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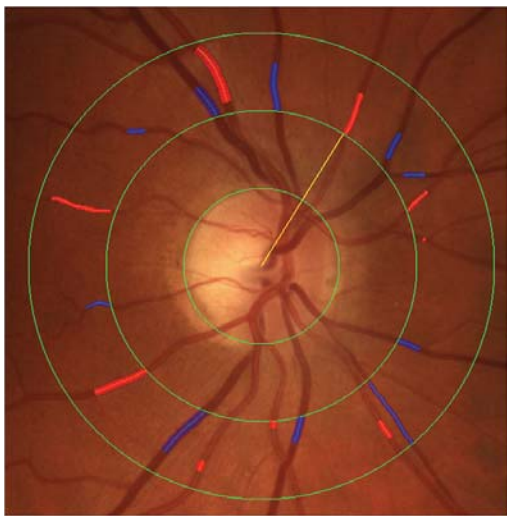
BRAIN AND CIRCULATION RESEARCH GROUP

Retinal vascular calibres: Risk factors and methodological aspects of retinal vascular imaging

The Tromsø Eye Study – a part of the Tromsø Study



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Retinal vascular calibres: Risk factors and methodological aspects of retinal vascular imaging

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2013

Writing is thinking

- Å skrive er å tenke

Toril Moi

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Therese
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Sammendrag

Hjerte og kar-relaterte sykdommer er viktige årsaker til sykdom og død. Forandringer i de små blodårene i kroppen er i mindre grad undersøkt enn i de store blodårene. Øyet gir en unik tilgang til å studere de små blodårene i kroppen og vi ønsket å undersøke hvordan kjente risikofaktorer for hjerte/kar-sykdom er assosiert med diameteren til netthinnens blodkar. Jern er en foreslått risikofaktor for hjerte/kar-sykdom og vi ønsket også å undersøke hvordan markører for kroppens jern-lager samt nivå av hemoglobin i blodet er assosiert med kar-diameteren i netthinnen.

De to første del-arbeidene er knyttet til data fra Tromsøundersøkelsen. Vi har målt diameteren av netthinnens arterier og vener på øyebunnsfotografier tatt av 6353 deltagere i den sjette Tromsøundersøkelsen (Tromsø 6, 2007-2008). I tverrsnittsundersøkelser fra Tromsø 6 fant vi at blodtrykk og røyking var de risikofaktorene som hadde størst effekt på kar-diameteren. Vi fant at blodtrykk og alder var forbundet med tynnere blodkar mens røyk, overvekt og ugunstig kolesterol-verdier var forbundet med videre blodkar. Vi fant videre at det var kjønnsforskjeller i disse forholdene, der overvekt og HDL-kolesterol hadde større effekt på kar-diameteren hos menn enn hos kvinner.

Det var 2993 deltagerne med kar-målinger i Tromsø 6 som også hadde deltatt i den femte Tromsøundersøkelsen (Tromsø 5, 2001-2007) da det ble utført målinger av kroppens jern-lager (serum ferritin og transferrin-metning) og hemoglobin. Menn har høyre nivå av både hemoglobin og jern og vi fant at hemoglobin var forbundet med tykkere vener hos både menn og kvinner mens serum ferritin var forbundet med tykkere vener bare hos menn.

Vi gjorde også en metode-relatert studie der vi undersøkte hvordan serie-fotografering og lyseksponering påvirker diameteren til netthinnens blodkar. Vi undersøkte 32 friske personer og fant at diameteren øker under fotografering med flere bilder i en serie samt at diameteren er større dersom øyet er eksponert for lys i forkant av fotograferingen.

Summary

Cardiovascular disease is a major cause of death and morbidity in developed countries. The eye offers a unique window to the study of the microvasculature in vivo, which has been less investigated than the macrovasculature. We wanted to investigate the relationship between the traditional cardiovascular risk factors and retinal vascular calibres. Further, iron is a proposed cardiovascular risk factor and we wanted to investigate whether measures of iron stores and hemoglobin are related to retinal vascular calibre.

The two first papers in this thesis are based on data from the Tromsø Study. We have measured the diameter of the retinal arterioles and venules on retinal images from 6353 participants of the sixth survey of the Tromsø Study (Tromsø 6, 2007-2008). We found that blood pressure and smoking were the factors with the most pronounced effect on the retinal vascular calibre. Blood pressure and age were associated with narrower retinal vessels and mainly affecting arteriolar calibre. Smoking, overweight (body mass index) and unfavourable lipid-profile were associated with wider retinal vessels, mainly affecting venular calibre. The effect of low HDL cholesterol and high BMI with venular widening was significantly stronger in men than in women.

Further, 2993 participants with retinal vascular calibre measurements in Tromsø 6 also participated in the fifth survey (Tromsø 5, 2001-2002) when measurements of iron stores (serum ferritin and transferring saturation) and hemoglobin were performed. Men have higher levels of both iron stores and hemoglobin. We found that serum ferritin was associated with wider retinal venules in men. Hemoglobin was associated with wider retinal venules in both men and women.

Cameras used for retinal photography commonly use light in the visual spectrum for the imaging technique and include several exposures with flash illumination to capture different fields of the retina. We examined 32 healthy volunteers and found that retinal venular calibre increased during an image sequence of 6 images and that the venular calibre was wider after light exposure compared to dark exposure before imaging.

List of papers

Paper 1

von Hanno T, Bertelsen G, Sjølie AK, Mathiesen EB. *Retinal vascular calibres are significantly associated with cardiovascular risk factors: the Tromsø Eye Study.* Acta Ophthalmol. 2013 Apr 29. doi: 10.1111/aos.12102. [Epub ahead of print]

Paper 2

von Hanno T, Bertelsen G, Broderstad AR, Wilsgaard T, Mathiesen EB. *Serum ferritin and hemoglobin are independently associated with wider retinal venular caliber. The Tromsø Study 2001-2008.* Invest Ophthalmol Vis Sci. doi:10.1167/iovs.13-12204. 2013;54:7053-7060.

Paper 3

von Hanno T, Sjølie AK, Mathiesen EB. *Retinal vascular calibre and response to light exposure and serial imaging. Acta Ophthalmol.* 2013 Jul 4. doi: 10.1111/aos.12213. [Epub ahead of print]

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Abbreviations

ARIC	Atherosclerosis Risk in Communities Study
AVR	arteriole-to-venule ratio
BDES	Beaver Dam Eye Study
BMES	Blue Mountain Eye Study
BMI	body mass index
CHS	Cardiovascular Health Study
CRAE	central retinal artery equivalent
CRVE	central retinal vein equivalent
HbA1c	glycosylated hemoglobin
HDL	high-density lipoprotein
hsCRP	high-sensitive C-reactive protein
ICC	intraclass correlation coefficient
LDL	low-density lipoprotein
MESA	Multi-Ethnic Study of Atherosclerosis
MRI	magnetic resonance imaging
SCORM	Singapore Study of the Risk Factors for Myopia
SD	standard deviation
SE	standard error
SiMES	Singapore Malay Eye Study

1 Background

1.1 The eye and its blood supply

The eye is a ball-shaped organ surrounded by connective tissue, i.e. cornea and sclera (figure 1). Light passes through the clear refractive media (the cornea, anterior chamber, lens and vitreous gel) to the sensory photoreceptors of the retina, and impulses are conducted to the central nervous system by the optic nerve, optic tract and optic radiation to the primary visual cortex.

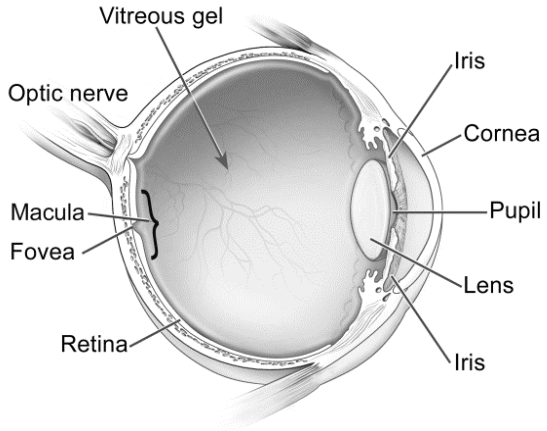

Figure 1. The schematic eye. Source: www.nei.nih.gov/photo

The eye is supplied with blood by the ophthalmic artery, which arises from the internal carotid artery and enters the orbita through the optic foramen. The ocular branches of the ophthalmic artery are the central retinal artery of the retina, the short posterior ciliary arteries, the long posterior ciliary arteries, the anterior ciliary arteries and the muscular arteries. The central retinal artery enters the optic nerve about 1.25 cm behind the eyeball and runs forward in the centre of the nerve and divides into the retinal branches at the level of the nerve head. The venous drainage of the eye is through the orbital superior ophthalmic vein, which communicates anteriorly with the facial angular vein, and drains to the cavernous sinus.

1.2 A historical perspective on retinal imaging

The anatomy and physiology of the eye is fundamental to our sense of sight. Our visual ability, to bring the image of the world into our eyes and the retinal photoreceptors, and eventually cerebral cognition, depends on clear media in the eye. Our ability to see “out”

makes it possible to see into the eye and its retinal structures. In 1850, the German physician and physicist Hermann von Helmholtz, invented the ophthalmoscope, which allowed the ophthalmologist to look inside a person's eye and the living retina.[1] This was a great achievement for the understanding, diagnosis and treatment of eye-diseases.

The history of retinal photography, allowing for simplified documentation, measurements, evaluation of progression and illustration, dates back to the late 1800s when Jackman and Webster described a technique for photography of the human living retina.[2] With the introduction of electronic flash and 35 mm cameras in the 1950s, the modern era of ophthalmic photography was started. Further achievements came with digital photography (figure 2) in the 1990s, with images immediately available without darkroom delay and simplified image-storing, which made the images readily accessible and allowed for measures and monitoring with higher precision. The quality of retinal images and development of new image modalities are in ongoing progress.

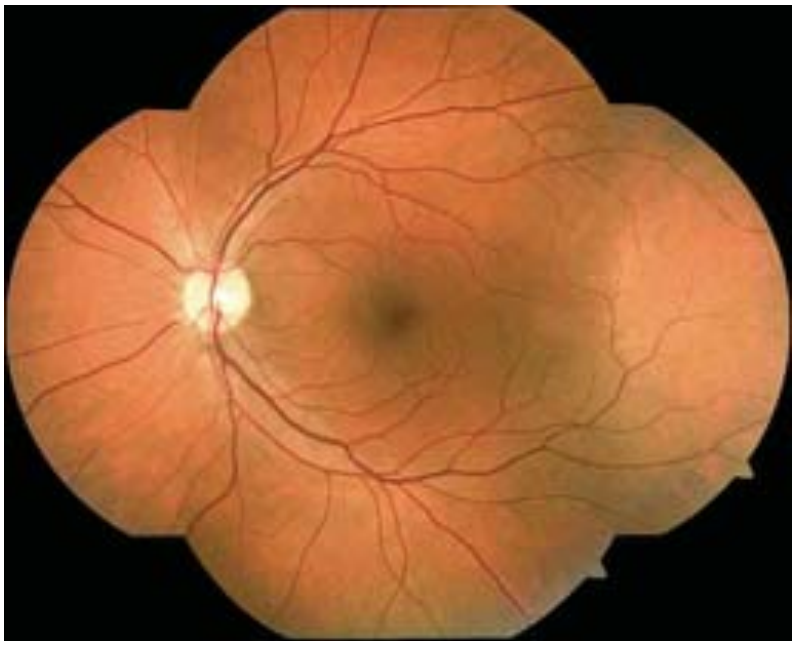

Figure 2. Composite retinal image. The Tromsø Eye Study 2007–2008.

1.3 Assessment of the microvasculature through the eye

The retinal arteries are arteriolar in nature.[3] The retinal blood vessels are part of the microvasculature which consists of vessels less than 0.1-0.3 mm. The microvasculature is a considerable part of the circulatory system but is less investigated than the macro-

circulation. The eye is a unique window to the study of the microvasculature in vivo, as it requires non-invasive methods. The retinal vessels may contribute to the study of anatomy, physiology and pathology of the microvasculature, shared with other organ systems.

In 1859 Liebreich was the first to describe fundus changes related to blood pressure.[4] In 1947 Wagner and colleagues presented a classification of retinal lesions in the presence of vascular hypertension, based on presence of generalized arteriolar narrowing and/or focal vascular abnormalities (arteriolar narrowing, arteriovenous crossing changes, arteriolar sheeting and changes in the arteriolar light reflex), hypertensive retinopathy (microaneurisms, intraretinal haemorrhages, cotton-wool spots and hard exudates) and optic disc swelling.[5] A few years later Scheie suggested to evaluate hypertensive changes (arteriolar narrowing, bleedings, exudates and papilledema) and arteriolar sclerosis (evaluated by the light reflex and arteriovenous crossing changes) in two separate five stage classification schemes.[3]

1.4 Assessments of the retinal blood vessel diameters

Generalized arteriolar narrowing is one element of the definition of classical retinal hypertensive changes. Subjective evaluation of this phenomenon was proven to have low reproducibility and more quantitative methods were seeked.[6]

In 1974 Parr et al. developed a method for a summarized measurement of the arteriolar calibre of all arterioles in a concentric zone around the optic disc, to obtain a single measure of retinal arteriolar calibre.[7] This measure was suitable for comparison of different eyes, independent of the number and branching pattern of the retinal arteries. The key issue in this procedure was to establish the relationship between the calibre of the parent trunk arteriole to the calibre of its two branches.[8] The summary measure was called the central retinal artery equivalent (CRAE) and the word “equivalent” was chosen to emphasize that the measure was derived from retinal arterioles and that pathology affecting this measure would not necessarily correspond to alteration in the true calibre of the central retinal artery. In 1999 Hubbard et al. derived a formula for venules, allowing for the corresponding venular summary measure, the central retinal vein equivalent (CRVE).[9] Hubbard et al. also showed that the procedure could be simplified by arbitrarily combing the largest and the smallest vessels throughout the pairing algorithm.[9]

In 2003 Knudtson et al. developed revised formulas for the relationship between the calibre of the parent trunk to the calibre of its two branches[10], based on an empirically derived branching coefficient:

Branching Coefficient (BC) $= (W_1^2 + W_2^2)/ W^2$ (Blum, 1919) (1)

Giving:

BC for arterioles = 1.28 (95%CI: 1.25, 1.32) $\Rightarrow \hat{W} = 0.88*(W_1^2 + W_2^2)^{1/2}$ (2)

BC for venules = 1.11 (95%CI: 1.08, 1.14) $\Rightarrow \hat{W} = 0.95*(W_1^2 + W_2^2)^{1/2}$ (3)

(W1: the narrower branch; W2: the wider branch; W: the parent branch; $\hat{W}$: the estimate of the parent branch)

As the summary measures were restricted to measurement on the six widest of each vessel type, the method is often referred to as the "Big 6" method. This method correlates highly with the Parr-Hubbard method, but has the advantage of being more robust against the variability of the number of vessels. It is easier to implement as only the six widest vessels are included, and is independent of scale and magnification as the formulas do not include constant terms.[10]

The Retinal Analysis software was developed at the Fundus Photograph Reading Center, University of Wisconsin (Madison, WI, USA) for the ARIC study.[9,11] This software, and the updated version IVAN, performs semi-automated calibre measurements on retinal photographs in a concentric around the optic nerve head (Zone B) and summarizes the measurements as CRAE and CRVE (figure 3). The IVAN software has been used in several large epidemiologic studies. Other software solutions are also available.[12]

Retinal Analysis/IVAN has high reproducibility with good inter- and intra-grader reliability.[9,11] Results from the Individual Variability Study showed high repeatability of grading of images in the same individuals, in images captured 15 minutes and 3 weeks after the original image.[11] Measurements on right and left eye are highly correlated.[11,13,14] The pulse related variation of arteriolar and venular calibre is 3.5-4.3% and 3.1-4.8% respectively[15,16] but contributes less to measurement variation than the inter-image variation.[17] The refraction and axial length of the eye imposes measurement variation through the magnification effect

on retinal images but is has been demonstrated that refraction has no appreciable effect on the association between CRAE and blood pressure.[13,18]

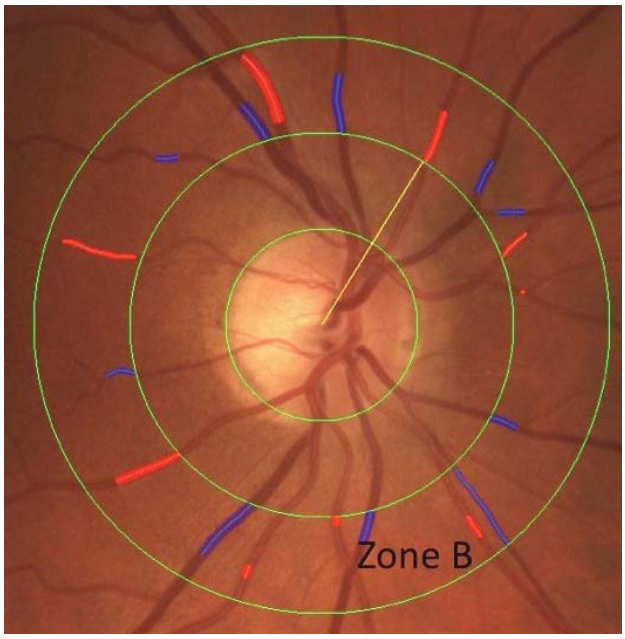

Figure 3. Fundus image showing grid centred on the optic nerve head and with measured retinal arterioles (red) and venules (blue), by the use of the IVAN software. All vessels coursing through the area of one-half to one disc diameter from the optic disc margin (Zone B) are traced and measured by the software and, if needed, corrected or modified by the grader. Calibres of the six widest vessels of each type are summarized as central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE). Image from the Tromsø Eye Study.

