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Outi Uimari

EPIDEMIOLOGICAL AND FAMILIAL RISK FACTORS OF UTERINE LEIOMYOMA DEVELOPMENT

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EPIDEMIOLOGICAL AND FAMILIAL RISK FACTORS OF UTERINE LEIOMYOMA DEVELOPMENT

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Abstract

Uterine leiomyomas are the most common benign tumours in females. They are myometrial neoplasms, may present single or multiple, and may be located in various sites of the uterus. Leiomyomas distort the uterine cavity and the uterus itself, causing abnormal vaginal bleeding, reduced fertility and also pelvic pressure and pain symptoms. The aim of this study was to elaborate current knowledge on familial uterine leiomyomas and to explore the possible association between uterine leiomyoma and cardiovascular disease risk factors, and also the association between leiomyomas and endometriosis.

The natural history of familial uterine leiomyoma study showed significant differences between familial and non-familial leiomyoma cases, familial cases having more severe clinical characteristics. They presented with multiple uterine leiomyomas and were more often symptomatic. They were also diagnosed at a younger age.

The prevalence study on uterine leiomyomas and endometriosis offered confirmation of an association between the diseases. Uterine leiomyomas and endometriosis seem to decrease female fertility independently of each other.

Uterine leiomyomas related to the hereditary leiomyomatosis and renal cell cancer (HLRCC) tumour syndrome were studied in regard to their clinical characteristics and immunophenotype. The study provided evidence that women with HLRCC may be identified through distinct leiomyoma clinical characteristics, and routine-use IHC of CD34 and Bcl-2. Distinguishing these leiomyoma cases from sporadic ones may identify families affected by *fumarate hydratase* (*fumarase, FH*) mutation.

Uterine leiomyoma and cardiovascular disease risk factors were studied in The Northern Finland Birth Cohort 1966 (NFBC1966). The study showed an association between leiomyomas and raised cardiovascular disease risk factors, serum lipids and metabolic syndrome in particular. These findings may suggest that there are shared predisposing factors underlying both uterine leiomyomas and adverse metabolic and cardiac disease risks, or that metabolic factors have a role in biological mechanisms underlying leiomyoma development.

This study provides novel information on clinical characteristics of familial uterine leiomyomas and on the immunophenotype of HLRCC-related leiomyomas. The study also offers significant confirmation of associations between uterine leiomyomas and both endometriosis and several CVD risk factors.

Keywords: Bcl-2, cardiovascular risk, CD34, endometriosis, epidemiology, familial, FH, glucose metabolism, HLRCC, lipid metabolism, natural history, population-based birth cohort studies, subfertility, uterine leiomyoma/fibroids

Uimari, Outi, Epidemiologisia ja familiaalisia riskitekijöitä kohdun leiomyoomien kehittymiselle.
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Tiivistelmä

Kohdun leiomyoomat ovat naisten yleisin hyvänlaatuinen kasvain. Ne ovat myometriumin neoplastisia muutoksia ja ne ilmenevät joko yksittäisinä tai monilukuisina, ja ne voivat sijaita missä tahansa kohdun myometriumia. Leiomyoomat muuttavat kohdun ja kohtuontelon säännöllistä muotoa. Lisäksi ne aiheuttavat vuotohäiriöitä, alentunutta hedelmällisyyttä, ja lantion alueen painetta ja kipua. Tämän tutkimuksen tavoitteena oli laajentaa nykyistä tietämystä suvuittain esiintyvistä kohdun leiomyoomista ja selvittää mahdollista leiomyoomien ja kardiovaskulaaritautiriskin assosiaatiota, ja lisäksi selvittää leiomyoomien ja endometrioosin assosiaatiota.

Suvuittain esiintyvien kohdun leiomyoomien taudinkulkua selvittävässä tutkimuksessa osoitettiin merkittäviä eroja suvuittain ja ei-suvuittain esiintyvien leiomyoomien välillä. Suvuittain esiintyvien leiomyoomien kliininen taudinkuva oli vaikeampi, leiomyoomia oli kohdussa useampia ja ne aiheuttivat useammin oireita ja lisäksi ne diagnosoitiin nuoremmalla iällä.

Kohdun leiomyoomien ja endometrioosin yleisyyttä selvittävä tutkimus antoi lisävahvistusta sille havainnolle, että nämä taudit assosioivat keskenään. Tutkimustuloksen mukaan leiomyoomat ja endometrioosi vähentävät naisen hedelmällisyyttä toisistaan riippumatta.

Perinnöllinen kohdun leiomyomatoosi ja munuaissyöpä (hereditary leiomyomatosis and renal cell cancer, HLRCC) -kasvainoireyhtymään liittyvän kohdun leiomyoomia selvittävän tutkimuksen tuloksien mukaan HLRCC-naisten kohdun leiomyoomien kliiniset ominaisuudet poikkeavat satunnaisesti esiintyvien leiomyoomien ominaisuuksista. Naisella HLRCC voitaisiinkin tunnistaa näiden poikkeavien ominaisuuksien perusteella, sekä immunohistokemiallisilla värjäyksillä CD34 ja Bcl-2. *Fumaraattihydrataasi* (*fumaraasi, FH*) -geenin mutaatiota kantava suku voitaisiin siten tunnistaa yksittäisen HLRCC leiomyoomatapauksen avulla.

Pohjois-Suomen syntymäkohortti 1966 (Northern Finland Birth Cohort 1966, NFBC1966) tutkittiin kohdun leiomyoomia ja kardiovaskulaarisairauden riskitekijöitä. Tutkimustuloksien perusteella kohdun leiomyoomat assosioivat koholla olevien kardiovaskulaarisairauden riskien kanssa, erityisesti seerumin lipidien ja metabolisen syndrooman suhteen. Näiden tutkimustulosten perusteella voidaan esittää, että leiomyoomien ja terveydelle epäedullisen metabolian ja kardiovaskulaaritaudin riskien taustalla on mahdollisesti joitain yhteisiä altistavia tekijöitä, tai että metabolisilla tekijöillä on rooli kohdun leiomyoomien tautimekanismissa.

