After the adjustment, a clear and occasionally a twofold, improvement of the correlations between different muscle morphology variables and insulin sensitivity was noted (Table 1). As the intra-individual variations attenuates the relationships, we found it appropriate to adjust the relationships for these variations inasmuch as we found it correct to adjust the relationships for known confounders like obesity and physical activity. However, different methods have to be applied for respective adjustments.

**Interrelationships between muscle morphology, physical activity and IRS (II)**

It has been convincingly demonstrated that higher physical activity is associated with lower cardiovascular morbidity and mortality \(^{110, 115}\) as well as with decreased incidence of type 2 diabetes \(^{72}\). This is consistent with the findings from the present cohort population, where the level of physical activity at age 50 years was inversely associated to cardiovascular mortality 20 years later (hazard ratio=0.78, 95% CI: 0.67 to 0.92) \(^{203}\). Physical activity affects cardiovascular morbidity by modulating several cardiovascular risk factors, which are components of insulin resistance syndrome (IRS). In population-based studies, where the associations between physical activity and the features of IRS have been evaluated, the impact of muscle morphological characteristics on that association has been suggested but has not been assessed.

In this cross-sectional investigation of a population-based sample of 70-year-old men, a lower proportion of type I fibers, lower capillary density and a higher prevalence of type IIB fibers were related to lower self-reported physical activity as well as to components of IRS like elevated serum TG and PAI-1 levels, elevated supine heart rate, and low HDL concentration. Insulin sensitivity was closely associated with self-reported physical activity level independent of possible confounders. However, the relationship between physical activity level and several other components of IRS like serum TG, PAI-1 activity and heart rate were confounded by muscle fiber distribution and capillary density. An association of serum TG and HDL cholesterol with the proportion of type I fibers has been reported earlier \(^{96}\). In addition, in our study, serum TG and HDL cholesterol also
showed a significant association to the percentage of type IIB fibers and capillary density. Thus, it might be that at a higher level of PA, the heart rate is lower mostly due to an increase in parasympathetic tone \(^{109}\). Simultaneously, fiber distribution is shifted toward more oxidative fibers \(^{66, 67}\) and new capillaries are recruited \(^{19, 66}\), which leads to higher LPL activity with a simultaneous increase in the capacity of serum TG extraction and an elevation in HDL cholesterol levels \(^{94, 95}\). A consistent increase in HDL levels has been demonstrated only when exercise training is intense and prolonged. In Study II, HDL cholesterol also showed an increasing trend over PA levels, but this did not reach statistical significance.

Associations between muscle morphological features and PAI-1 have not been described previously. In the present study, the strongest association was observed between PAI-1 and capillary density. In fact, it has been experimentally shown that PAI-1 may be an important factor inhibiting angiogenesis in vivo \(^{204}\). Additionally, increased PAI-1 activity is associated with endothelial dysfunction \(^{205}\), which in turn is related to capillary density (Study IV). Thus the significant inverse relationship between capillary density and PAI-1 activity might have its origin in endothelial impairment.

The increasing level of PA was not associated with blood pressure, serum levels of total or LDL cholesterol or to measures of obesity. This is in accordance with previous reviews \(^{83-86, 102, 206}\).

Differences in muscle morphology between the adjacent categories of PA were most pronounced between the first and second category of physical activity. Hence, the largest difference was seen between sedentary men and men who did at least some leisure-time walking. The same findings were observed when glucose uptake, insulin sensitivity index and BMI were compared between adjacent categories of PA. Thus, the higher proportion of type I fibers, capillary density and lower proportion of type IIB fibers with an increasing level of PA were paralleled by higher insulin sensitivity. This verifies the findings in Study I concerning the association between muscle morphology features and insulin sensitivity.