Arteriole-to-venule ratio (AVR: ratio of the calibre of arterioles to venules) was proposed by Wagener et al. as a marker of generalized arteriolar narrowing. The rationale behind this was that arteriolar calibre was considered easier to assess *relative to* venular calibre, and that venular calibre was considered to be fairly constant, irrespectively of blood pressure, age and other factors.[5] Thus, reduced AVR would mirror arteriolar narrowing. However, with the evolution of new high-resolution imaging and software assisted measurement tools of retinal calibre, it became evident that venular calibre was not at all that inert.[19] In 2007 Liew et al. showed that in linear regression models with AVR, CRAE and CRVE as explanatory variables and blood pressure and white blood cell counts as outcome variables, models with AVR had lower predictive power than models with its two components CRAE and CRVE entered together.[20]

1.5 Retinal vascular calibre and cardiovascular disease

Prospective data show that narrower retinal arterioles and wider retinal venules may be independent predictors of cardiovascular disease.

It is well documented that change in retinal vascular calibre is associated with both clinical and sub-clinical stroke and the results are most consistent for wider venular calibre.[21-23] Analysis of pooled data from six studies found that wider retinal venular calibre was independently associated with incident fatal and nonfatal stroke.[21] Retinal venular widening has also been associated with MRI-defined white matter lesions and lacunar infarction.[24,25] Retinal arteriolar narrowing was associated with incident lacunar infarction in one study.[24]

There is also good evidence that retinal vessel calibre changes may independently predict ischemic heart disease.[22,26] Analysis of pooled data from six population based studies found that retinal arteriolar narrowing and venular widening were associated with incident ischemic heart disease in women but not in men.[26] This result indicates that microvascular pathology is of greater importance in development of ischemic heart disease in women than in men.

Analysis of pooled data from two population based studies found that retinal arteriolar narrowing and venular widening were associated with cardiovascular mortality in persons younger than 70 years.[27] Both smaller arteriolar calibre and wider venular calibre were independent predictors of mortality from ischemic heart disease, while stroke-mortality was independently predicted by wider venular calibre.

Studying the retinal vessels may contribute to the understanding of the pathological processes in cardiovascular disease. Furthermore, it is possible that evaluation of the retinal vessels may have a future role in cardiovascular screening and risk stratification.

1.6 Retinal vascular calibre and traditional cardiovascular risk factors

The relationship between established cardiovascular risk factors and retinal vascular calibre has been investigated in epidemiologic studies in Europe, USA, Australia and Asia, but not in a general population in a Nordic country.

Most previous publications on this topic had a cross-sectional design.[22] A summary of results from the following population based cross-sectional studies are shown in Table 1:

Atherosclerosis Risk in Communities Study (ARIC), the Rotterdam study, the Multi-Ethnic Study of Atherosclerosis (MESA), Singapore Malay Eye Study (SiMES), Cardiovascular Health Study (CHS), Blue Mountain Eye Study (BMES), Beaver Dam Eye Study (BDES) and the Funagata study. Further are included results from the children cohort in Singapore Study of the Risk Factors for Myopia (SCORM). Results from paper 1 in this thesis are included in the table to facilitate comparison.

Blood pressure has been investigated in all the adult populations and is consistently negatively associated with CRAE.[19,28-34] The effect of blood pressure on venular calibre seems to be smaller, with higher blood pressure being significantly associated with smaller CRVE in some studies, while insignificant in others. The ARIC study demonstrated that the association between blood pressure and CRVE changed direction to positive with adjustment for the fellow vessel equivalent. (i.e. CRAE).[28]

Further, smaller CRAE is independently predicting incident hypertension, also when accounted for baseline blood pressure.[35-38] The other way around, BMES demonstrated that baseline blood pressure was associated with thinner arterioles at follow up, independent of other cardiovascular risk factors, also when accounted for blood pressure at follow up.[39]

Age has consistently been negatively associated with both CRAE and CRVE.[28-32,34,40]

Current smoking has been consistently associated with wider CRAE and CRVE,[19,28-31,40] with stronger effects for current smoking than for previous.[28,40] Results from the ARIC study demonstrated that the association between smoking and CRAE lost the statistical significance with adjustment for venular calibre.[28] In the BMES it was demonstrated that smokers were more likely to have relatively large changes in CRVE in either direction during a 5 year follow up, i.e. both venular narrowing and widening (≥1SD from the mean difference during 5 years).[40]

BMI has consistently been associated with wider CRVE while the association with CRAE has been insignificant in most studies.[28-31,40] In the Rotterdam study only age and sex adjusted analyses were presented and the results differ from the other studies, which may indicate that the association with retinal vascular calibre is confounded by other risk factors.[19]

Table 1. Association between cardiovascular risk factors and retinal vascular calibres in population based studies.[a]

Study	Size	Male sex		Age		BP		BMI		Cholesterol						Smoking		HbA1c		Adj.[b]	Adj. FV[c]	Sex-diff. explored
										HDL		LDL		Total								
ARIC [28]	8794			A-	V-	A-	V+[d]	A-[d]	V+	A-	V ns	A ns	V+			A ns	V+			m	yes / (no)	yes
SiMES [29]	3019	A-	V ns	A ns	V-	A-	V ns	A ns	V+	A ns	V-			A ns	V ns	A+	V+	A ns	V+	m	no / (yes)	yes
MESA [30,41]	5979	A-	V ns	A-	V-	A-	V ns	A ns	V+	A ns	V-	A ns	V+			A+	V+	A ns	V+	m[e]	no	no
CHS [31]	1992	A ns	V ns	A-	V-	A-	V-	A ns	V+	A ns	V ns			A ns	V +	A+	V+			u	no	no
BMES [33,40,42]	3006	A-	V-	A-	V-	A-	V-	A ns	V+	A -	V-	A ns	V ns	A ns	V ns	A+	V+			m	no / yes	yes[f]
BDES [34]	4247			A-	V-	A-	V ns													m	no	yes
Funagata [32,43]	921			A-	V-	A-	V-			A ns	V ns									m	no[g]	no
Rotterdam [19]	5674					A-	V-	A-	V ns	A-	V-			A ns	V ns	A+	V+			as	no	no
SCORM [44,45]	768[h]	A ns	V ns					A ns	V+											m[i]	no	yes
Tromsø 6 [46]	6353	A-	V ns	A-	V-	A-	V-	A ns	V+	A ns/-[j]	V-	A ns	V+			A+	V+	A+	V ns	m	no / yes	yes

A, arteriolar calibre; V, venular calibre; BP, blood pressure; BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; HbA1c, glycosylated hemoglobin; Adj., adjusted analyses; Adj. FV, analysis adjusted for fellow vessel calibre; Sex-diff. explored, sex-differences explored; ARIC Atherosclerosis Risk in Communities Study; MESA The Multi-Ethnic Study of Atherosclerosis; SiMES Singapore Malay Eye Study; CHS Cardiovascular Health Study; BMES Blue Mountain Eye Study; BDES Beaver Dam Eye Study; SCORM Singapore Cohort Study of the Risk Factors for Myopia.

a) Referred results are given as +, - and ns, where + denotes positive, - negative and ns non-significant association between the risk factor and retinal calibre. b) Adjustment; m=multivariable, as=age and sex, u=unadjusted. c) Adjustment for fellow vessel equivalent, '( )' results described, numbers not given. d) Difference in results related to the adjustment for fellow vessel calibre. e) Results for HbA1c were from unadjusted analyses. f) For age and blood pressure. g) HDL cholesterol adjusted for fellow vessel calibre. h) Children. i) Adjusted for age, sex, race, parental income and education, axial lenght/spherical equivalent, birth weight and length. j) Non-significant in women, significant negative association in men.

In the BMES they found that wider venular calibre was associated with incident obesity (defined as BMI > 30 kg/m^2) after 5 years, while the association was attenuated to non-significant with adjustment for baseline BMI. Further they showed that wider venular calibre was associated with significant weight gain 5 years later (defined as BMI increase of ≥4 units), these analyses were not adjusted for baseline BMI, which possibly may have confounded the results. CRAE was not associated with incident obesity or weight gain in any of the analyses.

High LDL and low HDL cholesterol has been associated with wider CRVE in some studies, while not as consistently as with many of the other risk factors, and LDL cholesterol seems not to be associated with CRAE.[19,28-31,40,42,43] Further, total cholesterol was investigated in four studies and except for a positive associations with CRVE in CHS (unadjusted analyses), total cholesterol was not significantly associated with CRAE or CRVE.[19,29,31,42]

HbA1c was assessed in two studies only and was positively associated with CRVE, while non-significant with CRAE in both.[29,41] The association between parameters related to diabetes and retinal vascular calibre is complex, as it is dependent of multiple factors as duration of diabetes, blood sugar control and presence and grade of retinopathy, which are factors that are inter-connected. There is good evidence that severity of diabetic retinopathy is positively associated with CRVE.[41,47-50] Prevalent diabetes has been associated with increased CRAE in several studies[41,47,48] while not in all,[31] and may also be dependent of presence and severity of retinopathy.[51] The Rotterdam study found that wider venules were independent predictors of incident impaired glucose tolerance.[52]

In a children cohort there was no difference in CRAE and CRVE between boys and girls.[44] In adults, male sex has quite consistently been associated with thinner CRAE while there seems to be no difference in CRVE between men and women.[29-31,40]

Sex differences in the association between one or more cardiovascular risk factor and retinal vascular calibre have been evaluated in five of the nine studies. In the SiMES they found that the association between blood pressure and narrower arterioles was stronger in men than in women, while they reported no other sex-interaction in how cardiovascular risk factors were associated with retinal vascular calibre.[29] The ARIC study researchers reported “some sex

differences, particularly with high-density lipoprotein cholesterol level, endothelial dysfunction markers, and current alcohol consumption", while results were not specified.[28] In BDES and BMES they did not find sex-differences in the association between age and blood pressure versus retinal vascular calibre.[33,34] In SCORM they did not find sex-differences in the association between BMI and wider venular calibre in children.[45]

1.7 Iron – a possible risk factor for cardiovascular disease

In 1981 Sullivan launched the hypothesis that iron is involved in the pathogenesis of atherosclerosis and that chronic iron depletion in menstruating women could contribute to the reduced risk of cardiovascular disease in pre-menopausal women.[53]

Iron is essential for the metabolism in all cells. It is tightly regulated in the body as free iron in soluble form is unstable and tends to react with oxygen to form toxic free radicals. About 65% of iron is bound to hemoglobin, 10% is a constituent of myoglobin, cytochromes and iron-containing enzymes, and 25% is bound to the iron storage proteins (ferritin and hemosiderin).[54] Absorption of iron in the duodenum is closely regulated to maintain homeostasis as iron loss occurs only passively by blood loss, sweat and loss of cells from the intestine, skin and urine.[55] In plasma, iron is bound to the glycoprotein transferrin. The systemic regulation of available iron is through the hormone hepcidin, secreted by the liver. Hepticin inhibits iron-transport out of the cells, through degradation of the iron exporter ferroportin.[56] Excess iron is primarily stored in the liver and reticuloendothelial macrophages. Serum ferritin is a useful measure of iron storage while its function and source remains to be defined.[55,57]

It has been hypothesized that iron in certain conditions may be pathologically liberated in an active noxious form, which can induce oxidative damage and oxidation of the pro-atherogenic factor LDL cholesterol.[58-61] Animal studies have shown that iron overload is associated with oxidative stress and atherosclerosis.[62,63] In humans, serum ferritin level has been demonstrated to be directly correlated to oxidant damage of atherosclerotic plaques.[64] In blood donors, high frequency donation was associated with enhanced vascular function (flow-mediated vasodilatation) compared to low frequency donation.[65] However, epidemiologic findings have been conflicting in whether iron status is correlated to incident cardiovascular disease and mortality (Table 2).[66-83]

Table 2. Markers of iron status and hemoglobin and the risk of cardiovascular disease, diabetes mellitus and mortality: Prospective population based studies.[a]

	Size	Men / women	Age	Follow up time	Outcome (incident)	Exposure		
						Ferritin	Transf. sat.	Hb / Htc
KIHD [66]	2682	m	42 - 60	5	AMI	+		ns
MONICA Iceland [67]	2036	m / w	25-74	8.5	AMI	ns		
BRHS [68]	7346	m	40-59	9.5	IHD			+
Bruneck [69]	855	m / w	40-79	5	CVD or CVM	+		
Turku [70]	344	m / w	65+	13	CVM	ns		
Rotterdam [71]	60/112 [b]	m / w	>=55	-	AMI	ns [c]		
SU.VI.MAX [72]	9917	m / w	35-60	7.5	IHD	ns		
Tromsø [73]	6541	m	20-49	20	ACM			+
Aberdeen [74]	396	m / w	75+	5-6	ACM	ns		
NHS [75]	242/483 [b]	w	60 (SD 6.5)	9	IHD	ns		
MONICA Denmark [76]	2874	m / w	30 - 70	10	CVD , IHD	ns		
NHANES I [77]	4518	m / w	45-74	14.6	AMI, IHD, CVM, ACM		ns -	ns
NHANES II [78]	1604	m / w	45-74	12-16	ACM, CVM	ns		
NHANES I / NHEFS [79]	10714	m / w	25-74		ACM		+	
NHANES III [80]	12258	m / w	>=20	12-18	ACM	ns	ns	
NHANES III [81]	5695	m / w	>50	12-18	ACM, CVM	ns	-	
CGPS / CCHS [82]	45 159	m / w	47-80	2-18	ACM		+	
Meta DM type 2 [83]	Meta-analysis of 6 studies			2.8-10	DM type 2	+		

Tranf.sat., transferrin saturation; Hb, hemoglobin; Htc, hematocrit; AMI, acute myocardial infarction; IHD, ischaemic heart disease; CVD, cardiovascular disease; ACM, all cause mortality; CVM, cardiovascular mortality; DM, Diabetes mellitus; KIHD, Kuopio Ischaemic Heart Disease Risk Factor Study (Finland); HHS, Helsinki Heart Study (Finland); BRHS, British Regional Heart Study; Bruneck, Bruneck Study (Italy); Turku, Turku study (Finland); Rotterdam, Rotterdam Study (Netherlands); NHANES, National Health and Nutrition Examination Survey Follow-up Study (USA); NHEFS, NHANES I Epidemiologic Follow-up Study (USA); SU.VI.MAX, SUpplementation en VItamines et Minéraux AntioXydants (France); Tromsø, Tromsø Study (Norway); NHS, Nurses Health Study (USA); MONICA, Monitoring of Trends and Determinants in Cardiovascular Disease; CGPS, Copenhagen General Population Study (Denmark); CCHS, Copenhagen City Heart Study (Denmark);

a) Referred results are given as +, - and ns, where + denotes increased risk, - reduced risk and ns non-significant association. b) Nested or matched case-control study.
c) Near significant + (p=0.078).

A recent population based cross-sectional study showed that higher level of hemoglobin was associated with wider retinal arterioles and venules independent of other cardiovascular risk factors.[84] Proposed mechanisms were blood viscosity and systemic factors related to oxygenation, e.g. lung disease and smoking. Iron body store is correlated to level of

hemoglobin and may possibly be an additional pathway underlying the correlation between retinal vascular calibre and level of hemoglobin. As far as we know there are no previous publications on the relationship between the level of iron body stores and microvascular calibre.

1.8 Retinal blood circulation and the effect of light

There is good evidence that light stimulation is a modulator of retinal blood flow.[85] Tissue perfusion depends on perfusion pressure and the total peripheral resistance to blood flow. In the eye the perfusion pressure is determined by systemic blood pressure and intraocular pressure.[86] The peripheral resistance depends on qualities of the blood e.g. viscosity, vessel length, vessel branching pattern and total vascular capacity, the latter which is related to the square of the vascular calibre which is regulated in the microcirculation.[87,88]

The inner retina (towards the retinal surface) is supplied by the retinal vessels, while the outer retina (photoreceptors) has a dual supply with diffusion from both the retinal and the choroidal circulation. Although the retinal photoreceptors are more metabolically active in dark than in light conditions it is not well understood how changing light conditions affect retinal blood flow.[87,89-92] Literature data are scarce and the few studies that have been conducted on retinal vessels show apparently contradictory findings.[93-98] This may partly be related to methodology, as rapid changes in blood flow due to changes in light exposures is challenging. Light exposure following dark adaptation seems to induce a rapid transient increase in the retinal blood flow velocity, reaching peak value at 30-60 seconds.[95] The studies of Feke et al.[98] and Riva et al.[97] demonstrated increased flow velocity in retinal vessels in dark, while Riva et al.[95] could not reproduce this result with infrared laser Doppler. As pointed out by the authors, the earlier results could have been affected by the effect of *change* of light as the former technique required light when measuring. Two other studies have demonstrated increased flow velocity in the central retinal artery in dark, by using Doppler ultrasound.[93,96] This technique is well suited for dark-measurements, while light-measurements were performed on closed eyes after light exposure. Flicker light stimulation induces increased venular and arteriolar diameter and the effect is reduced with smoking, hyperglycemia, essential systemic hypertension and diabetes.[99-104] Further, the oxygen saturation in the retinal arterioles and venules has been demonstrated to be higher in dark than in light while the physiologic mechanisms behind this finding is not known.[105]

The IVAN calibre measurements are performed on still images, and retinal photography is suitable for clinical and epidemiological studies as the technique of image capturing is non-invasive and cameras are easily accessible and easy to use.

Light exposure prior to imaging may vary according to artificial and natural room illumination. Few studies have investigated how light affects the retinal vascular calibre. Feke et al. (1983) found an arterial dilatation of 2-3% (assessed in three subjects) and a venous dilatation of 6-8% (assessed in one subject) in dark and Riva et al. (1983) found a venous dilatation of 5-8% (assessed in three subjects) in dark.[97,98] Contrary, Barcsay et. al. found a faint retinal venular constriction in dark, with use of an infrared illumination technique.[94]

Cameras used for retinal photography commonly use light in the visual spectrum for the imaging technique and include several exposures with flash illumination to capture different fields of the retina. We did not find any previous work exploring whether retinal vascular calibre is affected by sequential image capturing.