Tämä tutkimus on tuottanut uutta tietoa suvuittain esiintyvien kohdun leiomyoomien kliinisestä taudinkuvasta ja HLRCC:n liittyvien leiomyoomien immunofenotyypistä. Lisäksi tämä tutkimus esittää lisävahvistusta kohdun leiomyoomien ja endometrioosin assosiaatiolle sekä useille kardiovaskulaaririskitekijöille.

Asiasanat: alentunut hedelmällisyys, Bcl-2, CD34, endometrioosi, epidemiologia, familiaalinen, FH, glukoosimetabolia, HLRCC, kardiovaskulaaririski, kohdun leiomyooma, lipidimetabolia, taudinkulku, väestopohjaiset syntymäkohorttitutkimukset

Look up at the stars and not down at your feet.
Try to make sense of what you see,
and wonder about what makes the universe exist.
Be curious.

Stephen Hawking

To Mika, Oona and Aapo

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Flying above the skies between

Oulu and Oxford, January 2017 Outi Uimari

Abbreviations

17β-HSD	17β-hydroxysteroid dehydrogenase
ALDH1	aldehyde dehydrogenase
AMPK	AMP-activated protein kinase
AUC	area under the curve
BHD	Birt-Hogg-Dube
BMI	body mass index
CDK8	cyclin-dependent kinase 8
CFU	colony-forming unit
CI	confidence interval
CIM	carotid intima-media
COL4A5	collagen type IV α5
CVD	cardiovascular disease
CycC	cyclin C
DES	diethylstilbestrol
ECM	extracellular matrix
ERα	oestrogen receptor alpha
ET-1	endothelin 1
FAS	fatty acid synthesis
FASN	fatty acid synthase
FFPE	formalin-fixed paraffin-embedded
FH	fumarate hydratase
FLI	fatty liver index
FPG	fasting plasma glucose
G6PD	glucose-6-phosphate dehydrogenase
GGT	gamma-glutamyl-transferase
GnRH	gonadotrophin-releasing hormone
GREB1	growth regulation by oestrogen in breast cancer 1
GWAS	genome-wide association study
H&E	haematoxylin/eosin
HDL	high-density lipoprotein
HLRCC	hereditary leiomyomatosis and renal cell cancer
HMGA2	high-mobility group AT-hook 2
HPF	high-power frequency
HRT	hormone replacement therapy
hs-CRP	high-sensitivity C-reactive protein

ICD	International Classification of Diseases
IDF	International Diabetes Federation
IFG	impaired fasting glucose
IGF-1	insulin-like growth factor-1
IGFBP-3	insulin-like growth factor-binding protein 3
IGT	impaired glucose tolerance
IHC	immunohistochemistry
IVF	in-vitro fertilization
KEAP1	Kelch-like ECH-associated protein-1
LDL	low-density lipoprotein
MAPK	mitogen-activated protein kinase
MCUL	multiple cutaneous and uterine leiomyomatosis
MED12	mediator complex subunit 12
MED13	mediator complex subunit 13
miRNA	micro-RNA
MMP	matrix metalloproteinase
MP	main population
MRgFUS	magnetic resonance-guided focused ultrasound
MSC	mesenchymal stem cell
mTOR	mechanistic target of rapamycin
NF-κB	nuclear factor κB
NFBC1966	Northern Finland Birth Cohort 1966
NGT	normal glucose tolerance
NHS II	Nurses Health Study II
NICE	National Institute for Health and Care Excellence
NIEHS	National Institute of Environmental Health Sciences
NS	non-significant
NSAID	non-steroidal anti-inflammatory drug
OGTT	oral glucose tolerance test
OR	odds ratio
PR	progesterone receptor
PRE	progesterone response element
PrevDM	previously-known diabetes mellitus
PTEN	phosphate and tensin homologue
QUICKI	Quantitative Insulin Sensitivity Check Index
RA	retinoic acid
rAFS	Revised American Fertility Society

RCC	renal cell cancer
ScDM	screen-detected diabetes mellitus
SCORE	Systematic Coronary Risk Evaluation
SD	standard deviation
sFlt-1	soluble fms-like tyrosine kinase 1
SHBG	sex hormone-binding globulin
SMAD	mothers against decapentaplegic homologue
SORCS2	sortrilin-related VPS10 domain-containing receptor 2
SP	side population
SREBP-1	sterol regulatory element-binding protein 1
T	testosterone
TCAC	tricarboxylic acid cycle
TCF	T-cell transcription factor
TGF- β	transforming growth factor beta
TIMP	tissue inhibitor of matrix metalloproteinase
TMA	tissue microarray
TSC	tuberous sclerosis complex
UL	uterine leiomyoma
VDR	vitamin D receptor
WHO	World Health Organization
WHR	waist-hip-ratio

List of original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:

I Uimari O, Suomalainen-Konig S, Sakkinen N, Santala M, Nieminen P & Ryynanen M (2005) Natural history of familial myomas. Eur J Obs & Gyn and Reprod Biol 125(2): 255–258.

II Uimari O, Jarvela IY & Ryynanen M (2011) Do symptomatic endometriosis and uterine fibroids appear together? J Hum Reprod Sci 4(1): 29–33.

III Uimari O, Ahtikoski A, Kampjarvi K, Butzow R, Jarvela IY, Ryynanen M, Aaltonen LA, Vahteristo P & Kuismin O (2016) Clinical characteristics and histological features of uterine leiomyomas in Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC) syndrome. Manuscript.

IV Uimari O, Auvinen J, Jokelainen J, Puukka K, Ruokonen A, Jarvelin MR, Piltonen T, Keinanen-Kiukaanniemi S, Zondervan K, Jarvela IY, Ryynanen M & Martikainen H (2016) Uterine fibroids and cardiovascular risk. Hum Reprod 31(12):2689-2703.