Differences in relative proportion, fiber area and capillary supply across different levels of PA were most pronounced for type I fibers. These structural differences in skeletal muscle across the levels of physical activity were indicative of the nature of physical activity of the present study population, showing that this age group preferred low-intensity and endurance exercise. This was also supported by the observation that a higher PA level was not paralleled by a higher proportion of type IIA fibers, which have been shown to increase in number during intensified physical training \(^{19}\). The associations between skeletal muscle characteristics and physical activity remained significant after correcting for measures of obesity, glucose intolerance, hypertension, smoking and drug treatment. This indicates it was actually the level of physical activity \(\text{per se}\) and not the prevalence of concurrent disease states that was reflected in skeletal muscle characteristics. Additionally, this independent association reflects the subjects’ actual level of physical fitness and serves as a validation of the present questionnaire.
Alterations in the prevalence of type I fibers and muscle capillary supply might thus mediate the beneficial effect of physical activity on metabolic cardiovascular risk factors. Analysis of covariance, reflecting the contribution of different confounders to the relationship between physical activity and features of metabolic syndrome, shows the complexity of this association. Muscle morphology seems to have a particular impact on this association even though it shows some statistically different relationships to various components of the syndrome. With the help of multivariate analysis, however, we were able to separate the contribution of these confounders. We found that muscle morphology could explain 16% of the variation in insulin sensitivity and where the proportion of type IIB fibers showed an independent effect on insulin resistance. Considering the present findings, we suggest that muscle morphology should be considered as an essential part of the insulin resistance syndrome.

**Smoking**

According to earlier studies, smoking subjects presented a lower proportion of oxidative, type I fibers 207, 208. Since hereditary predisposition for different fiber patterns was ruled out, the authors referred to the impact of nicotine *per se* on normal neuromuscular interaction and to the adaptation of the muscle to a more sedentary lifestyle 207, 208. In addition to confirming these previous findings concerning the lower amount of type I fibers, we also observed a significantly lower muscle capillarization in smokers, which was not studied in these previous, fairly small studies. The evidence for the impact of cigarette smoking on endothelial function has been overwhelming and includes evaluation of the influence of different compounds, including nicotine, carbon monoxide and free radical components initiating the endothelial injury 209, 210, which can trigger the development of capillary rarefaction.

**Genetic considerations**

Due to the cross-sectional nature of the study, no conclusions about causality can be drawn. Nevertheless, we have evaluated to what extent these muscle morphological features contribute to the variation in IRS components that are also influenced by physical activity. More importantly, these interrelationships might characterize the lifestyle of these 70-year-old men. Furthermore, only 30% of the variations in muscle morphology are explained by environmental factors 65. Genetic factors control up to 45% of muscle fiber distribution 65, 211. Muscle morphology is also an important determinant of physical fitness. It is well documented that athletes with a large proportion of slow twitch (type I) and fast twitch aerobic skeletal muscle fiber (type IIA) are generally highly successful distance runners 212, 213. Indeed, the D/I polymorphism of angiotensin converting enzyme (ACE) gene, the presence of two insertion alleles (the II genotype), has been associated with better athletic performance 214, 215. Likewise, the opposite polymorphism, the DD allele, has been connected to insulin resistant states such as hypertension and type 2 diabetes 216, 217. Healthy offspring of hypertensive and
diabetic patients have shown both decreased insulin sensitivity and low physical fitness which parallels alterations in skeletal muscle pattern. One interesting possibility is that inherited abnormalities in skeletal muscle pattern limit the aerobic exercise capacity of patients with IRS resulting in a tendency towards a more sedentary lifestyle. Hence, it is tempting to hypothesize that some people are born with a more favorable skeletal muscle fiber pattern, which makes it easier for them to perform physical exercise and prevents the appearance of the features of the insulin resistance syndrome.