2 Aims of the thesis

- To investigate the relationship between the traditional cardiovascular risk factors and retinal vascular calibres in women and men.
- To investigate whether measures of iron stores and hemoglobin are related to retinal vascular calibre in women and men.
- To investigate whether retinal vascular calibre measurements on optical retinal photography are affected by light and dark exposure prior to photography and whether the retinal vascular calibres change during an imaging sequence of several images.

3 Methods

The participants of the studies included in this thesis were participants in the 6th survey of the population based Tromsø Study (paper 1 and 2) and healthy volunteers (paper 3).

3.1 The Tromsø Study and the Tromsø Eye Study

The Tromsø Study is a population based multipurpose health longitudinal study from the municipality of Tromsø, Norway, started in 1974. Six surveys have been carried out so far, and are referred to as Tromsø 1-6 (Tromsø 1: 1974, Tromsø 2: 1979-80, Tromsø 3: 1986-87, Tromsø 4: 1994-95, Tromsø 5: 2001-02, Tromsø 6: 2007-08). The Tromsø 4-6 have consisted of two visits, where visit 2 included a second extended examination in a selected subsample. The study selection criterion has been a combination of total and random samples of birth cohorts of the inhabitants of Tromsø. Inhabitants are identified through the Population Registry of Norway, and eligible persons are invited by mail. The study populations consist of both women and men, except for Tromsø 1 which was restricted to men. The participation rates have been high (>75% in Tromsø 1-5), but it was somewhat lower in Tromsø 6 (66%). The study adheres to the tenets of the Declaration of Helsinki and has been approved by the Data Inspectorate of Norway and Regional Committee for Medical and Health Research Ethics. From Tromsø 4 and onwards, all participants were asked to sign a written informed consent agreement prior to the examinations. Participants retain the right to withdraw the consent at a later stage.

In total 40051 subjects have attended at least one of the surveys, and repeated measurements are available in the majority of them.[106] Blood samples from each survey and DNA samples from the 3rd survey and onwards are stored in a biobank. The population is being followed up with registration of incident myocardial infarction, stroke, atrial fibrillation, diabetes, cancer and non-vertebral fractures. An overview of the data collected at each survey is given on the NESSTAR web-site http://tromsoundersokelsen.uit.no/tromso/. Further information about the Tromsø study, including information leaflets, consent forms and questionnaires for each study, is available at www.tromsoundersokelsen.no. The information leaflets from Tromsø 5 and 6 are included in the appendix.

The Tromsø Eye Study was included as a substudy of the Tromsø Study for the first time in Tromsø 6. The eye examinations included information on self-reported eye diseases, retinal photography, optical coherence tomography (OCT) and assessments of visual acuity and refraction errors.[107] In addition to measurements of retinal vascular calibres, the retinal photos have been graded for diabetic retinopathy and age-related macular degeneration.[107-109]

3.1.1 Population

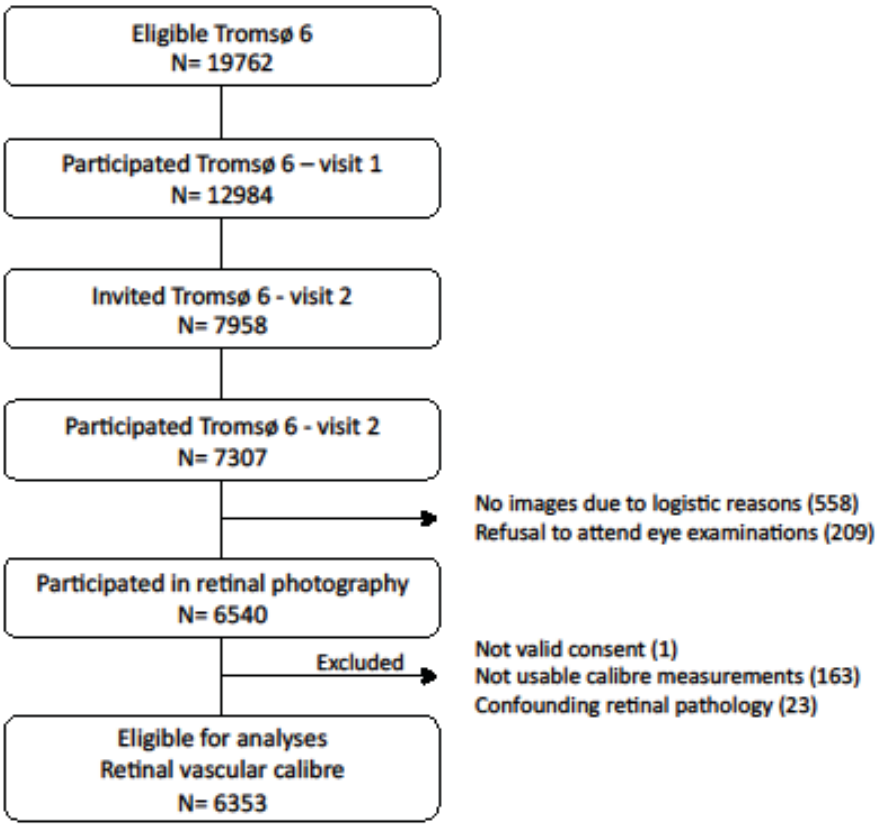

Figure 4. Flow chart illustrating the participation in retinal photography and retinal vascular measurements in the Tromsø Eye Study 2007-2008.

The eligible population for visit 1 in Tromsø 6 was: (1) All residents in Tromsø aged 40-42 or 60-87 years (n=12578), (2) a 10 % random sample of individuals aged 30-39 years (n=1056), (3) a 40 % random sample of individuals aged 43-59 years (n= 5787), (4) subjects who had attended the visit 2 (extended examinations) of Tromsø 4, if not already included in the three groups above (n=341). A total of 19762 were invited of whom 12984 (65.7%) attended (Figure 4).

The eligible population for visit 2 in Tromsø 6 was: (i) All subjects eligible for visit 1 aged 50-62 years or aged 75-84 years (n=7657), (ii) a 20 % random sample of men and women aged 63-74 years (n=942), (iii) subjects, if not already included in the two groups above, who had attended visit 2 of Tromsø 4 (n=2885). The study sample for visit 2 was predefined before the study start and included 11484 subjects, where 7958 were actually invited as only participants that attended visit 1 were invited to visit 2.[110] A total of 7307 attended visit 2 (91.8% of the invited), and in the eligible population (for visit 2) the overall attendance rate was 63.6%.

Figure 4 shows the participation in retinal photography and retinal vascular calibre measurements in Tromsø 6 / Tromsø Eye Study. There were 6540 participants with retinal photos of at least one eye. For the images to be considered as gradable, at least four vessels of each type (arterioles and venules) should be traceable. For images to be considered as usable, they should be gradable and six vessels of each type should be traceable, including all the six widest vessels of each type. We excluded 163 participants with no usable grading in either eye and 23 participants with confounding retinal pathology. One participant has withdrawn the informed consent (pr. 14.05.2012). This resulted in 6353 subjects eligible for the analyses on retinal vascular calibre. Due to missing values in one or more of the variables in the multivariable models (n=197), the final number of participants included in the regression analyses in paper 1 was 6156.

In paper 2 we included all participants in Tromsø 6 with retinal vascular calibre measurements on good quality retinal photos and who attended Tromsø 5, and who were non-pregnant in both surveys. Of the 6353 persons with retinal vascular measurements, 3309 participated in Tromsø 5. We excluded those with grading on images of poor quality (n=313) and those who were pregnant in either Tromsø 5 or Tromsø 6 (n=3), leaving 2993 subjects (1819 women and 1174 men) to be included in this study.

3.1.2 Refraction and retinal photography

Refraction was measured on both eyes with Nidek AR 660A auto refractor (Nidek CO.,LTD., Gamagori, Japan). Spherical equivalent refraction was calculated as spherical power plus half the cylindrical power in dioptres. Mydriasis was obtained by application of one drop of tropicamide 0.5% (Tropicamide, Chauvin Pharmaceuticals Ltd. Kingston Upon Thames,

Surrey, England) in both eyes. All participants were informed about the effects and potential risk of pupil dilatation and given the opportunity to withdraw from eye examination prior to application of tropicamide and still participate in the rest of the survey. Five field 45 degree retinal colour photographs with resolution 2196x1958 pixels were taken on both eyes with a Visucam PRONM (Carl Zeiss Meditec, Jena, Germany) digital retinal camera, with the disc-centred image taken as the second image on each eye.

3.1.3 Retinal vascular calibre measurements

Retinal vascular calibres were measured computer-assisted on the disc-centred images with IVAN software, the updated version of Retinal Analysis (Fundus Reading Center, University of Wisconsin, Madison, USA) (Figure 3). The protocol for grader-interaction on the automated measures was in accordance with previously validated protocols [9] with minor modifications according to Retinal Vascular Imaging Centre (RetVIC), Centre for Eye Research Australia, University of Melbourne (Appendix) The image conversion factor was determined based on image resolution and the reported average of the optic disc diameter of 1800 µm. Grading for the Tromsø Study was performed by Therese von Hanno (TvH), trained and certified at RetVIC in April 2010 (Appendix), and by three certified graders at the same centre. Graders were masked for any information on the subjects.[107] All vessels coursing through the area of one-half to one disc diameter from the optic disc margin (Zone B) were measured on one eye, preferably the right eye, and the six biggest of each type were summarized as the central retinal artery equivalent (CRAE) and the central retinal vein equivalent (CRVE) (Figure 3).[10] The left eye was measured only if the measurements from the right eye were not usable.

3.1.4 Cardiovascular risk factor measurements and blood samples

All examinations were performed by trained personnel. Blood pressure was measured three times at one-minute intervals on one arm after two minutes of seated resting with the use of an automatic device (Dinamap Vital Signs Monitor, Critikon, Tampa, FL, USA), and we used the mean of the last two measurements. In paper 1 we calculated *mean arterial blood pressure* (MABP), as one third of the systolic plus two thirds of the diastolic blood pressure. Weight and height were measured wearing light clothes and without shoes and BMI was calculated as kilograms per square meter.

Analyses of blood samples were performed at the Department of Laboratory Medicine at the University Hospital of Northern Norway. Participants were not requested to fast. Serum total, LDL and HDL cholesterol were measured by standard enzymatic methods. Glycosylated hemoglobin (HbA1c) was analysed with Bayer DCA 2000 (Bayer AG, Leverkusen, Germany) in Tromsø 5 and with Variant II (Bio-RadLaboratories Inc., Hercules, CA, USA) in Tromsø 6. HsCRP was measured by a particle-enhanced immuno-turbidimetric assay from Roche (Mannheim, Germany).

Blood samples for s-ferritin and transferrin saturation were drawn at Tromsø 5 visit 1 and samples for hemoglobin and hsCRP in Tromsø 5 visit 2 a few weeks later. Serum for analyses on ferritin, iron and transferrin was stored at -70ºC in 12 months before analyses on a Hitachi 917 analyzer (Boehringer, Ingelheim, Germany). Iron was measured by the ferrozine method and ferritin and transferrin were measured by a turbidimetric assay. In an effort to harmonize s-ferritin levels within Norway at the time, the laboratory used a factor of 0.82 for s-ferritin analysis.[111] Transferrin was reported in g/L and serum TIBC was calculated as s-TIBC µmol/L= 25.1 x s-transferrin. Transferrin saturation (%) was calculated as 100 x (serum iron/TIBC). Hemoglobin was measured with an automated blood cell counter (Coulter Counter, Beckman Coulter, Inc., Brea, CA, USA) within 24 hours.[112]

3.1.5 Data from questionnaires

Information on smoking, diabetes and cardiovascular disease was obtained from self-administered questionnaires. In paper 1, daily smoking was categorized as current, past and never, while in paper 2 it was dichotomised as current, yes/no.

3.1.6 Statistical analyses

For all analyses double-sided p-values <0.05 were considered statistically significant.

In paper 1 and 2 we used multivariable linear regression analyses (least squares regression) with retinal vascular calibre as dependent (outcome) variable and cardiovascular risk factors simultaneously (paper 1) and ferritin, transferrin saturation and hemoglobin separately (paper 2) as independent (exposure) variables:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + ... + \beta_n X_n$$

Y= fitted value of the dependent variable (retinal vascular calibre);

β_0= constant

β_1-β_n= regression coefficients for the independent variables $X_1 \ldots X_n$ in the regression model. The β gives the estimated change in the dependent variable with a defined change in the independent variable.

In paper 1 we further used multivariable logistic regression analyses (maximum likelihood):

$$Logit(p) = \ln(p/(1-p)) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + ... + \beta_n X_n$$

p=probability of a dichotom event (arteriolar narrowing/venular widening)

β_0= constant

β_1-β_n= regression coefficients for the independent variables $X_1 \ldots X_n$ in the regression model.

The e^{β} gives the estimated OR for the event with a defined change in the independent variable.

We performed sex-stratified analyses in paper 1 and 2. In paper 1 this was chosen as we found interaction between sex and exposure variables (HDL cholesterol and BMI), evaluated by including the appropriate cross-product term in the regression model. In paper 2 we chose sex stratified analyses as there was significant interaction between sex and s-ferritin and there was substantially lower level of s-ferritin and hemoglobin in women than in men.

In paper 2 we examined three multivariable regression models. Model 1 included adjustment for traditional cardiovascular risk factors (age, systolic and diastolic blood pressure, smoke, body mass index, HbA1c, total and HDL cholesterol). Model 2 included adjustment for hemoglobin in model for s-ferritin and vice versa, in addition to all the variables in model 1. Model 3 included adjustment for hsCRP in addition to the variables in model 1. Non-linear associations between hemoglobin, transferrin saturation, log-transformed s-ferritin and retinal vascular calibres were analyzed in age-adjusted first and second-degree fractional polynomial models with *fracpoly* in STATA. Interaction between exposure variables and prevalent hypertension, cardiovascular disease and diabetes was examined.

In paper 1 we performed supplementary analysis with additional adjustment for the fellow vessel calibre and spherical equivalent refraction, in separate models (not simultaneously). In paper 2 we performed supplementary analysis with additional adjustment for spherical equivalent refraction.

In paper 2 missing data were imputed by multiple imputation by chained equations (MICE) in STATA (*mi impute chained*) as this method may handle different types of variables (continuous, binary and categorical).[113-115] The imputed values are drawn from the posterior predictive distribution of the missing data, conditional on the observed data. Imputation was performed separately for women and men. We imputed 50 dataset and the regression analyses were performed by the Stata command '*mi estimate'* which combine estimates from each imputed set using Rubin's rule[113]: The combined estimate $\hat{\theta}$ is the average of the individual regression estimates from each of the 50 (m) datasets:

$$\hat{\theta} = \frac{1}{m}\sum_{j=1}^{m}\hat{\theta}_j$$

The total variance of $\hat{\theta}$ is determined by the within-imputation variance (W) and the between-imputation variance (B):

$$\mathrm{var}(\hat{\theta}) = W + \left(1 + \frac{1}{m}\right)B$$

W and B determined by: $W = (1/m)\sum_{j=1}^{m} W_j$ and $B = \left(1/(m-1)\right)\sum_{j=1}^{m}\left(\hat{\theta}_j - \hat{\theta}\right)^2$

Stata 12 (StataCorp, Texas, USA) was used for all statistical analyses, except ICC that was calculated in PASW Statistics 18 (SPSS).

3.2 Paper 3

In paper 3 we investigated if light exposure before imaging and sequential image capturing affect the retinal vascular calibre.

The study participants were recruited among healthy employees and students at Nordland Hospital in Bodø. Inclusion criterion was normal corrected vision on least one eye without any eye disease. Exclusion criterions were (1) reduced vision or eye disease on both eyes, (2) hypertension, (3) heart disease, (4) kidney disease, (5) diabetes, (6) myopia < -3 or hyperopia > +3 spherical equivalents. The study was approved by the Regional Committee for Medical and Health Research Ethics and adhered to the tenets of the Declaration of Helsinki.

We had no certain prior estimates of the variances in the response variables (change in retinal vascular calibre), the nearest was the study of Barcsay et al. with 11 participants and differences in venular calibre in dark vs. light with the range of -5.4% to 3.8%, suggesting a

SD of 2.3%.[94] This indicated that at least 27 participants were needed to test for a difference of 1.5% or more with level of significance of 0.05 and power 0.90.

Thirty-five persons were examined. Two persons were excluded due to elevated blood pressure (systolic blood pressure > 160 mmHg and/or diastolic blood pressure > 100 mmHg) and one person due to blood pressure lowering medication. This left 32 persons to be included in the study. All participants gave written informed consent.

Refraction was measured on both eyes with a Humphrey Automatic Refractor, modell 597. Visual acuity was tested using the built in Snellen charts. The pupil of one eye was dilated with one drop of tropicamide 0.5% (Tropicamide, Chauvin Pharmaceuticals Ltd. Kingston Upon Thames, Surrey, England). The dosage was repeated after 5 minutes. All participants were given written information ahead of the examination about the effects and potential risk of pupil dilatation and this information was repeated before application (Appendix). Intraocular pressure was measured with an Icare Tonometer (model TA01i, Helsinki, Finland) prior to dilatation. Blood pressure was measured with an automatic sphygmomanometer (WelchAllyn ProBP 3400, Skaneateles, NY, USA) in sitting position after 10 minutes of rest.

Retinal photos were captured on one eye by FF450 plus IR Fundus Camera, model 1087-185 (Carl Zeiss Meditec, Jena, Germany), an optical device using light in the visible spectrum and flash illumination at capturing. Flash energy level was set to 32 Ws. Images were disc-centred 50 degrees retinal colour photographs with resolution 2196x1958 pixels.