Contents

Abstract
Tiivistelmä
Acknowledgements **9**
Abbreviations **11**
List of original publications **15**
Contents **17**
1 Introduction **19**
2 Review of the literature **21**
2.1 Uterine anatomy and physiology 21
2.1.1 Myometrial anatomy and physiology 22
2.1.2 Benign myometrial tumours 23
2.2 Epidemiology and risk factors 27
2.2.1 Incidence 27
2.2.2 Life cycle and hormonal status 32
2.2.3 Familial aggregation 35
2.2.4 Ethnicity 35
2.2.5 Metabolic factors 37
2.2.6 Endometriosis 40
2.3 Uterine leiomyoma pathophysiology 41
2.3.1 Cellular origin of leiomyomas 41
2.3.2 Stem cells 42
2.3.3 Genetic features 44
2.3.4 Roles of oestrogen and progesterone 49
2.3.5 ECM and its significance 51
2.4 Histopathology 52
3 Aims of the study **57**
4 Materials and methods **59**
4.1 Subjects and materials 59
4.1.1 Women with uterine leiomyomas (Studies I, II, IV) 59
4.1.2 Women with HLRCC (Study III) 60
4.1.3 Uterine leiomyoma tissue samples (Study III) 62
4.2 Methods 63
4.2.1 Clinical characteristics analysis (Studies I, III) 63
4.2.2 Association analysis (Study II) 63
4.2.3 FH mutation analysis (Study III) 63

4.2.4 Morphological and immunohistochemical analysis (Study III) 63
4.2.5 Anthropometric and cardiovascular measurements (Study IV) 64
4.2.6 Serum sampling and measurements of glucose and lipid metabolism and other biochemical markers (Study IV) 65
4.2.7 Assessments of CVD risk, metabolic syndrome and fatty liver (Study IV) 65
4.2.8 Statistical analysis 66
5 Results and discussion **71**
5.1 Clinical characteristics of familial and HLRCC uterine leiomyomas (Studies I, III) 71
5.2 Histopathological features of HLRCC uterine leiomyomas (Study III) 72
5.3 Uterine leiomyoma and CVD risk (Study IV) 77
5.4 Association between uterine leiomyoma and endometriosis (Study II) 85
5.5 Limitations and strengths of the studies 88
6 Conclusions and future directions **91**
References **93**
Original publications **115**

1 Introduction

Uterine leiomyomas (also called *myomas* and *fibroids)* are benign lesions of uterine myometrial tissue composed of smooth muscle cells, vascular smooth-muscle cells, fibroblasts and extracellular matrix (ECM) (Holdsworth-Carson *et al.* 2014). They are the most common tumours in females, with an estimated cumulative incidence of nearly 70% among white women by the age of 50 (Baird *et al.* 2003). Uterine leiomyomas decrease the quality of life by causing significant morbidity among women of reproductive age. The related symptoms are heavy and prolonged menstrual bleeding, anaemia secondary to bleeding, pelvic pain and pressure, reduced fertility and other pregnancy complications (Stewart *et al.* 2016). Current treatment options for uterine leiomyomas are limited to hormonal treatments, surgery, uterine artery embolisation and magnetic resonance-guided focused ultrasound (MRgFUS) treatment. All therapies, however, are associated with substantial side-effects and risks. The mainstream medications, based on NICE (2013) *Fibroids* guidelines (levonorgestrel, progestogen, combined oral contraceptives, GnRH, ulipristal acetate, tranexamic acid and NSAIDs) focus on easing the symptoms rather than targeting the specific molecular disease mechanisms. The significant surgical need for uterine leiomyoma treatment is well reflected by the fact that uterine leiomyomas are the primary indication for hysterectomy (Farquhar & Steiner 2002, Brummer *et al.* 2009). Given all this, uterine leiomyomas bring about a significant financial burden for society (Soliman *et al.* 2015); for example of annual cost of 52.7 million euros in the UK alone (Fernandez *et al.* 2009).

The field of uterine leiomyoma biology has been transformed by crucial breakthrough discoveries through next-generation genetic studies in the very recent past revealing mutations in *MED12*, which is a subunit of the mediator complex that regulates global and gene-specific transcription (Allen & Taatjes 2015). The mutation has been recognised to have a frequency of 70% among uterine leiomyomas (Makinen *et al.* 2011b). This has led the way to the molecular classification of uterine leiomyomas (Mehine 2016), emphasising the likely possibility of several alternative disease mechanisms for leiomyoma development that yet await to be uncovered fundamentally.

Together with uterine leiomyomas, endometriosis is a common gynaecological disease affecting fertile-aged women. It is a chronic inflammatory disease in which tissue resembling endometrium is present outside the uterus, mainly on pelvic organs, causing pelvic pain and reduced fertility. The condition affects an estimated

5–10% of premenopausal women (Giudice & Kao 2004), with a substantial impact on the lives of sufferers (Nnoaham *et al.* 2011). Endometriosis has a genetic component with an estimated heritability of ~52% (Treloar *et al.* 1999), but overall the causes of endometriosis remain largely unknown.

Cardiovascular diseases (CVDs) comprise diseases of the heart, blood vessels and vascular diseases of the brain. Atherosclerosis, which is a pathological process in the walls of blood vessels, accounts for a major proportion of CVDs. Main risk factors of CVD are smoking, physical inactivity, unhealthy diets, alcohol usage, raised blood pressure, raised blood glucose, raised and abnormal serum lipid profiles and obesity (Tzoulaki *et al.* 2016).

The aims of this study were to elaborate current knowledge of familial uterine leiomyomas and to investigate whether or not detection of hereditary leiomyomatosis and renal cell cancer (HLRCC) patients could be improved by identifying typical clinical characteristics and histological features. The associations between uterine leiomyoma and cardiovascular disease risk factors were explored and the association between leiomyoma and endometriosis was also studied.

2 Review of the literature

2.1 Uterine anatomy and physiology

The uterus is a fibromuscular hollow organ situated in the female pelvis, in the sagittal plane between the urinary bladder and the rectum (Hoffmann *et al.* 2012). It is divided into two sections based on anatomical and functional relevance; the upper muscular body forms the *corpus*, and the lower fibrous part forms the neck of the uterus, the *cervix*. The transition between these two structures is called the *uterine isthmus*, where the endocervical canal transforms to the endometrial cavity. The fundus of the uterus is the top part above the level of entry of the fallopian tubes into the endometrial cavity (Hoffmann *et al.* 2012).

The uterus is supported in its anatomical location by endopelvic fascia, which is a connective tissue network that envelopes all pelvic organs and connects them loosely to the supportive musculature and pelvic bones (Barber 2005). The outer wall of the uterus is overlaid by *peritoneal serosa*, with the exception of the anterior side of the cervix, which is covered by the bladder, and lateral sides of the corpus and cervix, where they attach to the broad and cardinal ligaments (Hoffmann *et al.* 2012).