**Muscle morphology in glucose tolerant hypertensive subjects and its association with blood pressure (III)**

There are some reports about alterations in muscle fiber distribution of hypertensive subjects compared to healthy individuals. Nevertheless, in these studies the hypertensive groups have also shown a significant difference in body weight. Glucose tolerance or insulin sensitivity was not measured in these studies. Blood pressure level is associated to fiber distribution and that relationship is especially pronounced in subjects with a wide range of obesity. The amount of body fat is inversely related to the proportion of type I fibers and a higher proportion of type IIB fibers has been observed in the skeletal muscle of obese subjects. Alterations in fiber pattern are also associated with insulin sensitivity and physical activity level. These above reports support our findings that muscle fiber distribution was comparable in glucose tolerant hypertensive subjects and controls with the same level of obesity and physical activity. Our findings are consistent with recent population-based study in Norway. However, the association between blood pressure and muscle morphology was not confirmed in that study probably because of an extremely low percentage of type IIB fibers in this population. Normotensive individuals with magnified blood pressure level during exercise have an increased risk for developing hypertension. Interestingly, in one recent study individuals with relatively more type IIB fibers were prone to exaggerated blood pressure levels during exercise. Hence, one explanation for these observed relationships between fiber distribution and BP could be that type II fibers, particularly type IIB fibers, exhibit the lowest capillary density. Indeed, blood flow during exercise is positively related to the percentage of type I fibers. Peripheral resistance might thus be higher in individuals with a predominance of type IIB muscle fibers. A more uniform finding among hypertensive individuals is capillary rarefaction both in skeletal muscle and in other tissues. Normotensive offspring of hypertensive parents showed twice as high a prevalence of type IIB fibers with a lower number of capillaries around them in skeletal muscle than normotensive subjects with no family history of hypertension. Along with these changes in muscle morphology, these hypertension-prone men also showed a lower VO2max.
and insulin sensitivity. In Study III, these findings were confirmed in view of the fact that untreated hypertensive men showed a significantly decreased capillarization around skeletal muscle fibers compared to controls. This difference persisted even after adjustment for minor differences in obesity and physical activity. Capillary density in the muscle in the hypertensive subjects was closely associated with the increase in blood pressure over a 20-year period. One factor affecting the relationship between capillary density and the change in MAP during 20 years was the simultaneous increase in body mass index during 20 years. However, since this increase in BMI could also cause the rise in blood pressure, we did not find it proper to adjust this relationship for increase in BMI. The increase in BMI might be a common cause for both elevated blood pressure and decreased capillary density in skeletal muscle since obesity-linked hypertension also involves a simultaneous activation of the sympathetic nervous system, which leads to vasoconstriction and increased peripheral resistance. A microvascular constriction can reach to the point of non-perfusion of the vessel until its disappearance. The relationship between capillary density and development of high blood pressure might be mediated by endothelial dysfunction as well (Study IV and 121, 156, 166), which is a known phenomenon in hypertensive populations and particularly in untreated hypertensives.

**Muscle morphology and resting heart rate: the role of SNS (I, III)**

Those with the lowest capillary supply at age 70 years showed the highest resting heart rate both at age 70 years and 50 years (Study III). Relative proportions of type I and type IIB fibers were also significantly correlated with supine heart rate (Study II). All these findings were evident even after correcting for levels of obesity and physical activity. Correcting for physical activity level is of importance since it has been shown to slow the heart rate and to increase the capillary density in skeletal muscle. To our knowledge, similar correlations have not been detected previously. It has been proposed, that an increased sympathetic tone, responsible for the development of hypertension and insulin resistance, could concurrently magnify the expression of type II fibers. Hyperkinetic, early stage of hypertension is characterized by sympathetic overactivity decades before the manifestation of the disease. Microvascular network abnormalities are also observed in early stages, sometimes even before significant central hemodynamic alterations (for review see). There is some experimental evidence, which suggests higher sympathetic activity, or bradycardial pacing might induce alterations in muscle morphology and, along with that, affect insulin sensitivity. In rat, an infusion of β-adrenergic agonist caused the transformation from slow- to fast-twitch fibers. In addition, experimental studies done by Hudlicka et al. shows that long-term bradycardia (electrically or pharmacologically induced) produces an extensive capillary proliferation in the heart. The effect of sympathetic overactivity on both blood pressure and insulin resistance is
also related to enhanced $\alpha$–adrenergic tone that impairs the nutritional blood flow 233-235. The rat data suggest an imbalance in postsynaptic adrenoreceptor functions. It has been observed that postsynaptic $\alpha_1$-adrenergic functions became dominant while $\beta$-adrenergic functions are attenuated in arterial hypertension 236 which might promote the pressor effects of the sympathetic system. In experimental studies, infusion of prazosin, an agent blocking $\alpha_1$-adrenoreceptors which are predominantly found in capillaries, was found to cause an increase in capillary density explained by augmented blood flow with this agent 63, 221, 237. Interestingly, $\alpha_1$-adrenergic blocking agents can improve insulin mediated glucose uptake as much as 20% 238-240. Thus, higher sympathetic drive might initiate some structural alterations in the skeletal muscle.

Observations show that resting heart rate might be genetically programmed as well. The HERITAGE Family Study showed that the maximal heritability of resting HR was 34% when adjusted for BMI 241, 242. In addition, skeletal muscle pattern is partly explained by genetic factors 65, 211. Hence, genetical endowment could serve as an additional explanation of the correlation of high heart rate and some alterations in skeletal muscle structure.