The study protocol is presented schematically in figure 5. Prior to imaging the fundus camera was prepared. To facilitate short delay before capturing of the first image after dark exposure, fluorescent markers were placed on chin and forehead for position according to markers on the camera device. An external red lead light for the other eye, placed out of sight of the examined eye, was positioned to have the image disc-centred. The camera was focused according to the user manual. Dark exposure was then achieved with the room darkened, eyes closed and head turned away from two monitors in the room. After 10 minutes the head was positioned according to the markers on the face before the eyes were opened and the other eye fixating on the lead light. Camera light control was then turned on faintly allowing for a final adjustment if needed, before the first image was captured. Time from light was turned on in the camera until the first image was captured, was measured on

12 consecutive participants with the median of 6.3 seconds, range 2.9-10.8 seconds. A total of 6 images were captured approximately 10 seconds apart. Light exposure was then performed with participants positioned 30 cm in front of a daylight lamp (Philips EnergyLight HF 3319, ultraviolet-free) and looking continuously at the centre, with light intensity adjusted to give 1000 Lux at this distance (INS DX-200 Digital Luxmeter). After 10 minutes 6 fundus images were captured approximately 10 seconds apart. The pupil-size was measured immediately after both imaging sequences with aid of a pupil gauge with circular templates with diameter steps of 1 mm, range 2-9 mm.

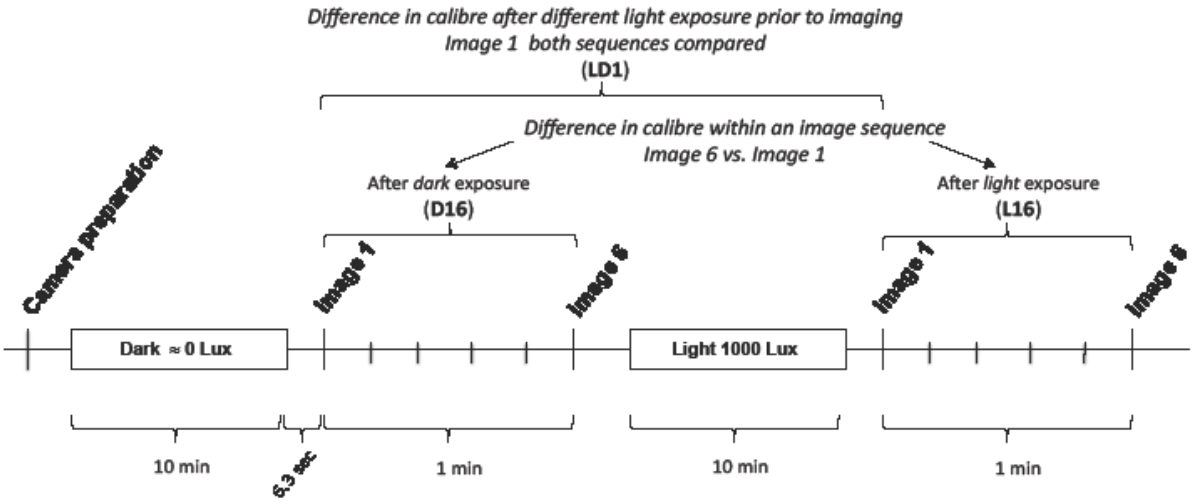

Figure 5. Schematic illustration of the study protocol.

Retinal vascular calibre measurements were performed as described in section 3.1.3. Image 1 and image 6 were graded in both sequences, i.e. sequence after dark exposure and sequence after light exposure. The four images from one participant were not graded in a row and the grader was masked for information on capturing sequence number and light exposure prior to imaging. All images were graded by TvH.

We used a one sample double-sided t-test for the difference between repeated measurements (at two points/exposures), testing against the null-hypothesis of zero difference. We used a paired double-sided t-test to test whether the change in calibre during an image sequence of 6 images was different with prior dark exposure versus light exposure. Shapiro-Wilk was used for test of normal distribution of the test variables.

4 Results – summary of papers

Paper 1

Retinal vascular calibres are significantly associated with cardiovascular risk factors: the Tromsø Eye Study

The aim of the study was to describe the association between retinal vascular calibres and traditional cardiovascular risk factors in a cross-sectional design.

The study included 6353 participants in Tromsø 6.

High age and blood pressure were associated with thinner retinal arterioles. High HbA1c and smoking were associated with wider arterioles. Low HDL cholesterol was associated with wider arterioles in men only. Blood pressure had the strongest effect on arteriolar calibre, with a decrease in calibre of 3.6 µm (women) / 4.1 µm (men) per SD increase in mean arterial blood pressure.

High age and blood pressure were associated with thinner retinal venules. Smoking, high BMI, high LDL cholesterol and low HDL cholesterol were associated with wider venules. The effect of BMI and HDL cholesterol on venular calibre was significantly stronger in men than in women. Current smoking was the most important factor for venular calibre, where smokers had 13.2 µm (women) / 15.2 µm (men) wider calibre than non-smokers.

In regression models with adjustment for the fellow vessel calibre, the association between arteriolar calibre and smoking was no longer significant and that of venular calibre and blood pressure changed direction.

Paper 2

Serum ferritin and hemoglobin are independently associated with wider retinal venular caliber. The Tromsø Study 2001-2008

The aim of the study was to investigate if markers of iron body stores and hemoglobin were associated with retinal vascular calibres measured 6 years later.

The study included 2993 participants in Tromsø 5 who had retinal vascular calibre measurements in Tromsø 6.

Men had higher levels of hemoglobin and s-ferritin than women.

Hemoglobin was positively associated with retinal venular calibre at follow up in both men and women, independent of traditional risk factors (mean difference highest compared to lowest sex-specific quartile: men 5.99 µm, p=0.001; women 7.28 µm, p<0.001). Hemoglobin was not associated with arteriolar calibre. In men the effect of hemoglobin on vascular calibre was stronger in participants without hypertension. This was evident in both venular and arteriolar calibre (p for interaction=0.003 and 0.041, respectively).

S-ferritin was positively associated with retinal venular calibre at follow-up in men but not women, independent of traditional risk factors (mean difference highest compared to lowest sex-specific quartile: men 4.21 µm, p=0.013; women -0.21 µm, p=0.89). The association in men was attenuated, but still significant, after adjustment for hemoglobin. S-ferritin was not associated with arteriolar calibre.

Transferrin saturation was not associated with retinal vascular calibres.

Paper 3

Retinal vascular calibre and response to light exposure and serial imaging

The aim of the study was to investigate whether retinal vascular calibre measurements on optical retinal photography are affected by light and dark exposure prior to photography and whether the retinal vascular calibres change during an imaging sequence of several images.

The study included 32 healthy adults who underwent digital optical retinal photography in two separate image sequences of 6 images during 1 minute; one sequence with 10 minutes of dark exposure and one with 10 minutes of light exposure prior to imaging.

Retinal venular calibre (CRVE) was wider with prior light exposure (2.7%, p=0.0001), than with prior dark exposure. Within each sequence there was a venular dilatation from first to last image, both with prior light exposure (1.7%, p=0.0003) and prior dark exposure (3.1%, p<0.0001), with the change less pronounced with prior light exposure (p=0.016). Retinal arteriolar calibre (CRAE) showed no significant change in either outcome.

5 Discussion – methodological considerations

5.1 Internal validity

The internal validity of a study refers to how the study reflects the actual study population. This is determined by the accuracy in estimations of associations, which means that the estimates are made with little error. *Random errors* are usually not a threat to the validity, though it will reduce precision, while *systematic errors* may bias and confound the estimates in a study. In epidemiologic studies this is usually caused by selection bias, information bias or confounding.[116] The following discussion is organized according to these three concepts, though some aspects may have several possible effects.

5.1.1 Selection bias

Those attending a study or included in analyses may be different than those that are not. If there are systematic differences in the characteristics between the two, this may bias the results. For such a bias to occur, the relation between exposure and outcome must be different for among those who participate, compared to the non-attending eligible population.[116,117]

5.1.1.1 Tromsø study population

In the Tromsø study, the selection criteria and use of the official population registry, combined with relatively high attendance rates are important factors to control selection bias. We have limited access to information on non-attendees. There is a tendency towards lower participant rates in the younger age groups and in men (www.tromsostudy.com). There are higher attendance rates in married subjects; In Tromsø 6, 59% of the attendees were married whereas 41% in non-attendees.[106] In the older age groups, the participant rates were lower. The *healthy participant effect* describes that the sickest and oldest are less likely to meet in surveys. People attending surveys may also tend to be more health-interested and this may contribute to the healthy participant effect. Due to legal restrictions, given by the Norwegian Data Inspectorate, we are allowed to study only those individuals that have given consent, thus we are precluded from studying the non-attending eligible population, by collecting information from e.g. the death registry or hospital journals.

However, analyses from Tromsø 4 demonstrated lower mortality in consistent attendees through Tromsø 2-4 compared to those invited but attending only in Tromsø 4.[106] This may indicate that those attending are healthier, or possibly that attending such a study may contribute towards a healthier behaviour.[118]

5.1.1.2 Missing retinal images and exclusion of retinal vascular calibre gradings

A total of 767 participants did not have retinal photos taken as part of visit 2 in Tromsø 6. In 558 participants this was due to logistic reasons and random events and has most probably not contributed to selection bias as participants were invited in random order. In 209 participants we have no images as they refused to attend the eye examinations. These subjects were slightly older (65.0 vs. 63.4 years), had slightly higher HbA1c (5.80 vs. 5.73 %) and higher frequency of self-reported eye-diseases.[107] This may mirror a healthy participants effect, though the differences were small.

Of the 6540 with retinal images, we excluded 38 participants with fewer than six of each vessel type, as in that case the revised formulas are not validated.[10] The majority of cases were due to the anatomic variation of sparse branching pattern proximal to zone B (Figure 3). Still we may not exclude that in a few of these cases, generalized vascular narrowing was adding to the pattern, with the smallest vessels not being measurable. We excluded 23 subjects with confounding retinal pathology, e.g. fundus laser treatment, retinal vein occlusions and myelinisation affecting vessels in zone B, as we considered they would only add noise to the data. The numbers were small and thus probably not important in regard to selection bias.

Table 3 shows the risk factor levels in the 6353 participants eligible for analyses on retinal vascular calibre compared to the 953 participants at visit 2 in Tromsø 6 who were excluded due to lack of images, unusable grading or confounding retinal pathology. Those excluded were older and tended to have a higher prevalence of diabetes. The proportion of daily smokers was higher in excluded men, while the prevalence of self-reported cardiovascular disease was higher in excluded women. Thus there is a possible selection bias, while we have no strong reasons to believe that these differences have essentially affected the results.

Table 3. Risk factor levels in the participants eligible for analyses on retinal vascular calibre compared to the rest of participants in Tromsø 6 visit 2.[a]

	Women			Men		
	Eligible	Not eligible	P value	Eligible	Not eligible	P value
Number	3627	538	-	2726	415	-
Age (year)	63.1 (9.1)	66.2 (10.4)	<0.0001	63.2 (8.9)	66.1 (9.9)	<0.0001
MABP (mmHg)	97.3 (13.5)	98.6 (13.8)	0.04	101.5 (12.3)	101.2 (12.3)	0.66
BMI (kg/m2)	26.8 (4.6)	26.5 (4.6)	0.11	27.4 (3.6)	27.2 (4.0)	0.19
LDL chol. (mmol/L)	3.7 (1.0)	3.7 (1.0)	0.34	3.6 (1.0)	3.5 (1.0)	0.02
HDL chol. (mmol/L)	1.7 (0.4)	1.7 (0.5)	0.72	1.4 (0.4)	1.4 (0.4)	0.99
HbA1c (%)	5.7 (0.6)	5.8 (0.7)	0.003	5.8 (0.7)	5.8 (0.8)	0.03
Current smoking	19.6 (699)	20.0 (106)	0.82	16.6 (448)	20.8 (85)	0.04
Myocardial infarction	3.6 (126)	6.0 (31)	0.008	10.4 (278)	13.7 (56)	0.05
Angina	4.7 (167)	8.5 (44)	<0.001	8.3 (220)	8.2 (33)	0.97
Stroke	2.0 (70)	4.6 (24)	<0.001	4.0 (107)	5.4 (22)	0.19
Diabetes	7.9 (280)	10.8 (57)	0.02	9.8 (261)	12.2 (50)	0.12

MABP, mean arterial blood pressure; BMI, body mass index; LDL chol., low density lipoprotein cholesterol; HDL chol., high density lipoprotein cholesterol; HbA1c, glycosylated hemoglobin.
a) Total N=7306 with valid consent. Results given as mean (SD) or percentage (number)

5.1.1.3 Loss of follow up

Data for paper 2 were collected at two different surveys 6 years apart. Eligible for inclusion were subjects who had participated both in Tromsø 5 and in the retinal vascular calibre measurements in Tromsø 6. Apart from non-attending eligible subjects in Tromsø 6, those lost at follow up were participants that had moved or died before the follow up survey. The emigrants have probably not contributed to selection bias, while the dead may have, as we can assume that they were older and had a higher morbidity load, thus contributing to the healthy participant effect. However, we have no strong reasons to believe that the exposure-outcome associations differ between participants compared to non-participants.

5.1.2 Information bias

Information bias is caused by systematic errors in the measurement of exposure and/or outcome and may possibly affect the results.[117]

5.1.2.1 Image quality

Image quality may result both in information and selection bias as well as confounding. Of the 6540 with retinal images, 29 subjects had ungradable images. Further, in the 96 subjects

with grading considered not usable because not all of the six widest vessels of each type could be traced, 80 had grading on poor quality images. Of the 6353 subjects included for analyses in paper 1, 418 had grading on poor quality images. Image quality was marked as poor when the grader considered it likely to affect the measurements. We believe that the most frequent reason for poor quality and ungradable images was probably cataract. However, this could not be confirmed as the study did not include slit lamp examination or anterior segment imaging. Cataract is associated with diabetes, chronic renal failure, smoking and recent studies have demonstrated increased risk with obesity and statin use.[119-123] We have performed supplemental analyses in paper 1, excluding the 418 with grading on poor quality images, and this did not substantially change the results (not shown).

In paper 2 we excluded subjects with poor quality images (n=313). Measurement error is more likely with poor quality images and we suspected cataract to possibly bias or confound the results. We suspected this as we found that the estimates for the association between s-ferritin and retinal venular calibre in men were increased by >25% by excluding subjects with poor quality images, leaving the standard errors only minimally increased in spite of reduced number of participants (complete case analyses, results not shown). There is evidence that increased iron content in the lens is associated with oxidative damage and cataract formation,[124] but whether this is related to level of s-ferritin is unknown.

5.1.2.2 Measurement of retinal vascular calibre – validity

The IVAN method for retinal vascular calibre measurement has been validated in several ways as described in the introduction, proving to be a method with high reproducibility.[9,11,13,14,17,18] The grading centre (RetVIC, section 3.1.3) performs regular assessment of their graders. We tested the reproducibility of the grading performed by TvH (intra- and inter-reliability and temporal drift), which gave ICC above 0.93 for all of the tests in the Tromsø Study, with results illustrated in figure 6. The test results gave assurance of good reliability of the performed grading of retinal vascular calibre in Tromsø 6.

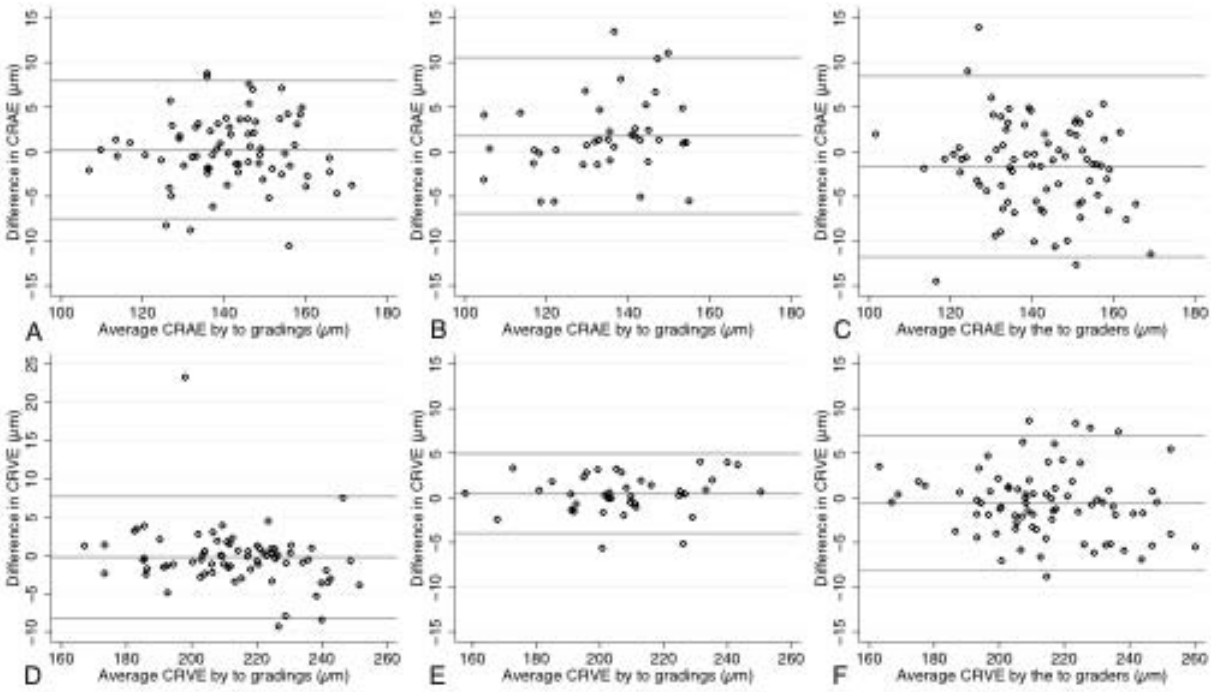

Figure 6. Bland-Altman plots of intra-grader-reliability (A and D), temporal drift (B and E) and inter-grader reliability testing the grading performed by TvH versus that to the coordinator at RetVIC (C and F). Lines give the mean difference between the two gradings and the 95% limits of agreement (mean difference ± 1.96 standard deviation of the difference).