The uterus is formed of three layers; an inner layer of mucosa, the *endometrium* which is the lining of the endometrial cavity, the *myometrium*, which is the thick muscular wall, and the *perimetrium*, which is a serous layer of the visceral peritoneum, covering the outer surface of the uterus (Michael & Pawlina 2011).

The blood supply to the uterus arrives from several sites of the vascular system. The corpus receives blood supply bilaterally from the ascending branches of the uterine arteries and from the medial and uterine branches of the ovarian arteries. The descending uterine arteries or the cervical branch supply blood to the cervix (Farrer-Brown *et al.* 1970). Uterine lymphatic drainage passes to the obturator and internal and external iliac nodes. Additionally, some lymphatic ducts from the uterine corpus may circle down the round ligaments to the superficial inguinal nodes, whereas others may flow down the uterosacral ligaments to the lateral sacral nodes (Hoffmann *et al.* 2012). The uterovaginal plexus covers the uterine innervation. The nerve fibres go round the uterine arteries, descending past the cardinal ligament connective tissue (Hoffmann *et al.* 2012).

The primary function of the uterus is gestational – to nurture the implanted embryo. The uterus allows the fertilized ovum, a multicellular blastocyst, to

implant. In early pregnancy the uterus performs placental-like functions for embryonal tissue and structural development, until the embryo can develop its own placenta (Teixeira *et al.* 2008). The pregnant uterus has unique characteristics shown in the peripartum period as it undergoes dramatic functional changes necessary for completion of a normal pregnancy. The uterus enlarges its capacity to contain the fetus and placenta for 38 weeks for growth and development. The enlargement requires myometrial hypertrophy and hyperplasia, angiogenesis and vascular remodelling, resulting in a uterine net weight increase of more than tenfold. During most of a pregnancy, uterine function is not to contract in order to maintain the gestation to full term. At the end of pregnancy, however, it is smooth muscle contractions that deliver the infant. The uterus undergoes phasic contractions at parturition, which soften and dilate the cervix and then expel the fetus. Each stage is associated with periodically increasing intrauterine pressures. Lastly, the uterus undergoes a tonic contraction and expels the placenta. These functional changes occur over months, then days, hours, and finally minutes (Young 2007).

2.1.1 Myometrial anatomy and physiology

The uterus is mostly formed of smooth muscle, i.e. myometrium with three layers; an outer layer made of longitudinal myometrial cells, a middle layer made of crisscrossing muscle fibres, and inner circular fibres around the uterine cavity. The innermost layer of the myometrium, the *sub-endometrial* layer, is suggested to be of embryonic Müllerian-duct origin, while the outer layers appear to originate from non-Müllerian tissue. Additionally, these layers seem to have distinct physiological properties, as the sub-endometrial myometrium has been observed to contract the during menstrual cycle, (‘endometrial waves’) (Ijland *et al.* 1996), having vital importance in sperm and embryo transport and implantation, whereas outer myometrial contractions are mostly involved in more intense uterine activity such as abortion and parturition of the fetus (Aguilar & Mitchell 2010).

Smooth muscle, including myometrium, has some unique characteristics in comparison with skeletal muscle that are of significance in parturition as regards the strength of uterine contractions. Smooth muscle cell shortening is far greater during contraction than that of striated muscle cells. The exerted forces are multidirectional instead of aligned with the axis of striated muscle fibres. Smooth muscle plexiform arrangement allows greater shortening and force-generating capacity, as the thick and thin filaments are found in long, random bundles throughout the cells. Lastly, the uterine fundal myometrium generates greater

multidirectional force compared with that of the lower uterine segment, creating versatile expulsive force towards the birth canal (Cunningham *et al.* 2010).

2.1.2 Benign myometrial tumours

Uterine myometrial cells may become neoplastic, forming stiff nodular tumours named *uterine leiomyomas* (also called *myomas* and *fibroids)* that are composed of four key cell types: smooth muscle cells, vascular smooth muscle cells, fibroblasts and leiomyoma-associated fibroblasts, and the extracellular matrix (ECM) (Holdsworth-Carson *et al.* 2014). The blood supply to leiomyomas arrives mainly from uterine arterial branches, but ovarian and round ligament arteries may also play a role (Gomez-Jorge *et al.* 2003). The perileiomyoma arteries are functional end arteries that can be obstructed by targeted embolisation by micro-particle injection, without blocking the antegrade blood flow in the main uterine artery (Stewart 2015). Leiomyomas present as single or multiple, and may be located in various sites of the uterus. The size of these tumours varies from initial development to 10–20 cm (Abdul Ghaffar *et al.* 2008).

Anatomical classification of uterine leiomyomas

Uterine leiomyomas can be classified according to their location and growth direction in the uterus. *Subserosal* leiomyomas originate from myometrial cells close to the serosa and directing outward towards the abdominal cavity, creating the characteristic irregular feel of the enlarged myomatous uterus (Figure 1). Subserosal leiomyoma is called *pedunculated* when it is attached only by a stalk to its progenitor myometrium. *Intramural* leiomyomas originate from middle -layer myometrial cells, growth kept within the uterine walls (Figure 1). *Submucous* leiomyomas originate from myometrial cells close to the endometrium, growing and protruding towards the uterine cavity (Figure 1). Submucous leiomyomas are further classified by the European Society of Hysteroscopy to offer aid for endoscopic resection evaluation: type 0, leiomyoma mass located entirely in the uterine cavity with no myometrial extension; type I less than 50% located within the myometrium; type II more than 50% of the mass surrounded by the myometrium (Wamsteker *et al.* 1993, American Association of Gynecologic Laparoscopists: Advancing Minimally Invasive Gynecology 2012). Cervical leiomyomas arise from cervical myometrial cells. Rare intraligamentary leiomyomas present separately from the uterus, usually originating from round

ligament smooth muscle fibres (Colak *et al.* 2013). However, most myomatous uteri are of mixed type, presenting with multiple leiomyomas of varying size and with different/overlapping uterine locations (Stewart 2001). The International Federation of Gynecology and Obstetrics (FIGO) has established a classification system (PALM-COEIN) for causes of abnormal uterine bleeding in nongravid women of reproductive age (Munro *et al.* 2011). Uterine leiomyomas can be staged according to their location relative to mucosal and serosal surfaces within the uterus, aiming to improve leiomyoma description: submucosal (SM) 0, pedunculated intracavitary; SM-1, <50% intramural; SM-2, ≥50% intramural, other (O) 3, contacts endometrium 100% intramural; O-4, intramural; O-5, subserosal ≥50% intramural; O-6 subserosal <50% intramural; O-7, subserosal pedunculated; O-8 other (specify e.g. cervical, parasitic); and hybrid leiomyomas (impact both endometrium and serosa) are documented with two numbers, separated by a hyphen, the first refers to the relationship with the endometrium while the second refers to the relationship to serosa (Munro *et al.* 2011).