Sometimes the role of heart rate as a sympathetic marker has been questioned. This is challenged by both experimental and clinical data showing that sympathetic activation and inactivation are usually associated with increases and reductions in heart rate, respectively 243. Moreover, heart rate has been correlated to plasma levels of epinephrine and muscle sympathetic nerve activity in population studies 244, 245. More properly defined, heart rate is a marker of the balance between sympathetic and parasympathetic activity in the heart reflecting variations in sympathetic drive.

**Capillary density and endothelial function in healthy elderly men (IV)**

Capillary density in skeletal muscle and serum FFA level seem to be independent determinants of endothelial function as they were significantly associated with changes in insulin-mediated LBF, together explaining 71% of its variation (Study IV). On average, insulin-induced leg blood flow increased by 15% with a large range of leg blood flow response to insulin from −23% to 93% in these 15 healthy 70-year-old men. Systemic hyperinsulinemia appears to augment skeletal muscle blood flow by 20-90% 156, 158, 246-249, but this is in certainly not a universal finding 250-253. In healthy, elderly individuals age itself does not seem to affect the fiber type composition or capillary supply expressed as the number of capillaries per square millimeter in muscle tissue 38, 254. Some loss of capillaries around fibers has been reported because of reduced muscle fiber area in aged subjects 255. According to observations, the vasoactive effect of insulin involves a balance between its local vasodilatory capacity and its vasoconstrictive central
sympathoexcitatory effect \(^{160}\). Data from studies in which insulin is infused systematically show that there is a parallel stimulation of the SNS, which has hemodynamic consequences both centrally and in the periphery. Evidence for this mechanism comes from recordings of muscle sympathetic nerve activity under conditions of insulin stimulation \(^{256}\). Furthermore, it is theoretically possible that under hyperinsulinemia, the effects of the vasoconstrictive protein endothelin (ET-1) become apparent \(^{257-260}\). Ageing is also followed by an increase in SNS tone \(^{261}\). Elderly subjects may actually attain vasoconstriction in response to physiological elevations of plasma insulin, resulting in a decrease in LBF of up to 15% \(^{170}\). This was nicely illustrated in one recent study. In this study the resting limb blood flow between young and old healthy individuals was compared. The age-related impairment in blood flow was no longer significant after correction for muscle sympathetic nerve activity, which was 74% higher in the elderly \(^{186}\). This finding is in accordance with those of the present study, in which the supine heart rate showed an inverse association to LBF changes.

Experimental observations show that an acute increase in the circulating FFA level in response to exogenous lipid and heparin infusion produces impairment in endothelial function \(^{262, 263}\). This finding is consistent with the present study, as well as with a recent observation in a larger sample from the present cohort population, in which the insulin-induced changes in LBF were inversely associated with serum concentration of FFA \(^{183}\). Furthermore, observations show increased levels of FFA lead to increased neurovascular tone by increasing \(\alpha\)-adrenoreceptor reactivity \(^{264}\). Higher resting heart rate, which was associated with capillary rarefaction in Study III, was significantly related to elevated levels of serum FFA in a larger sample from the present population \(^{183}\). Because there was no correlation between capillary supply and serum FFA level, the associations of these two parameters with insulin-mediated changes in LBF most likely involve different mechanisms. However, both mechanisms might have their origin in increased sympathetic activity because both of the parameters were associated with heart rate. Thus, the combination of high FFA levels in serum and elevated sympathetic tone would explain why men with the highest FFA levels did not respond to insulin with vasodilation, but instead showed a decrease in blood flow caused by stimulation of vascular adrenergic tone.

The entire debate regarding the contribution of insulin-mediated skeletal muscle blood flow to glucose uptake is intense. In a number of studies, the decrease in glucose disposal was determined by impaired insulin-mediated blood flow \(^{156, 246, 265, 266}\). Others, however, have questioned this association \(^{253, 267, 268}\). According to Baron \textit{et al.} \(^{265}\), 20-30% of insulin-mediated glucose uptake is blood flow dependent. Differences in the doses of insulin used and the methodology of blood flow measurement have been claimed to account for some of these divergent findings \(^{269, 270}\). On the other hand, these discrepancies might also depend on a subjects’ age and disease states such as hypertension, the duration of high blood pressure, obesity and insulin resistance. All these diseases have been characterized
by both capillary rarefaction 98, 117, 119, 140 and blunted blood flow in response to insulin 156, 161, 162, 246, 249. Therefore, the number of capillaries present in skeletal muscle might be one factor to explaining whether these two actions of insulin – glucose uptake and vasodilation - are associated or not.