Repeatability of the grading in paper 3 was assessed by re-grading 60 random images and gave ICC of 0.98 for CRAE and 0.99 for CRVE. Mean (SD) difference between first and second grading was -0.42 (3.15)µm for CRAE and -0.41 (2.57)µm for CRVE.

In paper 3 we found that retinal venular calibre was significantly increased in the last compared to the first image of an image sequence of 6 images. In the Tromsø Eye Study the disc-centred image, used for retinal vascular calibre measurements, was taken early in the image sequence (second image) which probably has limited a possible bias.

There is a pulse related variation of 3.5-4.3% in retinal arteriolar calibre and 3.1-4.8% in retinal venular calibre.[15,16] Error due to this was probably randomly distributed due to the cyclicity of the pulse, but the lack of pulse synchronization is a limitation of the present studies. Even after controlling for pulse cycle, grading of different images of the same subject has been shown to be the factor imposing most variance in calibre measurements, probably related to both biological changes and differences in camera and eye position.[17]

In paper 3 an important strength is that the grader was masked for information on light exposure and image sequence number, which is impossible with techniques using real time measurements.

5.1.2.3 Measurement of exposures variables – misclassification

Information on smoking, diabetes and cardiovascular disease was obtained from self-administered questionnaires. Smoking habits are prone to under-reporting and there may be differences in reporting between groups, which may lead to differential misclassification and possible bias associations or lead to residual confounding.[125,126] Smoking is an important determinant of retinal vascular calibre and is involved in the pathogenesis of a diversity of diseases, and should ideally be biochemically confirmed. Self-reported diabetes is limited by the high fraction of unrecognized diabetes in the older population and we have compensated for this as we included those with HbA1c ≥ 6.5% as having prevalent diabetes.[127]

The measurements of blood pressure, height and weight in the Tromsø study were standardized and performed by trained personnel. Though blood pressure was measured following a specified protocol (thrice over a short time interval), it can be considered as a point measurement and thus prone to misclassification as blood pressure may vary considerably during the day. Still, in this type of study, with a high number of participants, it is the best possible as long term ambulatory measurements would not be feasible. Blood samples were analysed at a hospital laboratory adhering to quality control systems. Freezing of serum affects s-ferritin measurements only to a small extent.[128,129]

5.1.3 Confounding

Confounding may be considered as confusion of effects (figure 7). When investigating a possible causal relationship between an exposure and an outcome it is important to consider confounding factors, i.e. factors that are causally related to the outcome *and* associated (causally or non-causally) with the exposure variable. Additionally the confounder shall not be an intermediate between the exposure and the outcome.[116,130]

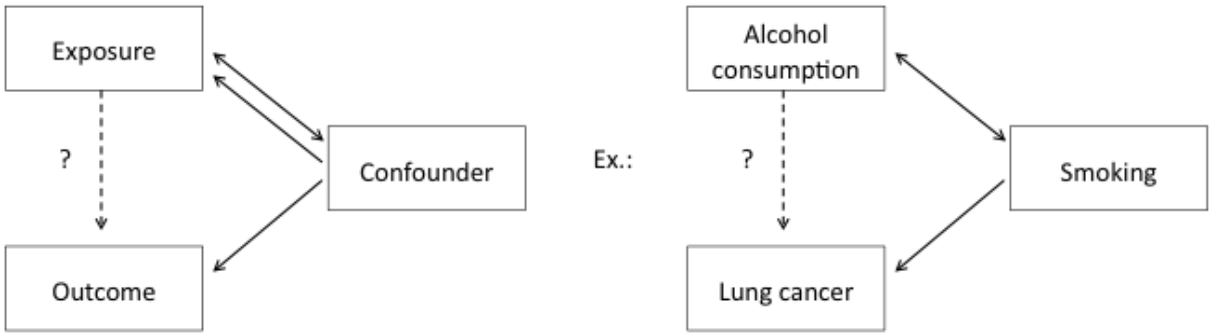

Figure 7. Definition of confounding. Unidirectional arrow indicates that the association is causal; a bidirectional arrow indicates a non-causal association.

In observational studies, it is considerably important to evaluate confounding in the analytic stage as the population is non-selected and contains a variety of different groups and levels of exposures. We used multivariable regression analysis as it is a convenient strategy, allowing for adjustment for several factors at the same time. An alternative strategy is stratified analyses, though only convenient for one or very few confounding factors at the same time or in combination with multivariable regression analyses. We stratified by sex in both papers, mainly to handle interaction, secondary it is useful to handle confounding by sex.

5.1.3.1 Serum ferritin, hemoglobin and hsCRP

In paper 2 we found that s-ferritin and retinal vascular calibre was associated while the causal relationship is not established. S-ferritin is causally associated with hemoglobin and is a possible confounder in the association between hemoglobin and retinal venular calibre (Figure 8 A). Regression estimates for this association were unchanged with adjustment for s-ferritin, thus not supporting that s-ferritin acts as a confounder in this relationship. In regard to the direction of association between s-ferritin and hemoglobin, we would consider hemoglobin not as a confounder, but possibly on the pathway in the relationship between s-ferritin and retinal venular calibre (Figure 8 B). Adjustment for hemoglobin attenuated the regression estimates (24%) supporting that part of the association was mediated through the effect of hemoglobin. Still there was a significant association, independent of level of hemoglobin, supporting an additional pathway.

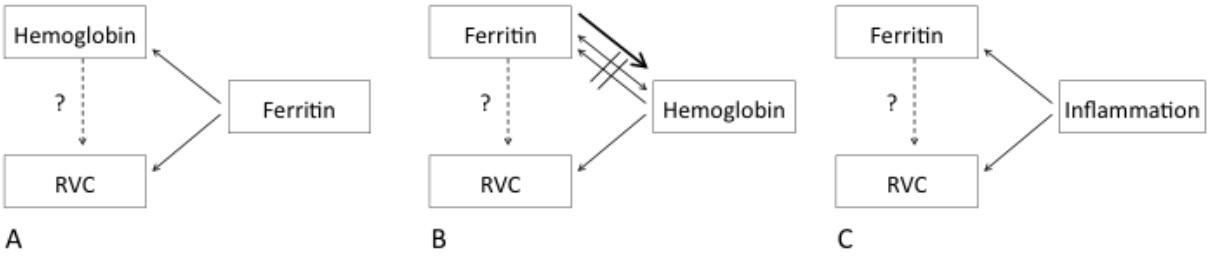

Figure 8. (A) Ferritin as a possible confounder in the relationship between hemoglobin and retinal vascular calibre (RVC). (B) Hemoglobin as a possible intermediate in the association between s-ferritin and retinal vascular calibre. (C) Inflammation as a possible confounder in the relationship between ferritin and retinal vascular calibre. Unidirectional arrow indicates that the association is causal; a bidirectional arrow indicates a non-causal association.

S-ferritin is an acute phase protein increasing with states of infection and inflammation and interpretation is thus problematic in populations in which the prevalence of infection or inflammatory diseases is high, which is not the case in Norway. Studies have demonstrated that markers of inflammation are associated with retinal vascular widening.[30,131,144] Regression estimates for the association between s-ferritin and retinal venular calibre were unchanged with adjustment for hsCRP, i.e. hsCRP did not act as a confounder (Figure 8 C), suggesting that the association is not solely reflecting a possible relationship between low-grade inflammation and retinal venular widening.

5.1.3.2 Adjustment for refraction

Refraction and axial length imposes a magnification effect on images with increasing magnification with hyperopia.[132] Adjustment for refractive error (spherical equivalent refraction) in analytic model did not essentially change the results and left the estimates for most traditional cardiovascular risk factors unchanged. Age was positively associated with refraction and adjustment clearly tended to increase the effect of age with more than doubling of the estimates, indicating that some of the age-effect was hidden by the magnification from refraction, thus refraction acted as a negative confounder. Concerning s-ferritin, adjustment for refractive error in analytic model tended to slightly attenuate the estimates (10%) and narrowing the standard errors, though did not essentially change the results. Association between hemoglobin and retinal vascular calibre was unaffected by adjustment for refractive error.

5.1.3.3 Changes in light exposures

Light exposure following dark adaptation seems to induce a rapid transient increase in the retinal blood flow velocity, reaching peak value at 30-60 seconds.[95] Though the neurovascular coupling is slower in the retina than in the brain,[85] the possibility of relatively rapid changes in retinal blood flow due to changes in light exposures is challenging when investigating the effect of light, and there is a possibility of confusion of effects. Our study-design in paper 3 involved use of an optical photography device that used light in the visible range, which limits our ability to conclude on retinal vascular calibre during dark, as our first image was taken after a few seconds of light exposure. But the method allows assessment of differences in calibre after standardized prior dark and prior light exposure. In our study we put emphasis on shortening the time from full darkness to the first image was captured to avoid the transition described by Riva et al.[95]

5.1.3.4 Changes in blood pressure

In paper 3 we cannot exclude systematic changes in blood pressure during examination, as it was measured only once prior to imaging. Change in blood pressure could have confounded the correlation between arteriolar calibre and light exposure/image sequence as retinal arteriolar calibre shows vasoconstriction with rising blood pressure.[133] However, an effect on venular calibre is unlikely as venular blood flow rate and calibre is stable when acute changes of arteriolar blood pressure occur within the range of mean arteriolar blood pressure less than 115 mmHg.[134]

5.2 External validity

The external validity of a study refers to what extent the results are valid for people outside the study population. The age and sex distribution in the Tromsø Study reflects the Tromsø population in general. The Tromsø population is mainly of Caucasian origin (97.6%) and the generalizability could be restricted by ethnicity. The risk factor levels are comparable to other Western populations and results are likely to be applicable to similar adult Caucasian populations, with high living standards and level of education and equality in social services. The generalizability of the associations between s-ferritin, hemoglobin and retinal vascular calibre may be restricted to populations with low prevalence of anemia, infection and inflammatory diseases.

In paper 3 we included healthy volunteers only, and the results may not be valid for other groups, e.g. smokers and persons with overweight, dyslipidemia and diabetes.

5.3 Some statistical considerations

5.3.1 Association versus causation

"Association is not causation" and a cause must precede the effect.[117] In paper 1 we investigated the relationship between established cardiovascular risk factors and retinal vascular calibre in a cross-sectional design. This is a point measure and we are precluded from inferring about a causal relationship. Even with a longitudinal design, as in paper 2, we are not able to definitely conclude about the temporality as we have available retinal photos and retinal vascular calibre measurement only at the latest survey, and may not investigate how potential risk factors are associated with *change* in the calibre.

5.3.2 Complete case analyses versus imputation

Missing data will occur in nearly all dataset in medical and epidemiological research and all statistical analyses make assumptions about the missing data. Rubin and Little introduced the concept of three categories of missing mechanisms: (i) *missing completely at random* (MCAR) – the probability of missingness is unrelated to both observed and unobserved data; (ii) *missing at random* (MAR) – the probability of missingness does not depend on the unobserved (missing) data, conditional on the observed data (measured and included in the statistical model) or (iii) *missing not at random* (MNAR) – the probability of missingness depends on the unobserved data.[113,115]

In paper 1 we used complete case analyses, losing 197 participants (3%) due to missing values on one or more of the variables in the regression model. Complete case analyses assume the missing data to be MCAR. This is the strongest possible assumption and is nearly never true, though it has been the most frequent strategy in epidemiologic research. As the percentage of incomplete cases was small, we believe that it is unlikely that this had substantial impact on the results.

In paper 2 our exposure variables of interest were measures of iron status and hemoglobin. S-ferritin was missing in 5%, transferrin saturation in 4% and hemoglobin in 16% of the participants. Blood samples for hemoglobin were drawn at visit 2, to which 148 participants

were not eligible. In addition, due to logistic reasons samples for hemoglobin in Tromsø 5 collected after 1:00 pm the day before weekend were not analyzed, resulting in missing values of hemoglobin in 12% of the participants.[112] Considering the known missing mechanisms (described in detail in paper 2, Supplemental Materials) we did not suspect this to substantially bias the study-sample. The primary motivation for performing imputation of missing data was to gain power in the analyses, especially the model including both s-ferritin and hemoglobin where the percentage missing was greatest (23%). Additionally we found that age was negatively and hsCRP was positively associated with missingness of hemoglobin, thus performing imputation (including age and hsCRP in the imputation model) has possibly corrected some selection bias in the data.

5.3.3 Multivariable model building

In paper 1 we performed supplementary analysis with additional adjustment for the fellow vessel calibre. Liew et al. have proposed that modelling arteriolar and venular calibre simultaneously may give less confounded results, though they later on pointed to the possibility of over-adjustment when including the fellow vessel calibre in the regression model.[20,28] Kaushik et al. have described an alternative way to handle this adjustment, based on a residual method.[135-137] CRAE and CRVE are correlated and important reasons for this are probably shared genetic and environmental influences, normal anatomic variation and measurement error from e.g. magnification effect, poor focus, cataract and background pigmentation.[12,20,28,138] Adjustment for the fellow vessel calibre may possibly control for normal anatomic variation in vessel calibre and some sources of measurement errors, e.g. cataract, refraction and axial length. In our study we had the possibility to adjust for refraction. It is possible that adjustment for the fellow vessel calibre may hide or disturb relevant associations, as genetic and environmental factors affecting both arteriolar and venular calibre are relevant to assess when evaluating the importance of microvascular changes in the pathogenesis of cardiovascular disease. Further, it is possible that disturbances in the autoregulation or vascular changes due to autoregulation may be important when investigating the relation between retinal vessels and cardiovascular pathology. We expect that adjustment for the fellow vessel calibre could hide such associations as part of the correlation between CRAE and CRVE may also be related to

covariance due to vascular autoregulation in the retina due to the role of retinal arterioles as end-arteries and resistance vessels which mediate this regulation.[87]

5.3.4 Assessment of the relative contribution of the risk factors

To assess the relative contribution of each cardiovascular risk factor to the explained variance in retinal vascular calibre, we chose to use the partial R^2 and not the standardized regression coefficients . The latter gives the estimate of a standardized change in the dependent variable, with 1 SD change in the independent variable, and is useful for comparing the effect of different continuous independent variables (1) within the same study and (2) in different studies.[139] It is not a meaningful measure with dichotomous independent variables as the predictor will be either present or not. The use of standardized regression coefficients has been criticised and it is important to appreciate its limitations as the SD is determined by the sample distribution and thus is dependent on and sensitive to the study design.[140,141]

6 Discussion – results

Prospective data from population based studies indicate that narrower retinal arterioles and wider retinal venules may be independent predictors of cardiovascular disease.[21,22,26,27]

We found that all the examined traditional cardiovascular risk factors were associated with retinal vascular calibre (Table 1). The amount of effect of each risk factor differed between arteriolar and venular calibre, with blood pressure mainly affecting arteriolar calibre while factors associated with vascular widening were most evident in venular calibre. Retinal vascular calibres by age and sex are shown in Table 4, which shows that both arteriolar and venular calibre decreases with age.

Table 4. Retinal arteriolar and venular calibre by age groups and sex.[a]

	CRAE			CRVE		
Age (years)	n	Women	Men	n	Women	Men
<50	140	146.6 (1.18)	147.2 (1.72)	63	213.6 (1.60)	215.5 (2.27)
50-55	517	145.1 (0.67)	141.0 (0.72)	409	214.5 (0.95)	212.5 (0.98)
55-60	510	142.5 (0.62)	138.0 (0.65)	462	212.9 (0.88)	211.3 (0.96)
60-65	1005	140.7 (0.45)	137.2 (0.55)	722	212.8 (0.66)	212.2 (0.82)
65-70	598	140.1 (0.62)	136.3 (0.74)	364	210.8 (0.89)	209.7 (1.11)
70-75	391	139.0 (0.73)	136.9 (0.70)	361	209.7 (1.04)	210.8 (1.05)
75-80	288	138.2 (0.80)	135.2 (0.92)	238	206.2 (1.30)	206.4 (1.37)
>80	178	137.7 (0.99)	135.9 (1.35)	107	201.8 (1.63)	203.1 (1.89)
P [b]		< 0.001	< 0.001		< 0.001	< 0.001

Crae: central retinal artery equivalent; Crve: central retinal vein equivalent

a) Results given as mean (SE). b) p for linear trend over age-groups.

The effect of the risk factors differs in direction, and probably this reflects different pathological processes. The link between hypertension and retinal arteriolar narrowing is well documented and is related to chronic arteriosclerotic changes and adaptive labile calibre changes to regulate perfusion pressure.[142] Retinal arterioles are end-arteries and resistance vessels and retinal autoregulation of perfusion is mediated through calibre changes, involving myogenic responses, metabolic signals and endothelial function.[87] Thus, disturbed autoregulation may also affect retinal arteriolar calibre. Pathohysiological mechanisms of retinal venular calibre changes are less understood. Wider retinal venular calibre is associated with reduced brachial flow-mediated vasodilatation, which indicate endothelial dysfunction as a possible mechanism.[143] Systemic inflammatory markers have

consistently been found to be associated with retinal venular widening, which supports that inflammation may be a link between venular widening and cardiovascular disease.[22] In the Rotterdam study, inflammatory markers were also associated with retinal arteriolar widening, though to a lesser degree than in venular widening.[144]

We found that blood pressure had a substantial effect on retinal arteriolar calibre and this is in accordance with previous studies.[19,28,35,36,145]

Further, smoking was important for explaining the variance in arteriolar calibre. Adjustment for the fellow vessel calibre was profoundly affecting the results, as the association between smoking and increased arteriolar calibre was no longer significant with adjustment for venular calibre. Liew et al. argued for this adjustment as they found the results more consistent with the literature, which reports that smoking is associated with hyaline and fibrous thickening of the myocardial and renal arterioles.[28,146,147] Another possible interpretation is that the observed association between smoking and wider retinal arterioles may reflect autoregulatory disturbances or response to smoke-induced vessel wall changes. Experimental studies have shown reduced flicker light-induced retinal vasodilatation and increased hyperoxia-induced reduction in vascular diameter in smokers.[99,148] This supports that part of the association between smoking and vessel calibre may be related to physiological effects or disturbances and not only anatomical changes.