Symptomatology of uterine leiomyomas

Most uterine leiomyomas cause no symptoms and remain undiagnosed, but many women experience significant reduction in quality of life due to leiomyoma-related symptoms. In general, the bigger the leiomyoma or the more there are, the greater the likelihood of symptoms (Wegienka *et al.* 2003). The most common leiomyoma-related symptom is abnormal vaginal bleeding, usually presenting as heavy menstrual bleeding. Submucosal leiomyomas may cause substantial bleeding problems due to their location rather than size, but also intramural and subserosal leiomyomas are suggested to have the same propensity to the same extent (Wegienka *et al.* 2003, Olufowobi *et al.* 2004). This may be related to dilatation of venules, as the tumour exerts pressure and impinges on the uterine venous system, which causes venous dilatation within the myometrium and endometrium (Wegienka *et al.* 2003). Dysregulation of a number of local vasoactive growth factors in myomatous uterus has also been suggested to have a crucial role in vasodilatation. This, together with dilated venules, might exhaust the usual haemostatic mechanisms (Stewart & Nowak 1996).

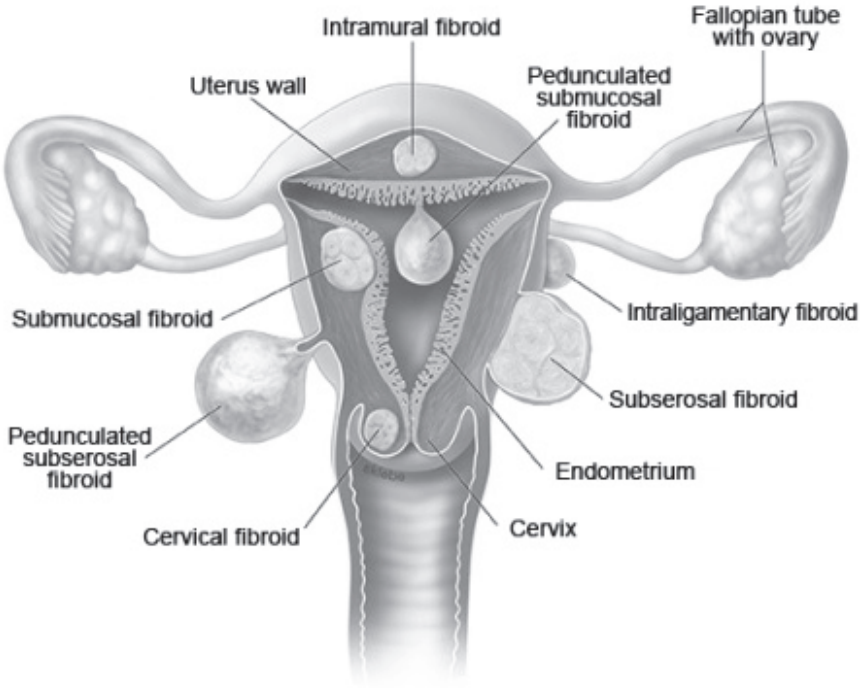

Fig. 1. Anatomical classification of uterine leiomyomas. The figure was retrieved on the 23rd of Nov 2016 from the Informed Health website and is printed with the copyright holder's permission (https://www.informedhealth.org/uterine-fibroids. 2622.en.html).

An enlarged uterus with uterine leiomyomas can cause pelvic pain and pressure sensation. Clinical studies show that gynaecological pain is related to leiomyomas (Vollenhoven *et al.* 1990, Lumsden & Wallace 1998, Stewart 2001). 42% of patients with pelvic pain that go through laparoscopy are diagnosed with leiomyomas, while the figure for patients going through abdominal hysterectomy is 74% (Carter 1994, Tay & Bromwich 1998). Also, 41% of hysterectomy patients with presurgical leiomyoma diagnosis have been reported to experience pelvic pain (Kjerulff *et al.* 1996). Leiomyoma-associated pain can be either non-cyclical, or cyclical as dysmenorrhoea (Buttram & Reiter 1981), or dyspareunia (Lippman *et*

al. 2003, Moshesh *et al.* 2014). More rarely, uterine leiomyomas cause acute pain, but this might be due to rapid growth and change in blood supply, or tumour degeneration and necrosis. Torsion of a subserosal pedunculated leiomyoma can also cause acute pain (Gupta & Manyonda 2009). Pelvic pain during pregnancy is more frequent among women with uterine leiomyomas. The pain is related to both leiomyoma size and uterine position (Rice *et al.* 1989, Exacoustos & Rosati 1993).

Uterine leiomyomas are associated with reduced fertility. They are estimated to be a single factor as regards infertility in less than 10% of infertility cases (Bajekal & Li 2000). However, no consensus exists. As leiomyomas may distort the uterus and enlarge and elongate the cavity, alter the contours and surface area of the cavity, obstruct tubal ostia or the cervical canal, or displace the cervix in the vagina, it is commonly accepted that the anatomical location of the leiomyoma is a crucial factor in fertility outcomes, with submucous, intramural and subserosal leiomyomas in decreasing significance order. Large leiomyomas (>5cm) and those situated close to the cervix or near the tubal ostia are more likely to cause a problem (Ubaldi *et al.* 1995). Uterine function may be affected by submucous and intramural leiomyomas that can cause dysfunctional and altered uterine contractility and thus interfere with sperm migration, ovum transport and embryo implantation (Hunt and Wallach 1974, Buttram and Reiter 1981, Vollenhoven 1990). Uterine leiomyomas may be associated with implantation failure due to overlying endometrial damage such as vascular disturbance, endometrial inflammation, ulceration, thinning and atrophy, and by an altered biochemical environment which may impair implantation (Deligdish & Loewenthal 1970, Farrer-Brown *et al.* 1971, Buttram & Reiter 1981, Farhi *et al.* 1995).