These findings suggest that endothelial factors are important in modulation of capillary supply. Experimentally, growth of capillaries at least in the rat heart occurs under physiological circumstances during endurance exercise training, exposure to high altitude and/or cold, and changes in cardiac metabolism or heart rate elicited by modification of thyroid hormone levels 231. Capillary growth in all these conditions can be linked with increased blood flow, decreased heart rate, or both. This has been further proved by studies reporting that the effect of antihypertensive drugs on capillarization, mostly investigated in animals, might depend on their effect on blood flow (endothelium-dependent vasodilation) and on the presence of endothelial dysfunction as shown by Hudlicka et al. 237. Drugs that improve the endothelial function and blood flow 271, 272 have been reported to enhance capillary density as well 273-277.
Conclusions

• In the population-based sample of 515 elderly men an analysis between muscle morphology and measurements of insulin sensitivity showed a positive correlation of insulin sensitivity to the percentage of type I fibers as well as to capillary density and an inverse correlation to type IIB fibers irrespective of obesity and physical activity level. After additional correction of these relationships for within-subject variation up to a twofold improvement of the relationships was seen due to removal of the bias caused by variation in insulin sensitivity and muscle features.

• In a population-based sample of 475 men, a lower proportion of type I fibers, lower capillary density and a higher prevalence of type IIB fibers were related to lower self-reported physical activity as well as to components of IRS like elevated serum TG and PAI-1 levels, elevated supine heart rate and low HDL concentration.

• Insulin sensitivity was closely associated to self-reported physical activity level independent of possible confounders. However, the relationship between physical activity level and several other components of IRS like serum TG, PAI-1 activity and heart rate were dependent on muscle fiber distribution and capillary density.

• The proportion of type IIB muscle fibers together with BMI, glucose intolerance, PAI-1 activity, serum FFA concentration, HDL cholesterol level, drug treatment, PA level, and W/H ratio explained 55% of the variation in the insulin sensitivity.

• A sample of 43 untreated hypertensive men from this population showed a significantly decreased capillarization around skeletal muscle fibers compared to controls. Fiber type distribution was related to mean arterial pressure. Capillary density in the muscle of the hypertensive subjects was closely associated with the increase in blood pressure over a 20-year period.

• Cross-sectional analysis showed that capillary rarefaction was correlated to supine heart rate. Retrospectively, men with the lowest capillary density in skeletal muscle showed the highest heart rate 20 years ago, which is supports the hypothesis of involvement of increased sympathetic tone in reduction of the capillary net in skeletal muscle.

• In a healthy sample of 15 elderly men, capillary density in skeletal muscle and serum FFA level seemed to be independent determinants of endothelial function because both of these apparent independent determinants were significantly associated to the change in insulin-mediated LBF and explained the majority of LBF variation during hyperinsulinemia.
Future perspectives

Reduction in capillary supply and alterations in fiber pattern (partly due to genetics) in skeletal muscle were linked to higher SNS activity and to sedentary lifestyle explaining a considerable part of insulin sensitivity and other features of IRS. Additionally, capillary rarefaction was an independent determinant of impaired endothelial function. As these alterations in skeletal muscle structure are present in the offspring of subjects with insulin resistant states they might serve as prerequisites for further development of IRS. The changes in muscle morphology features also characterize the developed states of hypertension and type 2 diabetes. Accordingly, we suggest that muscle morphology should be considered an essential part of the metabolic syndrome.

Skeletal muscle structural adaptations may prove to be a critical component in preventing diseases such as coronary artery disease, type 2 diabetes, and obesity. Endothelial function suggests a promising target for interventions aimed at decreasing the death from coronary heart diseases and its related disorders. The present cross-sectional study gives a glimpse or idea of how habitual physical activity might affect health. It would be an important challenge to move beyond “correlational studies” and to identify responsible molecular/genetic mechanisms in population-based settings. As physical activity has one of the most powerful impacts on change in skeletal muscle morphology affecting a number of common health hazards, including an impact on civilization pandemics like type 2 diabetes, it has to be more intensively promoted in order to return to the more natural, active lifestyle of our ancestors.
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The cover illustration is a myosin ATPase stain (preincubation pH=4.58) of skeletal muscle fibers.

Ann Hedman

Uppsala and Tallinn in July 2001
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