Apart from blood pressure, age and smoking the effects on arteriolar calibre were small or absent. Low HDL cholesterol was associated with wider arterioles in men. The direction of association was consistent with three other studies, while the association has been insignificant in several other studies (Table 1).[19,28,30,42] The direction of association may seem surprising given the negative (inverse) association between HDL levels and risk of cardiovascular disease and because reduced arteriolar calibre is associated with ischemic heart disease and mortality.[26,27,149,150] The effect was small and in opposite direction compared to the effect of blood pressure and age, pointing to a different mechanism, possibly related to endothelial dysfunction.[100]

HbA1c was also associated with wider arteriolar calibre. The association between parameters related to diabetes and retinal vascular calibre is complex, as it is dependent of multiple factors as duration of diabetes, blood sugar control and presence and grade of

retinopathy, which are factors that are cross-connected.[41,47-51] Mechanisms behind the retinal vessel calibre changes in diabetes probably include disturbed autoregulatory responses in the small vessels.[87,151] Nguyen et al. showed that in persons with diabetes, wider retinal arterioles and venules were associated with impaired flicker-light induced vasodilatation, which is considered to be a sign of endothelial dysfunction.[100]

A recent meta-analysis including 9 adult populations found that overweight and obesity were associated with narrower arteriolar calibre.[152] In women we found no effect of BMI on arteriolar calibre while in men there was a corresponding near-significant negative association. Thus BMI may pull in the same direction as blood pressure and age in the effect in arteriolar calibre, opposite to the effect on venular calibre. Enhanced myogenic activation and endothelial dysfunction are suggested mechanism.[152]

Wider venular calibre may be a marker of burden of several risk factors and there has been suggested a connection to inflammation and endothelial dysfunction.[22,143] Smoking was the most important factor for explaining the variance in venular calibre and results are in accordance with other studies.[19,28,40] Some of the effect of smoking may be secondary to arteriolar widening and a possible increased retinal perfusion. The association was attenuated but was still substantial with adjustment for arteriolar calibre. The involvement of endothelial dysfunction is supported by an experimental study finding that flicker induced vasodilatation in veins was diminished in smokers.[99]

Further, high BMI and unfavourable lipid-profile has been associated with retinal venular widening which may involve inflammation and endothelial dysfunction.[22] Our results, with a positive association between BMI and venular calibre were consistent with the meta-study on obesity.[152] The association to lipid-profile has been less clear in previous studies, which may be due to small effects and varying size of the explored populations as well as methodological differences, especially concerning adjustment for the fellow vessel calibre.[19,28-31,40] We found that both high LDL and low HDL cholesterol was associated with venular widening, consistent with several of the previous studies.

Contrary to the risk factors discussed above, blood pressure and age was associated with reduction in venular calibre. On the other hand, concerning blood pressure, there was a change in direction of association when adjustment for arteriolar calibre was included, as

was also demonstrated in the ARIC study. Liew et al. argued for this adjustment as blood pressure is an important risk factor and retinal venular widening a predictor of cardiovascular disease.[28] We believe that the effect of this adjustment must be interpreted with caution as it probably reflects that the effect of blood pressure is considerably stronger on arteriolar than venular calibre.

The study size of the Tromsø Study allowed for sex-stratified analyses, and we found that the association between low HDL cholesterol and high BMI with venular widening was significantly stronger in men than in women. To the best of our knowledge, effect modification by sex has not been fully explored in previous studies. In the ARIC study, sex differences in the association with HDL cholesterol, endothelial function markers and alcohol consumption were reported, without results specified.[28] In the SiMES they described interaction between sex and blood pressure only, with stronger effect of blood pressure on arteriolar narrowing in men than in women, which we did not observe.[29]

The biological reasons and implications for the observed sex differences are not known and interpretation should be done with caution. Although the onset of ischemic heart disease is approximately 10 years later in women, it is accompanied with more morbidity and worse prognosis and it has been hypothesized that microvascular pathology in ischemic heart disease is more important in women than in men.[26,153-155] Our results indicate that such gender differences probably are not related to the direct microvascular effect of the traditional risk factors investigated, as we found that either there was no gender specificity or that in fact BMI and HDL cholesterol imposed greater effect on the retinal calibre in men than in women. Further studies should aim at seeking biological explanations for such a possible microvascular gender difference in clinical disease in other factors, as hormonal influences, possible sex differences in lifestyle or unrecognized risk factors.

Level of iron-stores has been a suggested cardiovascular risk factor and we found that high s-ferritin was associated with retinal venular widening in men. A possible mechanism is through endothelial dysfunction. This is supported by a study on blood donors, where high-frequency donors had decreased body iron stores and enhanced flow-mediated vasodilatation, a marker of endothelial function, compared to low-frequency donors.[65]

Iron stores remain stable throughout adulthood in men, except for a decline in the oldest age groups, while iron stores are lower in premenopausal women than in men, but increase after the onset of menopause.[156,157] In our study, it seems like ferritin levels ≥105 µg/L (the two highest quartiles) drive the association in men. Women with ferritin levels >100 µg/L (4.quartile) had slightly thinner venular calibre than those in the lower quartiles, though not significantly. Rather than hormonal influences, we hypothesize that the gender difference is related to higher lifetime iron stores in men than women.

Hemoglobin was independently associated with venular widening, in both men and women, but not with a slight arteriolar widening as demonstrated in the cross-sectional baseline Beaver Dam Eye Study.[84] Level of s-ferritin was associated with retinal venular calibre even with adjustment for level of hemoglobin, though it was attenuated. This indicates that part of the effect of s-ferritin on retinal venular calibre is independent of the effect of hemoglobin. On the other hand the association between hemoglobin and venular calibre was mainly unchanged with adjustment for s-ferritin and blood viscosity and factors related to oxygenation are among possible pathways for this independent association.

The effect of hemoglobin on retinal vascular calibre was stronger in men without hypertension. Hypertension is associated with narrower vessels and increased vascular resistance. Possibly the observed increased effect of hemoglobin in non-hypertensive men may be related to viscosity with increased effect in vessels with less vascular resistance due to arteriosclerosis.

Since the first publication in 1992 of a positive association between s-ferritin and risk of incident myocardial infarction,[66] the relationship between iron stores and incident cardiovascular disease and mortality has been investigated in several studies, with conflicting results (Table 2).[66-83] The clinical relevance of the association between s-ferritin and retinal venular widening in men remains to be investigated.

In paper 3 we found that venular calibre increased during the imaging sequence. We hypothesize that this change is related to background and flash illumination during imaging. This effect may at least in part be related to the phenomenon of increasing retinal vessel calibre during flicker light illumination.[104,158] As described above, there has been demonstrated disturbed vascular reactivity in the retinal vessels in smokers and patients

with diabetes or hypertension, with decreased flicker light induced retinal vasodilatation.[99,101,151] Further, the vascular reactivity was found to be negatively correlated with venular calibre in persons with diabetes while not correlated in persons without diabetes.[100] Hence, the vascular reactivity may be different between groups and this transition we observe during an imaging sequence may bias retinal vascular calibre measurements and reduce the differences.

7 Conclusions

All traditional cardiovascular risk factors were associated with retinal vascular calibre. Age and blood pressure were associated with narrower retinal vessels while smoking, overweight, high LDL and low HDL cholesterols with wider retinal vessels. The effect of low HDL cholesterol and high BMI with venular widening was significantly stronger in men than in women while the biological reasons and implications for this sex difference are not known. Microvascular pathology is possibly more important in women than in men, but the observed difference in effect of the traditional cardiovascular risk factors do not add to explain such differences in clinical disease as the effect was stronger in men than in women.

Iron is a candidate cardiovascular risk factor which may also be related to sex differences in the age of onset of ischemic heart disease. In this population high s-ferritin was associated with wider retinal venular calibre in men. We hypothesize that the gender difference is related to higher lifetime iron stores in men than women.

Hemoglobin was independently associated with venular widening in both women and men. The effect of hemoglobin on retinal vascular calibre was stronger in men without hypertension. Possibly the observed increased effect of hemoglobin in non-hypertensive men may be related to viscosity with increased effect in vessels with less vascular resistance due to arteriosclerosis.

Retinal venular calibre was wider with light exposure prior to imaging and increased slightly during the imaging sequences, less pronounced after prior light than dark exposure. This may possible bias retinal calibre measurements if there are differences between groups.

8 Implications for public health

Cardiovascular disease is a major cause of death and morbidity in developed countries. The eye offers a non-invasive direct access to the retinal microvasculature which shows characteristics probably shared with the microvasculature in other organs. The macrovasculature has been more extensively investigated than the microvasculature and this study supports that established cardiovascular risk factors are all related to microvascular changes. The study of the retinal vessels may further contribute to the understanding of the pathological processes involved in cardiovascular disease, eventually possibly contributing to refined risk evaluation and targeted therapies.

9 Further research

The Tromsø study population is being followed up with registration of incident cardiovascular disease and diabetes and the relevance of retinal microvascular changes for these outcomes should be explored in further studies.

It is possible that microvascular pathology in cardiovascular disease plays a more important role in women than in men. Future studies should further explore gender related microvascular differences, seeking biological explanations in other factors, as hormonal influences, possible sex differences in lifestyle or unrecognized risk factors.

S-ferritin and hemoglobin were associated with retinal vascular calibre. Future studies should investigate whether s-ferritin and hemoglobin are related to change over time in retinal vascular calibre, as well as whether change over time in s-ferritin and hemoglobin is related to retinal vascular calibre. Furthermore, one should aim at exploring the relevance for clinical cardiovascular disease.

To more clearly determine the relationship between retinal vascular calibre and serial imaging there is a need of further studies with measurements at different points of the capturing sequence and with comparison between different exposure groups, e.g. smokers and persons with overweight, dyslipidemia, diabetes or hypertension.

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Paper 1

Retinal vascular calibres are significantly associated with cardiovascular risk factors: the Tromsø Eye Study

von Hanno T, Bertelsen G, Sjølie AK, Mathiesen EB

Acta Ophthalmol. 2013 Apr 29. doi: 10.1111/aos.12102

[Epub ahead of print]

Paper 2

Serum ferritin and hemoglobin are independently associated with wider retinal venular caliber. The Tromsø Study 2001-2008

von Hanno T, Bertelsen G, Broderstad AR, Wilsgaard T, Mathiesen EB

Invest Ophthalmol Vis Sci. doi:10.1167/iovs.13-12204
2013;54:7053-7060.

Paper 3

Retinal vascular calibre and response to light exposure and serial imaging

von Hanno T, Sjølie AK, Mathiesen EB

Acta Ophthalmol. 2013 Jul 4. doi: 10.1111/aos.12213

[Epub ahead of print]

Appendices

A 1. Vessel Measurement Grading (IVAN); General Protocol

A 2. Certification; Retinal Vascular Caliber Measurement: IVAN software

A 3. Information leaflet Tromsø Study 5th survey, participants

A 4. Information leaflet Tromsø Study 6th survey, participants

A 5. Information leaflet method-study (paper 3), participants

Vessel Measurement Grading (IVAN); General Protocol **A1**

Retinal Vascular Imaging Centre (RetVIC)
Centre for Eye Research Australia, University of Melbourne

1. Is the image gradable?

Yes, continue to 2.
No, choose REJECT-NEXT for saving data (no need to enter comment) and continue to grade the next image. **Eyes that have <4 acceptable measurements of either vessel type will be considered ungradable.**

2. Is the disc centred?

Yes, continue to 3.
No, then moved the disc until the inner green circle best fits around the optic disc.

3. Identify Vessel Types

Assess every vessel automatically measured by IVAN in Zone B. Using right mouse button, toggle vessel type (and colour) until each vessel is identified correctly as either arteriole or venule.

4. Delete Obvious Errors

5. Are the Largest SIX vessels of each type measured?

Yes, continue on to 6.
No, adding or redrawing Vessels according to the principles summarized below:

Start at inner green circle of Zone B.

a. Use the “Add Seed” tool along the length of the vessel
b. If a. proves unsuccessful, repeat this method on a high quality section of the vessel past the problem.
c. If b. is unsuccessful, try the “Draw” tool, measuring the whole length of the vessel in Zone B.
d. If c. is unsuccessful, repeat “Draw” tool once more.
e. If d. proves unsuccessful, use the “Width” tool starting close to the inncer circle (i.e. as near to proximal border of Zone B as possible)
f. If e. proves unsuccessful, repeat method on high quality section past the problem.
g. Finally, if all of the above methods (a.-f.) have failed to add a satisfactory measurement, delete any attempts on this vessel and move on to assessing the next vessel. This should be entered into the “Comment/Approve – next” and write “2” (arteriole) or “3” venule.
h. Only use branch pairs of major vessel if trunk cannot be measured by steps a.-f..

6. Adjustment of Vessel Measurements

It is essential to be systematic to eliminate grader variability. The results by this method might not be the mest measurements possible, but they will be the most consistent.

Try to follow the principles in order:

1 DON'T TOUCH	Minimise Manipulation Consider "don't touch" first and any modification the second
2 POOR QUALITY AND PATHOLOGY IMAGES	If edges are poorly defined in an image (blur, low contrast, ghosting) a higher sigma should be considered. Also, if there is anatomical reason for variation (such as nerve fibre reflex or pathology like vessel narrowing) a higher sigma should be considered.
3 CLEAN UP BRANCHING AND CROSSINGS	Truncate to 1 mm before bifurcation or Truncate or Proximal Chop 1 mm to crossing a major vessel (only of the vessel being crossed is bigger than ½ the width (evaluated visually) of active vessel) so as to exclude the crossover section.
4 CLEAN UP ENDS	Only Truncate/Proximal Chop if it is visually obvious (at normal/default Zoom): i.e. measurements only span part of the width of the vessel AND sigma is more than 8. Cut as close to error as possible. 1 mm away is a good minimum.
5 CLEAN UP OTHER ERRORS (MISTAKES OR ARTIFACTS)	For obvious measurement errors in the centre of the vessel AND the sigma is greater than 8, use either "Truncate" of "Proximal Chop" to obtain the longest segment of the vessel. Again, cut as close to error as possible. Only in very extreme cases of error (cannot obtain satisfactory measurement according to the above principles); delete vessel and remeasure (follow Adding or Redrawing Vessel Principles above)

7. Calculate and Save

Press the "Calculate" button and mentally note the connecting lines drawn by the program – these estimate the branching pattern for the major vessels. Consider adding a comment for any strange results. Follow the standardized grading codes as below:

Code	Comment
9	ungradable
1	poor image quality
2	not big 6 artery
3	not big 6 vein
23	not big 6 artery and vein
4	< artery
5	< vein
45	<6 artery and vein
7	confounding pathology
8	other (marked when included traced tiny vessels)

* for data to be considered gradable, at least 4 of each vessel type must be able to be traced
* for data for be considered usable (for big 6 method), the biggest 6 of each vessel type must be measured.

Centre for Eye Research Australia

Eye & Ear Hospital
caring in every sense

Certificate of Completion

This certificate is presented to

Therese von Hanno

For successful completion of all required training and assessment for

Retinal Vascular Caliber Measurement: IVAN Software

22nd April 2010

Certificate Record: TH1100422VMC

Lauren Hodgson	Ryo Kawasaki	Tien Yin Wong
Retinal Vascular Imaging Centre Operations Manager	Retinal Vascular Imaging Centre Grading Centre Manager	Retinal Vascular Imaging Centre Professor

THE UNIVERSITY OF MELBOURNE

A2

Welcome to the fifth round of the Tromsø Study!