When investigating in-vitro fertilization (IVF) outcomes, uterine leiomyoma location and size are the most important factors determining the impact of treatment. A distorted endometrial cavity clearly affects the outcome and it is widely accepted that submucosal leiomyomas decrease fertility and that their removal seems to improve pregnancy rates (Donnez & Jadoul 2002, Martin 2003, Manyonda *et al.* 2004, Pritts *et al.* 2009). As regards intramural leiomyomas, there is no clear evidence, despite a randomized clinical trial, that myomectomy would be beneficial to fertility (Casini *et al.* 2006).

Women with uterine leiomyomas have an increased risk of spontaneous miscarriage with age and BMI. This applies to small (<3cm) submucosal leiomyomas. Intramural or subserosal larger leiomyomas have been reported not to increase the risk of miscarriage (Promislow *et al.* 2004). The presence of submucosal or subserosal leioymyomas slightly increased the risk of preterm birth

in this study population. There appeared to be no delay in conception when compared with women without leiomyomas (Promislow *et al.* 2004). It was concluded that women with leiomyomas would most likely have normal pregnancy outcomes. However, given the limited amount of research on the effects of leiomyomas on reproductive outcomes, additional research is warranted (Laughlin *et al.* 2009).

2.2 Epidemiology and risk factors

2.2.1 Incidence

The reported incidence of uterine leiomyoma varies greatly, depending on the population under study in terms of sample size, ethnicity, age and study design, and leiomyoma detection method: self-reports, clinical assessment or ultrasonographic screening. The on-going prospective cohort study on premenopausal female registered US nurses, the Nurses' Health Study II (NHS II) holds data on self-reported uterine leiomyomas. An incidence of 10–15% (per 1,000 woman-years) is reported for white women (Marshall *et al.* 1997), while for European women the figures vary between 4.5% among British women (Zimmermann *et al.* 2012) to 26.3% among Italian women (Eskenazi *et al.* 2007) (Table 1). Somewhat similar incidence figures have been presented for Asian women (Marshall *et al.* 1997, Zimmermann *et al.* 2012). Leiomyoma incidence figures among African-American women differ significantly compared with other ethnicities. African-American women have self-reported leiomyoma incidences between 34.4% (per 1,000 woman-years) (Wise *et al.* 2005b) and 37–42% among those aged 35–44 years (Marshall *et al.* 1997) (Table 1).

There are two studies that have presented leiomyoma incidence figures based on clinical screening methods. Possibly the most commonly mentioned study in the published literature on leiomyoma frequency is a histological study on 100 consecutive hysterectomy specimens (Cramer & Patel 1990). The authors reported a 77% frequency of leiomyomas in uteri that were surgically removed for symptomatic leiomyoma and other reasons, and screened for leiomyomas by sectioning the uteri at 2 mm intervals. By way of this meticulous method, leiomyomas of size <2 mm to 1 cm were detected. No data is available on the patient characteristics or ethnic background, but the main result of this study is that uterine leiomyomas may be found in removed uteri at the same frequency as in

uteri removed due to leiomyoma-related symptoms and pre-surgical clinical leiomyoma diagnosis. Another study on leiomyoma frequency in women undergoing tubal sterilization revealed a 9.0% frequency among white women and a 16.0% frequency among African-American women (Chen *et al.* 2001). A total of 3,174 women were screened for leiomyoma findings during surgery, but a self-reported history of leiomyoma was also taken into account (Table 1).

To date, six studies have involved the use of ultrasonographic imaging as a screening method to detect uterine leiomyomas (Table 1). It is worth noting that these studies have revealed leiomyomas that had not yet been clinically diagnosed. The prevalence of leiomyomas among white women living in the USA has been reported to be 30–35% at age 35–44 years (Baird *et al.* 2003) and 38.5% at age 34–46 years (Bower *et al.* 2009) (Table 1). Italian women had a prevalence of 21.4% in a cohort study (Marino *et al.* 2004). Interestingly, Swedish women had a very low prevalence of only 5.4% in a random sample of asymptomatic women (Borgfeldt & Andolf 2000), suggesting that Scandinavian women have a relatively low leiomyoma prevalence (Table 1). The largest dataset arises from a study on women in the first trimester of pregnancy (n=4,271), which documents an 8% leiomyoma prevalence figure for white women aged 17 and older

Table 1. Uterine leiomyoma prevalences and incidences in previously published studies.

Author	Sample size *N*	Ethnicity	Age	Study population	Study design	Prevalence (P)/Incidence (I)
Self-reported cases						
Wise *et al*. 2005b	22,895	African-American	21-69	Black women enrolled in the USA	Cohort	I:11.5%; 34.4% (95%CI: 33.1-35.7)/1,000**
Eskenazi *et al*. 2007	956	Italian	20-	Women enrolled living in Seveso area	Cohort	P:26.3%
Marshall *et al.* 1997	95,061	All	25-44	Premenopausal female registered US nurses	Cohort	
	300,899**	White				I:Age 35-44 years: 10-15%/1,000**
	4,367**	African-American				I:Age 35-44 years: 37-42%/1,000**
	4,654**	Hispanic				I:Age 35-44 years: 15-17%/1,000**
	6,007**	Asian				I:Age 35-44 years: 8-15%/1,000**
Downes *et al.* 2010	1,756	European	≥18	Invited European women for internet-based survey	Cross-sectional	
	1,111	French				P:11.7%*
	857	German				P:14.2%*
	783	Italian				P:23.6%*
	751	Spanish				P:18.8%*
	912	Britons				P:12.2%*