-a collaboration between:

Department of Community Medicine, University of Tromsø
tlf: 77 64 48 16 (kl. 9 - 11) Tromsous@ism.uit.no

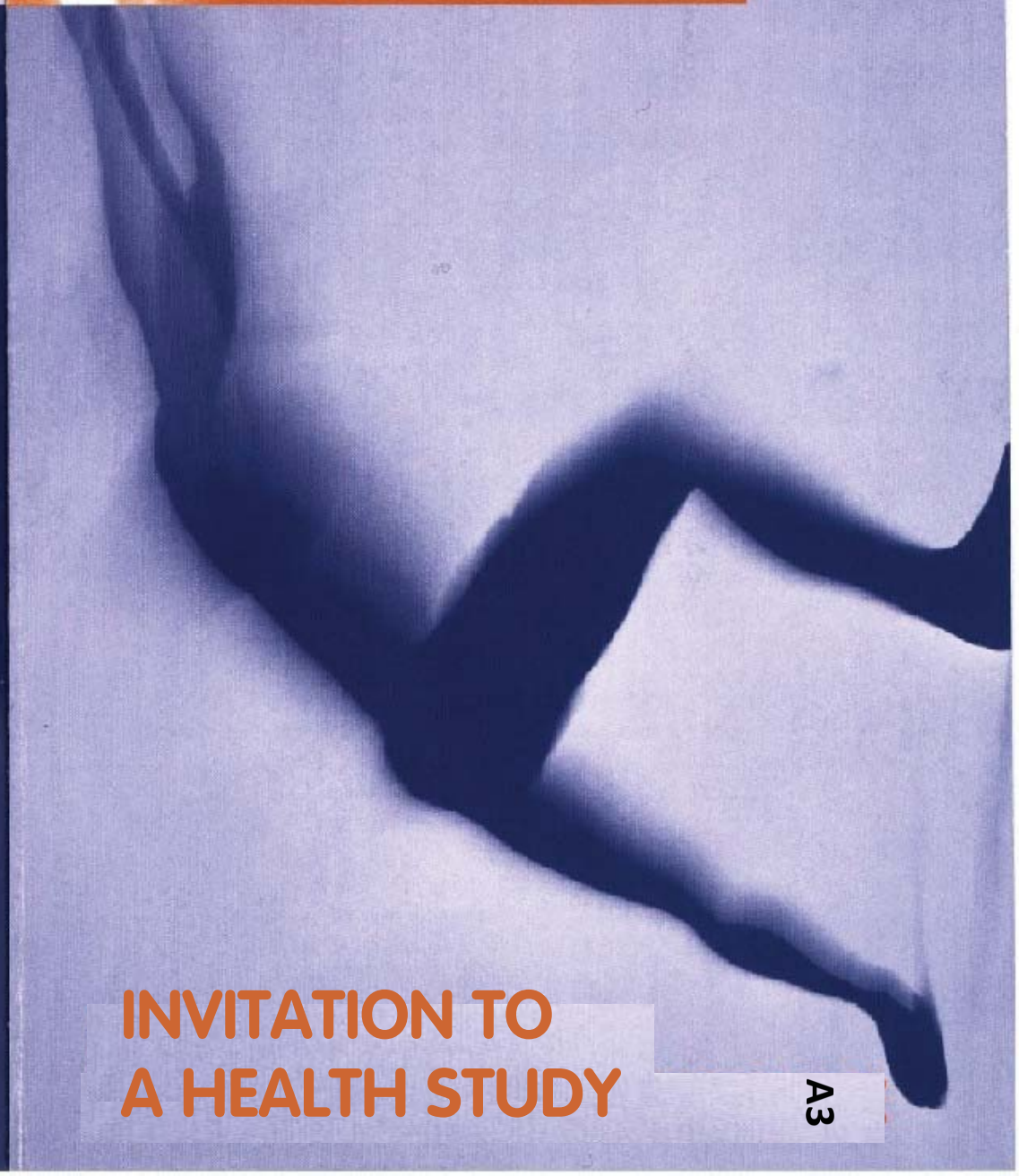
National Health Screening Service
tlf: 22 24 21 00 (kl. 9 - 15) post@shus.no

You will find more information about the health survey on the homepage of the National Health Screening Service

www.shus.no

IE 03600-1047-1 ■ ISBN 82-91210-01-4

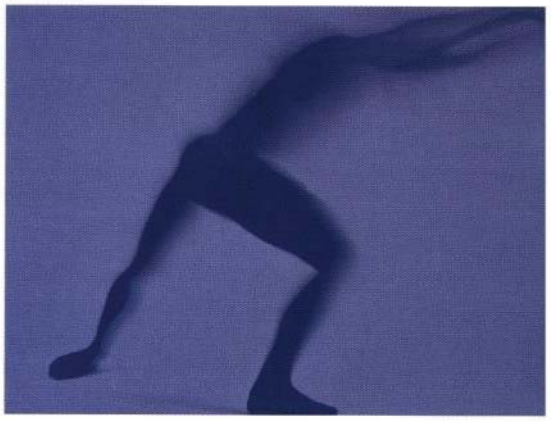
Take the chance!



INVITATION TO A HEALTH STUDY

A3

Would you like to participate in the fifth survey of the Tromsø study?



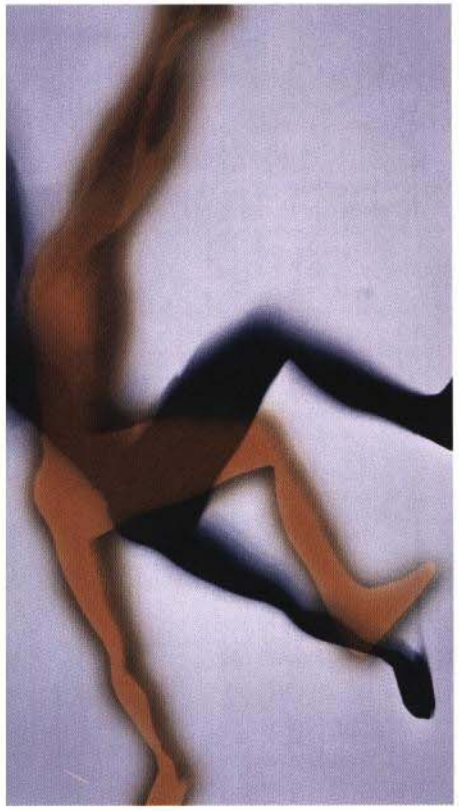
Why a new round of the Tromsø study?

Large health studies were conducted in Tromsø in 1974, 1979-80, 1986-87, and 1994-95. These surveys have given us important knowledge concerning cardiovascular epidemiology and other serious diseases, such as cancer.

The main purpose of another Tromsø study is to monitor any changes in the health of the population since last survey. We will analyze the information we have about a person, both personal data and results from analysis of frozen blood, and see if there are relationships to diseases that occur. This way we learn more on how cardiovascular diseases, cancer and other major diseases develop and how they can be prevented.

Why are we asking you to participate?

We ask everyone who participated in the Special Study in the Tromsø study in 1994-95 and a selection of others older than 29 years.



Where are you going to meet?

The survey will for the vast majority take place in Elizabeth Center in Tromsø. For some of the outer places in the municipality, the survey will take place locally. Those concerned are notified in this letter.

On the front page of the questionnaire that you receive with this letter are the opening hours for the health survey and when you have to attend the survey. If you cannot attend at that time, you are welcome any another time during the opening hours of the survey. There is no need to tell us about this – just show up when we are open.

What does the study include?

The Tromsø study is first and foremost a research project. Through following up as many as possible from the study of 1994-95, we gain valuable information of health and disease in the population of Tromsø.

Participants' general health status will be examined with regard to certain diseases and risk conditions. If you have a high risk of developing cardiovascular diseases you will be notified of this.

On the day of the examination you will be guided through the survey and there will be an opportunity to ask questions. Your height, weight and waist circumference is measured, as well as blood pressure, and a blood sample is taken. Your lung capacity is determined, in addition to simple tests of vision and strength. Tests to determine osteoporosis is are also conducted.
The blood sample may later be analyzed for fatty substances, blood sugar, indicators of infections, diet, hormones, liver- and kidney function, and bone markers.

Everyone who participated in the Special Study in 1994-95 is also offered to take part in another Special Study. This study provides information on the heart and the main arteries in neck and abdomen, and offers a more detailed analysis on tendency of osteoporosis. This survey is also located at the Elisabeth-center in Tromsø. A time will be scheduled for you and information is provided upon arrival.

The Questionnaires

With this letter a questionnaire is attached. We kindly ask you to complete this form at home and bring it on the day of the examination.

If you are unsure of how to answer a question, leave it blank. You will be aided at the examination.

Everyone who participates in the study will be given an additional questionnaire of other factors which might affect your health. The questionnaire is to be completed at home and sent to the National Health Screening Service in the enclosed envelope.

Future analysis of blood

The blood which is frozen will be used for medical research only, in order to find factors influencing disease. In most cases this means that data from people with a disease is compared to data from those without it. The comparison is done on already collected data and the new analysis from the frozen blood.

We might want to analyze parts of the DNA from the frozen blood cells. Because DNA is important for the regulating and development in human being, we need knowledge on DNA to understand why diseases evolve. Analysis of this kind are only conducted after the Data Inspectorate has given a permission and if The Regional Committee for Research Ethics has no objections to the analysis.

We need your consent

When you attend the study, you will be asked to sign a consent form where you agree to the following six points:

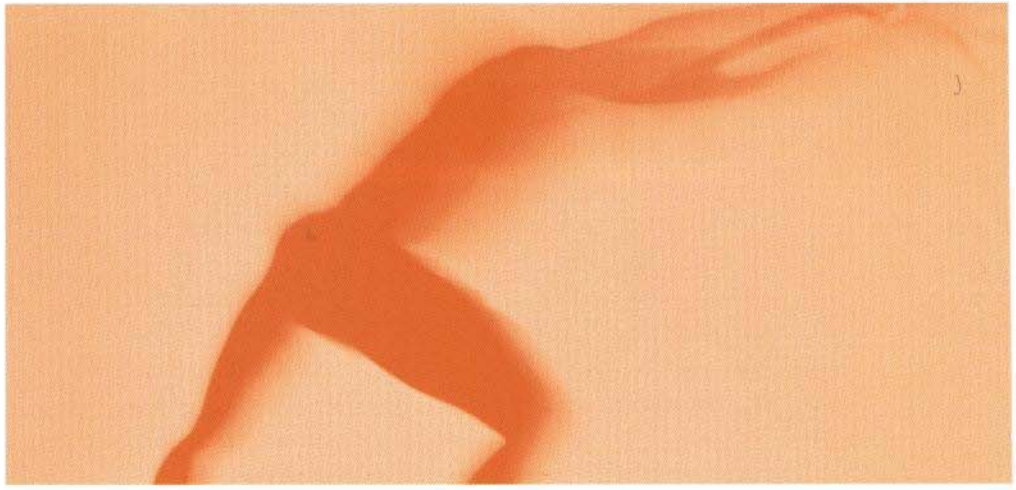
- That we may contact you with recommendations of follow ups, treatment or prevention of disease.
- That we may ask you to participate in similar studies in the future.
- That we may use the results for medical research.
- That the results, after legal approval from the Data Inspectorate, may be linked with information about you in other registries, to be used for research purposes. This might be registries including information on health, pension and disease, and also data on income, education and occupation, in addition to information from previous health studies in Tromsø. Examples of such registries are the Cancer Registry, the Cause of Death registry and population censuses. In these cases your name and social security number are removed when data is analyzed.
- That the blood sample may be stored and used for medical research. All use of this sample will only take place after approval from the Data Inspectorate and if The Regional Committees for Research Ethics has no objections.
- That the blood sample may also be used for analysis of DNA.

Even if you approve to this now, you are entitled to change your opinion later and also ask to have your profile deleted from the registry. You may also decline to consent to one or more of the points above. The Data Inspectorate has given consent to this fifth survey of the Tromsø Study, and the Regional Committee for Research Ethics has no objections. We keep your results confidential and safe. Everyone employed in the Tromsø Study has signed a confidentiality agreement.

When will you receive your results?

About four weeks after you participated in the study you will receive a letter wherein your recorded values for cholesterol, blood pressure and blood sugar are stated. You will also receive more information on the different risk factors.

People who are found to be at particularly high risk of developing cardiovascular diseases and diabetes will be recommended to seek further examination from their own doctor.



Tromsø 6
- en del av Tromsøundersøkelsen

A4

Vil du være med i den 6. Tromsøundersøkelsen?

» viktig forskning
» undersøkelse av egen helse
» forebygging av helseproblemer

Hva er Tromsøundersøkelsen?

Tromsøundersøkelsen er et stort forskningsprosjekt. Opplysninger som samles inn skal brukes til å gi oss kunnskap som kan bedre menneskers helse.

Den første Tromsøundersøkelsen ble gjennomført allerede i 1974, og dette er den sjette i rekken. Et viktig mål med undersøkelsen er å få kunnskap om hvorfor noen blir syke mens andre beholder god helse gjennom livet.

Visste du at ..?

Den som deltar på Tromsøundersøkelsen får også en enkel undersøkelse av sin egen helse.

Hva forskes det på i Tromsøundersøkelsen?

Tromsøundersøkelsen gjennomføres først og fremst for å kunne øke kunnskapen om de store folkehelseproblemene og forhold som påvirker disse, blant annet:

- Hjerte- og karsykdommer
- Lungesykdommer (f.eks. KOLS)
- Diabetes
- Stoffskiftesykdommer
- Kreftsykdommer
- Psykiske plager
- Demens
- Muskel- og skjelettplager

Undersøkelsen vil også bli benyttet til forskning om bruk og effekter av legemidler, trivsel, livskvalitet, livsstil, døgnrytme, smerter, sosial ulikhet, fysisk aktivitet, kosthold, bruk av helsetjenester og alternativ behandling. Det vil også bli undersøkt om miljøgifter kan påvises i blodet og om disse innvirker på helsa.

Videre vil det bli gjort forskning på kvinnesykdommer, sykdommer i fordøyelsesorganer, allergi, nyrer og urinveier, nervesystemet, sanseorganer og hud. Det vil også bli forsket på arbeidsuførhet som følge av disse sykdommene eller tilstandene. En del av prosjektene vil spesielt undersøke samspillet mellom arv, miljø, sykdom og helse. Til slike prosjekter vil det bli hentet ut DNA (arvestoff) fra blodprøvene.

Det er allerede planlagt mange forskningsprosjekter som skal benytte data fra Tromsøundersøkelsen. Du vil finne en liste over disse på vår internettside:

http://www.tromso6.no

Vil du delta?

Ved å delta på Tromsøundersøkelsen er du med på å bidra til forskning om hvordan sykdom kan forebygges og behandles, hva som fremmer god helse, og hva som er årsak til helseproblemer.

Hvorfor spør vi deg?

Alle som møtte til spesialundersøkelsene i Tromsøundersøkelsen i 1994 og 2001, og et tilfeldig uttrukket utvalg av personer som er over 30 år og som er innbyggere i Tromsø kommune, blir spurt om å delta.

Alle er viktige!

Hver deltaker er like viktig, enten du er ung eller gammel, frisk eller syk. Det har vært stort frammøte til de tidligere Tromsøundersøkelsene. Godt oppmøte er viktig for gode forskningsresultater. Det er en styrke for forskningen at de som har vært med i tidligere Tromsøundersøkelser møter fram på nytt.

Frivillig

Det er frivillig å delta. Det vil ikke få noen konsekvenser for deg dersom du ikke deltar eller velger å trekke deg fra undersøkelsen på et senere tidspunkt. Du må ikke gi noen begrunnelse dersom du ønsker å trekke deg fra undersøkelsen.

Visste du at ..?

Du kan delta på Tromsøundersøkelsen selv om det er deler av undersøkelsen du ikke ønsker å være med på.

Din helse

Cirka fire uker etter undersøkelsen vil du få et brev med resultatene fra målinger av kolesterol og blodtrykk. Dersom det er nødvendig, vil du bli anbefalt å ta kontakt med din fastlege. Det blir ikke gitt rutinemessig tilbakemelding om resultater av andre blodprøver eller målinger.

Dersom resultatet av prøvene viser at det er nødvendig med oppfølging av lege eller henvisning til spesialist, vil du bli orientert om det. Ved behov for henvisning til spesialist, vi vil sørge for at slik henvisning blir sendt.

Du kan reservere deg mot å få vite resultatene av prøvene dine. Men hvis et prøveresultat er slik at det er nødvendig med rask legebehandling, vil du uansett bli kontaktet.

Tromsøundersøkelsen er gratis. Trenger du videre undersøkelse / oppfølging av fastlegen eller i spesialisthelsetjenesten, betaler du vanlig egenandel.

Slik foregår undersøkelsen

Sammen med dette informasjonsskrivet ligger det et ark med praktiske opplysninger og beskjed om hvor og når du kan møte fram. Her står også åpningstidene for undersøkelsen. Hvis du vil delta og den foreslåtte tiden ikke passer, kan du komme en annen dag. Du trenger ikke melde fra om dette på forhånd.

Unngå før undersøkelsen

For at resultatene skal bli mest mulig korrekt, er det en fordel om du avstår fra alkohol og smertestillende medisiner 12 timer før undersøkelsen.

Påkledning

Vekt og høyde, liv- og hoftevidde måles med lett påkledning, men uten sko. For at det skal gå raskt å måle blodtrykk, er det en fordel om du har plagg som ikke strammer over armen og benet. Ha gjerne et kortermet plagg innerst.

Spørreskjema

Sammen med denne brosjyren har du fått et spørreskjema som du skal fylle ut og ta med til undersøkelsen. Hvis du er i tvil om hvordan du skal svare på et eller flere av spørsmålene, lar du det stå åpent. Personalet på undersøkelsen hjelper deg da med utfyllingen om du ønsker det.

Utfylte svar i spørreskjema er like viktig for forskningen som resultater fra blodprøver og undersøkelser.

Regelmessig bruk av legemidler

Ved frammøte til undersøkelsen vil du bli intervjuet om hva slags legemidler du har brukt regelmessig de siste fire ukene, og om noen av de legemidlene du har brukt siste 24 timer. Navn på legemidler du bruker fast kan besvares i skjemaet på forhånd. Ta gjerne med deg legemidlene du bruker ved frammøte til undersøkelsen.

Undersøkelser

Når du møter fram, vil kvalifisert helsepersonell veilede deg gjennom undersøkelsen og svare på spørsmål. Du vil bli intervjuet og få utlevert et nytt spørreskjema med en frankert svarkonvolutt. Spørreskjemaet kan også besvares mens du er tilstede på undersøkelsen, og du vil kunne få hjelp underveis. Hver enkelt undersøkelse varer bare noen minutter. Totalt vil undersøkelsen vare cirka en time.

De måler høyde, vekt, hoftevidde og livvidde, de måler blodtrykket og tar blodprøve av deg. I tillegg vil følgende undersøkelser bli gjort:

- Beintetthetsmåling (måling av beinmasse) i den ene armen med svake røntgenstråler. Målingene brukes til å undersøke risiko for beinskjørhet og brudd.
- Bakterieprøve fra nese og hals fra om lag halvparten av deltagerne, for å se etter gule stafylokokker, en bakterie som normalt finnes på hud og slimhinner hos mennesker, men som i enkelte tilfeller kan forårsake alvorlige infeksjoner. Prøven gjøres med fuktet vattpensel.
- Smerteølsomhet som måler hvordan kroppen reagerer på smerte. Du blir bedt om å holde hånden i isvann i opptil 1 minutt. Underveis registreres blodtrykk og du angir hvor mye smerte du kjenner. Du kan ta hånden ut av vannet før tiden er ute hvis det blir for ubehagelig.
- Hårprøve. Vi vil be om å få noen hårstrå for å undersøke forekomsten av spormetaller som kvikksølv.
- Fysisk aktivitet og kosthold. Vi planlegger at utvalgte deltakere vil bli bedt om å registrere fysisk aktivitet (aktivitetsmålere som skritttellere og lignende) og kosthold i en periode.

Blodprøver

Blodet fordeles på fem glass, men til sammen utgjør det ikke mer enn 45 milliliter, som er mindre enn en tidel av det en blodgiver gir. For de aller fleste vil det være tilstrekkelig med ett stikk. Disse analysene blir gjort:

- Måling av kolesterol og andre fettstoffer, blodsukker, blodlegemer, stoffskifteprøver, hormoner, markører for betennelsesreaksjoner, allergi, mage- og tarmfunksjon, lever- og nyrefunksjon samt muskel- og beinmarkører.
- DNA (arvestoff) vil bli lagret til bruk i forskningsprosjekter som er omtalt i denne brosjyren og som kartlegger sammenhengen mellom arv og miljø, sykdom og helse. DNA vil ikke bli brukt til andre formål enn forskning.
- Miljøgifter, blant annet sporstoffer, spormetaller og organiske stoffer. Forekomsten i blodet skal sammenlignes med tilsvarende målinger i andre befolkninger. Forskere vil studere om miljøgifter kan påvirke helsa vår.

Spesialundersøkelsen

Når første del av Tromsøundersøkelsen er gjennomført, kan du bli forespurt om å delta i en eller flere deler av Spesialundersøkelsen noen uker senere. Over halvparten vil bli spurt om dette. Hele Spesialundersøkelsen vil vare cirka en time, og

varigheten vil være avhengig av hvor mange deler du blir spurt om å være med på. Ved oppmøte til Spesialundersøkelsen vil det bli tatt ny blodprøve som skal brukes til samme formål som beskrevet for første del av undersøkelsen. Deler av blodprøven blir frosset ned for senere bruk i forskning som er beskrevet i denne brosjyren.

Hvilke undersøkelser gjøres i Spesialundersøkelsen?

- Ultralyd av blodårene (arteriene) på halsen. Undersøkelsen gjøres for å se etter forkalkninger og innsnevringer av årene. Undersøkelsen kartlegger også blodforsyningen til hjernen.
- Ultralyd av hjertet gjøres for å undersøke hjertets form og funksjon.
- Måling av beintetthet i rygg/hofte og kroppens fettmengde. Målingene brukes til å undersøke risiko for beinskjørhet og brudd, og for studier om sammenhengen mellom kroppsfett, beinmasse og brudd.
- Fotografering av øyebunn. Fotografiet vil vise tilstanden for blodkarene i øyet som også sier noe om blodkarene i kroppen. Ved øyestasjonen tas fotografi av øyebunnen din. Deltagerne får en øyedråpe i hvert øye en tid før fotografering for at pupillene skal utvide seg. Dette kan svi noe og synet kan forbigående bli noe uklart. Effekten går gradvis over, og etter en time er den borte. I tillegg vil det gjøres en enkel synstest som du vil få svar på umiddelbart.
- Tester av hukommelse gjøres ved hjelp av enkle spørsmål og omfatter også evne til gjenkjenning av ord og grad av fingerbevegelighet.
- EKG og blodtrykk. EKG er en registrering av hjerterytmen som også kan gi informasjon om hjertesykdom. Ved registrering festes ledninger til kroppen. Blodtrykket måles både på overarmen og ved ankelen.
- Pusteprøve. Dette er en enkel undersøkelse av lungefunksjonen. Du skal puste så hardt du klarer gjennom et munnstykke. Hvor mye luft som blåses ut pr. sekund, er et mål på lungefunksjonen din.
- Ny bakterieprøve fra nese og hals. Prøven utføres på samme måte som i første del av undersøkelsen.
- Urinprøve. Du vil bli bedt om å avlevere urinprøver fra de tre siste dagene før spesialundersøkelsen. Du gis alt nødvendig utstyr. Urinen blir lagret til bruk i forskning som er beskrevet i denne brosjyren.

For å sikre høy kvalitet på forskningsdata ønsker vi å undersøke et lite utvalg som møter til undersøkelsen to ganger med circa en ukes mellomrom. De som er aktuelle vil bli forespurt om dette ved frammøte.

Nye prosjekter

Noen deltakere vil i ettertid bli spurt om å delta i videre undersøkelser. Hvis dette gjelder deg, vil du få en forespørsel i posten. Du er ikke forpliktet til å delta selv om du har deltatt i andre deler av Tromsøundersøkelsen. Omtale av alle delprosjektene finner du på nettsiden vår:

http://www.tromso6.no

Forsikring og finansiering

Deltakere i Tromsøundersøkelsen er forsikret gjennom Norsk Pasientskadeerstatning.

Tromsøundersøkelsen er finansiert av Universitetet i Tromsø, Helse Nord HF samt ulike forskningsfond.

Etikk, personvern og sikkerhet

Du kan være trygg på at informasjon som gis til Tromsøundersøkelsen vil bli behandlet med respekt for personvern og privatliv, og i samsvar med lover og forskrifter. Alle medarbeidere som jobber med undersøkelsen har taushetsplikt. Opplysningene som samles inn vil bare bli brukt til godkjente forskningsformål.

Alle opplysninger om deltakere vil bli lagret på datamaskin. Navn og personnummer blir fjernet og erstattet med en kode. Kodenøkkelen oppbevares separat og kun noen få, autoriserte medarbeidere har tilgang til denne.

Den enkelte forsker får ikke tilgang til opplysninger som gjør det mulig å identifisere enkeltpersoner. Hver enkelt deltaker har en rett til å vite hvilke opplysninger som er lagret om en selv.

For alle prosjekter kreves det at prosjektlederen tilhører en kompetent forskningsinstitusjon.

Tromsøundersøkelsen har konsesjon fra Datatilsynet og er godkjent av Regional komité for medisinsk forskningsetikk, Nord-Norge.

Sammenstilling med andre registre

Opplysninger om deg fra den sjette Tromsøundersøkelsen kan bli knyttet sammen med opplysninger fra tidligere Tromsøundersøkelser. For enkelte prosjekter kan det være aktuelt å sammenstille opplysninger om deg med opplysninger fra barn, søsken, foreldre og besteforeldre hvis disse har deltatt i Tromsøundersøkelsen.

For spesielle forskningsprosjekter kan det være aktuelt å sammenstille informasjon fra Tromsøundersøkelsen med nasjonale helseregistre som Reseptregisteret, Medisinsk fødselsregistrer, Kreftregisteret, Norsk pasientregister og Dødsårsaksregisteret, og andre nasjonale registre over sykdommer som det forskes på i Tromsøundersøkelsen.

I tillegg kan det være aktuelt å innhente helseopplysninger fra primær- og spesialisthelsetjenesten til bruk i forskning på sykdommer og helseproblemer som er nevnt i denne brosjyren, for eksempel hjerte-karsykdom, diabetes og beinbrudd. I slike tilfeller innhentes nytt samtykke, eller annen type godkjenning (dispensasjon fra taushetsplikten).

Informasjon fra Tromsøundersøkelsen kan også bli sammenstilt med registre ved Statistisk sentralbyrå, for eksempel om miljø, befolkning, utdanning, inntekt, offentlige ytelser, yrkesdeltakelse og andre forhold som kan ha betydning for helsa.

Slike sammenstillinger krever noen ganger forhåndsgodkjenning av offentlige instanser, for eksempel Regional komité for medisinsk forskningsetikk, Datatilsynet eller NAV.

Bruk av innsamlede data i framtiden

Data fra Tromsøundersøkelsen vil kun bli brukt til forskning og vil ikke kunne brukes til andre formål.

Opplysninger og prøver som du gir, blir oppbevart på ubestemt tid til bruk i forskning til formål som nevnt i denne brosjyren. I noen tilfeller kan det bli aktuelt å gjøre analyser av blodprøver ved forskningsinstitusjoner i utlandet. Hvis dette gjøres, vil det skje i en slik form at våre utenlandske samarbeidspartnere ikke kan knytte prøvene opp mot deg som person.

Hva som er aktuelle problemstillinger i medisinsk forskning forandrer seg hele tiden. I framtiden kan data bli brukt i forskningsprosjekter som i dag ikke er planlagt, forutsatt at det er i samsvar med gjeldende lover og forskrifter. For alle slike nye prosjekter kreves det at prosjektet er godkjent av Regional komité for medisinsk forskningsetikk og Datatilsynet.

Tromsøundersøkelsen informerer om nye forskningsprosjekter på: http://www.tromso6.no
Her kan du også lese om forskningsresultatene fra Tromsøundersøkelsen. Forskningsresultater vil ellers bli publisert i internasjonale og nasjonale tidsskrifter, på faglige konferanser og møter. Det vil ikke være mulig å identifisere enkeltpersoner når forskningsresultatene offentliggjøres.

Samtykke

Hvis du vil delta i den sjette Tromsøundersøkelsen, må du gi skriftlig samtykke til dette. Personalet på Tromsøundersøkelsen vil kunne gi mer informasjon om undersøkelsen, og kan svare deg dersom du har spørsmål i forbindelse med samtykket.

Det er viktig å vite at selv om du sier ja til dette nå, kan du senere ombestemme deg. Du kan når som helst etter undersøkelsen trekke ditt samtykke tilbake. Allerede innsamlede data blir lagret videre, men kan ikke lenger knyttes til deg som person, og dine data vil ikke bli brukt i nye forskningsprosjekter. Du kan be om at blodprøven din blir ødelagt.

Hvis du vil trekke tilbake ditt samtykke, henvend deg til:

Tromsøundersøkelsen, Inst. for samfunnsmedisin
Universitetet i Tromsø
9037 Tromsø
telefon: 77 64 48 16
telefaks: 77 64 48 31
e-post: tromsous@ism.uit.no
internett: www.tromso6.no

Hvis vi i framtiden ønsker å forske på nye spørsmål som ikke er beskrevet i denne brosjyren, kan det bli nødvendig å be deg om et nytt samtykke.

Vil du delta?

Følgende tekst er en kopi av dokumentet du blir bedt om å signere når du møter fram til undersøkelsen:

Samtykke til bruk av helseopplysninger i forskning - den 6. Tromsøundersøkelsen

I brosjyren jeg har fått tilsendt, har jeg lest om undersøkelsens innhold og formål, og jeg har hatt mulighet til å stille spørsmål. Jeg samtykker herved i å delta i undersøkelsen [dato/signatur].

Tromsø-
undersøkelsen

Tromsøundersøkelsen
Institutt for samfunnsmedisin, Universitetet i Tromsø
9037 TROMSØ
telefon: 77 64 48 16
telefaks: 77 64 48 31
epost: tromsous@ism.uit.no
internett: www.tromso6.no

www.GnistDesign.no

A5

Forespørsel om deltakelse i forskningsprosjektet

"Hvordan påvirker lysforhold kardiameteren i netthinnen og tykkelsen av årehinnen? En metodestudie for okulære karanalyser"

Bakgrunn og hensikt
Dette er et spørsmål til deg om å delta i en forskningsstudie for å undersøke hvordan lysforhold påvirker målingen av blodårer i netthinnen og årehinnen i øyet hos friske forsøkspersoner.

Hva innebærer studien?
Undersøkelsen forgår ved øyepoliklinikken ved Nordlandssykehuset og tar 45-50 minutter. Synsstyrke, øyetrykk og fargesyn vil bli målt på begge øyne. Blodtrykket ditt måles. Det ene øyets øyets pupille vil bli utvidet ved bruk av øyedråpen Tropikamid. Deretter vil det bli tatt øyebunnsfotografi og optical coherence tomografi (oct) av netthinnen og årehinnen, etter tilpasning til forskjellig lysforhold.

Mulige fordeler og ulemper
Noen vil oppleve tilpasningen til skarpt lys som ubehagelig. Fotograferingen og oct-scanningen gir ikke ubehag. Øyedråpen Tropikamid gir forbigående nedsatt nærsyn på det dryppede øyet, dette vil normaliseres ila 2-3 timer. Man kan pga. den store pupillen bli lysømfintlig i noen timer etter avsluttet undersøkelse, dette kan avhjelpes av solbriller. I sjeldne tilfeller kan Tropikamid gi forhøyet trykk i øyet med smerter og tåkesyn. Dette vil inntreffe få timer etter dryppingen og man må i så fall søke lege umiddelbart.
NB! Du kan ikke kjøre bil før synet er normalisert igjen, ca. 2-3 timer etter undersøkelsen.

Hva skjer med bildene og informasjonen om deg?
Bildene tatt av deg og informasjonen som registreres om deg skal kun brukes slik det er beskrevet i hensikten med studien. Alle opplysningene og prøvene vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. En kode knytter deg til dine opplysninger og prøver gjennom en navneliste. Det er kun autorisert personell knyttet til prosjektet som har adgang til navnelisten og som kan finne tilbake til deg. Denne navnelisten vil bli slettet innen utgangen av 1.november 2013 slik at dataene da ikke lenger er mulig å knytte til deg.

Det vil ikke være mulig å identifisere deg i resultatene av studien når disse publiseres.

Frivillig deltakelse
Det er frivillig å delta i studien. Du kan når som helst og uten å oppgi noen grunn trekke ditt samtykke til å delta i studien. Dersom du ønsker å delta, undertegner du samtykkeerklæringen på siste side. Om du nå sier ja til å delta, kan du senere trekke tilbake ditt samtykke. Dersom du senere ønsker å trekke deg eller har spørsmål til studien, kan du kontakte Therese von Hanno, telefon 755 34000

Ytterligere informasjon om studien finnes i kapittel *A* – *utdypende forklaring av hva studien innebærer.*
Ytterligere informasjon om biobank, personvern og forsikring finnes i kapittel B – *Personvern, biobank, økonomi og forsikring.*

Samtykkeerklæring følger etter kapittel B.

Kapittel A- utdypende forklaring av hva studien innebærer

Kritererier for deltagelse
Deltagere må ha normalt syn (med brille/kontaktlinse) på minst ett øye og være uten kjent øyesykdom nå eller tidligere. Deltager må ha normal hørsel og førlighet og må kunne samarbeide for instruksjoner.

Følgende vil gjøre at man ikke kan delta i studien: Nedsatt syn eller kjent øyesykdom *på begge øyne*. Vanskelig regulert forhøyet blodtrykk. Alvorlig hjertesykdom. Alvorlig nyresykdom. Diabetes. Myopi (nærsynthet) <-3 eller hypermetropi (langsynthet) > +3.

Mulige bivirkninger
I svært sjeldne tilfeller vil utvidelse av pupillen kunne utløse forhøyet trykk inne i øyet innen 12 timer etter undersøkelsen. Dette vil ledsages av smerter og tåkesyn. Tilstanden er mulig å behandle men det er svært viktig at lege kontaktes umiddelbart dersom man får slike symptomer.

Kontaktperson: Therese von Hanno, mobiltelefon: 906 59 908.
Dersom det ikke oppnås kontakt med kontaktpersonen kontaktes vakthavende lege på øyeavdelingen, via sentralbordet på tlf. 755 34000.

Undersøkelser / Tidsskjema
Ett øye undersøkes (Høyre øye dersom normalt syn uten øyesykdom)
Gjennomgang av *Kriterer for deltagelse* (se Kapittel A over)
Synsstyrke, øyetrykk og fargesyn måles, pupillen dryppes ut.
Intervju: alder, kjønn, røykestatus (fast - ja/nei), blodtrykksbehandling (ja/nei), faste medikamenter (navn).
Blodtrykksmåling.

Etter 10 minutter etter at pupillen dryppes ut: 2 Oct-scan
Etter 10 minutter i mørkt rom med lukkede øyne: 1 Oct-scan
Etter 10 minutter i mørkt rom med lukkede øyne: 6 øyebunnsfotografier
Etter 10 minutter med eksponering av skarpt lys (1000 lux): 6 øyebunnsfotografier + 1 oct-scan

I alt vil undersøkelsen normalt ta 45-50 minutter.
Vi vil ha radio samt mulighet for å spille medbragt musikk på CD.

Kapittel B - Personvern, biobank, økonomi og forsikring

Personvern
Opplysninger som registreres om deg er utelukkende informasjon som innhentes under undersøkelsen. Det vil ikke bli utført kobling til andre informasjonskilder.

Billedbank
Bildene som blir tatt vil bli lagret avidentifisert på Nordlandssykehusets server.

Utlevering av materiale og opplysninger til andre
Ikke aktuelt.

Rett til innsyn og sletting av opplysninger om deg og sletting av prøver
Hvis du sier ja til å delta i studien, har du rett til å få innsyn i hvilke opplysninger som er registrert om deg. Du har videre rett til å få korrigert eventuelle feil i de opplysningene vi har registrert. Dersom du

trekker deg fra studien, kan du kreve å få slettet innsamlede prøver og opplysninger, med mindre opplysningene allerede er inngått i analyser eller brukt i vitenskapelige publikasjoner.

Økonomi
Studien er finansiert gjennom forskningsmidler fra Helse Nord.

Forsikring
Norsk pasientskadeerstatning.

Kontaktpersoner
Overlege/stipendiat Therese von Hanno, Nordlandssykehuset er ansvarlig for den praktiske gjennomføringen av undersøkelsen, og kan nåes på mobiltlf.nr.906 59 908, e-post: therese.von.hanno@nlsh.no eller tlf. via sentralbordet Nordlandssykehuset 75534000. Prosjektleder er prof. Ellisiv B. Mathiesen, Institutt for klinisk medisin, Universitetet i Tromsø, tlf. 77646418, e-post: ellisiv.mathiesen@uit.no.

Samtykke til deltakelse i studien

Jeg er villig til å delta i studien

(Signert av prosjektdeltaker, dato)

Anka

Anne Katrin Sjølie (1943-2013)

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