Author	Sample size *N*	Ethnicity	Age	Study population	Study design	Prevalence (P)/Incidence (I)
Self-reported cases						
Zimmermann *et al.* 2012	21,749	8 countries	15-49	Recruited via an online-panel	Cross-sectional	
	2,552	Brazilian	15-49			P:7.0%
	2,514	Canadian	15-49			P:5.5%
	2,543	French	15-49			P:4.6%
	2,558	German	15-49			P:8.0%
	2,519	Italian	15-49			P:9.8%
	2,524	South Korean	15-49			P:9.0%
	2,500	British	15-49			P:4.5%
	4,039	American	18-49			P:6.9%
Heinemann *et al.* 2003	10,077	German	18-65	Public enrolment	Cohort	I:5.0%
	396,000**					I:12.7%/100,000**
Clinically diagnosed cases						
Chen *et al.* 2001	3,174	All	17-44	Women undergoing tubal sterilization	Cohort	
	2,726	White				P:9%*
	448	African-American				P:16%*
Cramer & Patel 1990	100	Not reported	-	Consecutive hysterectomy specimens	Histological screening	P:77%*

Author	Sample size *N*	Ethnicity	Age	Study population	Study design	Prevalence (P)/Incidence (I)
Ultrasound screened cases						
Baird *et al.* 2015	1,696	African-American	23-34	Enrolled African-American women	Prospective patient series	I:22.3%*
Laughlin *et al.* 2009	4,271	All	17-	Women in the first trimester of pregnancy	Cohort	P:10.7% (95%CI: 8.5-13.6%)
	915	African-American				P:18% (95%CI: 13-25%)
	2,826	White				P:8% (95%CI: 7-11%)
	335	Hispanic				P:10% (95%CI: 5-19%)
	186	“other” (Asian)				P:13% (95%CI: 10-16%)
Baird *et al.* 2003	1,364	All	35-49	Random sample of premenopausal women	Prospective	
	840	African-American				I:Age 35-44 years: 55%
	524	White				I:Age 35-44 years: 30-35%
Marino *et al.* 2004	341	Italian	30-60	Invited cohort women	Cohort	I:21.4%
Borgfeldt & Andolf 2000	335	Swedish	25-40	Random sample of asymptomatic women	Prospective	P:5.4% (95%CI: 3.0-7.8%)
Bower *et al.* 2009	966	All	34-46	Women enrolled by geographical site	Population-based observational study	
	501	White				P:38.5%*
	465	African-American				P:66.9%*

*Confidence interval (CI) not reported

** woman-years

(Laughlin *et al.* 2009). Understandably, this study is not comparable to others as the dataset is biased on fertility characteristics. Nevertheless it again emphasizes the high prevalence of asymptomatic and clinically undiagnosed uterine leiomyomas. African-American women have been reported to have prevalence rates between 22.3% (newly diagnosed at baseline of the study) (Baird *et al.* 2015) and 66.9% (Bower *et al.* 2009), with pregnant women having a 18% prevalence (Laughlin *et al.* 2009).

2.2.2 Life cycle and hormonal status

Adolescence and menarche

There are numerous observations that uterine leiomyoma development and clinical picture are associated with female hormonal status. The clinical significance of leiomyomas is limited to reproductive years, as the cumulative incidence increases until menopause (Baird *et al.* 2003), after which the disease burden starts to decline as the leiomyoma tumours become smaller in size (Cramer & Patel 1990). Normally uterine leiomyomas do not emerge in prepubertal girls, but a few case reports have been published on adolescent girls and women (Fields & Neinstein 1996, Michala *et al.* 2010). These subjects were aged 13 to 21 years, median 15 years, most commonly presenting with abdominal pain and bleeding problems. Many investigators have reported an inverse association between age at menarche and uterine fibroid risk (Marshall *et al.* 1998a, Faerstein *et al.* 2001a, Yang *et al.* 2014), with five categories showing a gradual decrease in risk with increasing age at menarche from ≤9 to ≥16 years (Terry *et al.* 2010). The inverse relationship seems to be stronger as regards multiple fibroids (Velez Edwards *et al.* 2013). Results concerning interaction of race on this association are contradictory; a pregnancy cohort showed no interaction (Velez Edwards *et al.* 2013), whereas an ultrasound screening study revealed more African-American than Caucasian women having an earlier age at menarche (Dragomir *et al.* 2010).

Menstrual characteristics

Only a few studies have evaluated menstrual characteristics among women with uterine leiomyomas. There seem to be no statistically significant differences regarding time to regular cycles, usual menstrual cycle length, duration of

menstrual flow or the presence of menstrual irregularities at early reproductive age (Faerstein *et al.* 2001a, Terry *et al.* 2010). Later on, at above 22 years, women with very regular or always irregular cycles have a decreased risk of leiomyomas (Terry *et al.* 2010).

Parity, pregnancy and breastfeeding

Several studies have shown that parity is protective as regards uterine leiomyoma development. Parous women have a decreased risk of leiomyomas when compared with nulliparous women (Parazzini *et al.* 1996, Marshall *et al.* 1998a, Dragomir *et al.* 2010) and the risk seems to decline with number of births (Parazzini *et al.* 1996, Parazzini 2006), but stops after the third pregnancy (Marshall *et al.* 1998a). Furthermore, the association has been found to be independent of infertility history, as the relationship remains the same when comparing parous and nulliparous women with no fertility problems and when comparing parous and nulliparous women with fertility problems (Marshall *et al.* 1998a, Terry *et al.* 2010).

Further attempts to study the association between parity and uterine leiomyoma development have revealed that delivery in the mid-reproductive years, that is between ages 25 to 29 years, appear to be most protective as regards uterine leiomyoma development (Baird & Dunson 2003). Delivery before the age of 25 and after 29 years have a less strong effect, indicating that optimal uterine growth during pregnancy and postpartum remodelling may be crucial in connection with uterine leiomyoma tumorigenesis (Baird & Dunson 2003). The documented lower risk of leiomyomas among women with more recent pregnancies than with more remote pregnancies indicates that uterine remodelling has an effect on leiomyoma development (Marshall *et al.* 1998a, Wise *et al.* 2004). This hypothesis is supported by experimental studies on the Eker rat, an animal model as regards uterine leiomyomas. There was a drop in leiomyoma incidence from 71% in single-litter rats to 10% in multiple-litter animals (Walker *et al.* 2001). Furthermore, another group evaluated this relationship in a follow-up screening study on pregnant women with one initial leiomyoma (Laughlin *et al.* 2010). The women were systematically screened ultrasonographically in early pregnancy and then again three to six months postpartum. The study revealed 36% of leiomyomas to have resolved to undetectable in the postpartum screening, regardless of the initial tumour size, and the leiomyomas that remained were reduced in diameter by a median of 0.5 cm (Laughlin *et al.* 2010). Further analysis also revealed that miscarriages were associated with leiomyoma regression, but at a decreased rate

when compared with live births. The later the miscarriage, the greater was the regression (Laughlin *et al.* 2011), presenting possible gradual pregnancy-long effects on leiomyoma regression. The further analysis additionally revealed an association between leiomyoma regression and postpartum progestin use, with users having significantly less regression, indicating progestin involvement in uterine leiomyoma pathology. Other hormonal changes (use of other hormonal contraceptives), Caesarean delivery, fever or breastfeeding, were not associated with leiomyoma regression in this study (Laughlin *et al.* 2011).

Breastfeeding suppresses ovarian steroid production. Only a few studies have examined the relationship between uterine leiomyoma risk and breastfeeding. A small case-control study revealed a negative association, but not of statistical significance (Samadi *et al.* 1996). A prospective cohort study on black women showed no association with lifetime breastfeeding for up to two years (Wise *et al.* 2004), whereas a case-control study on Thai women showed a reduced risk after five years of lifetime breastfeeding (Lumbiganon *et al.* 1996). An inverse association between lifetime duration and exclusive breastfeeding and leiomyoma risk was reported in a large prospective cohort study (Terry *et al.* 2010). Interestingly, breastfeeding duration was not associated with leiomyoma regression in the prospective ultrasonographic screening study (Laughlin *et al.* 2011). This may be due to the role of oxytocin as a stimulator of leiomyoma cell growth (Busnelli *et al.* 2010), counteracting the hormonal suppression initiated by breastfeeding.

Hormonal contraception

Findings on hormonal contraceptive usage and risk of uterine leiomyomas are inconsistent, but tend to show an inverse association, with higher-dose progestin preparations in particular. Several studies have revealed a decreased risk with increasing duration of oral contraceptive use; risk reducing for up to seven years of usage (Chiaffarino *et al.* 1999), and a follow-up study showed a roughly 17% reduction in risk with each five years of use (Ross *et al.* 1986). Two groups of investigators have been able to differentiate associations among different contraceptive products and they report inverse associations between depot medroxyprogesterone acetate (Lumbiganon *et al.* 1996) and progestin-only injectables (Wise *et al.* 2004) vs. leiomyomas. However, some groups have observed no association (Parazzini *et al.* 1992, Samadi *et al.* 1996, Parazzini 2006),

while positive correlations have also been published (Parazzini *et al.* 1996). Interestingly, a large cohort study revealed a significantly elevated risk among women with a history of oral contraceptive use at ages of 13 to 16 years (Marshall *et al.* 1998a), but the indications for contraceptive use may have played a role in this observed association.

Menopause and hormone replacement therapy

Uterine leiomyoma dependency on ovarian steroid hormones is supported by observations that the incidence of leiomyomas declines in menopause (Ross *et al.* 1986, Parazzini *et al.* 1988, Parazzini 2006) and fewer women self-report leiomyomas in menopause (Samadi *et al.* 1996). Thorough histological screening of uteri, however, has shown that postmenopausal incidence does not differ from premenopausal incidence, but the leiomyomas are smaller in size and fewer in numbers (Cramer & Patel 1990) and thus less symptomatic.

2.2.3 Familial aggregation

Uterine leiomyoma heritability has been assessed by way of epidemiological family and twin studies in order to investigate whether genetic factors play a role in leiomyoma pathogenesis. First-degree family members in families with two or more verified leiomyoma cases have been observed to have a 2.2-fold higher frequency of leiomyoma than family members with one or no leiomyoma cases (Vikhlyaeva *et al.* 1995). A similar result was obtained among Japanese women (Sato *et al.* 2002). Twin studies have brought more confirmation of the suspected heritability of leiomyomas, with reporting of higher hospitalization rates for uterine leiomyomas (Luoto *et al.* 2000) and also higher hysterectomy rates in monozygotic than in dizygotic twins (Treloar *et al.* 1992).

2.2.4 Ethnicity

Studies in which populations have been separated into different racial and ethnic groups have documented major differences in uterine leiomyoma incidence rates and clinical severity, thus also strongly suggesting a heritable component in the disease mechanism. Most evidence arises from studies conducted in the United States that show repeatedly that African-American women have an increased risk of developing symptomatic leiomyomas compared with Caucasian women.

Reports on hysterectomy rates and indications have implied that African-American women are at a higher risk of hysterectomy (Meilahn *et al.* 1989), and, additionally, leiomyomas are the leading indication for hysterectomy (Kjerulff *et al.* 1993, Kjerulff *et al.* 1996, Palmer *et al.* 1999). Differences among ethnic groups have remained the same in reports performed at decade intervals (Palmer *et al.* 1999, Bower *et al.* 2009). Longer hospital stays, higher rates of complications in surgery and more severe symptoms at an earlier age (Kjerulff *et al.* 1993, Velebil *et al.* 1995, Kjerulff *et al.* 1996) again confirm the ethnic disparity in leiomyoma biology.

Further studies on uterine leiomyoma incidence have involved use of other case ascertainment methods in addition to surgery and hospitalization rates, in order to investigate the prevalence figures in more detail. Studies involving questionnaires for self-reporting and screening data (physical examination and ultrasonography) have been used to quantify incidence figures among black and white women. Data arising from the NHS II study confirm a higher rate of uterine leiomyomas among premenopausal black women independent of known leiomyoma risk factors (RR 3.25; 95% CI: 2.71–3.88) (Marshall *et al.* 1997), with a following study showing similar trends (Faerstein *et al.* 2001a). An extensive screening study, carried out independently of clinical symptoms, was published on cumulative incidence figures for black and white women (Baird *et al.* 2003). 59% of black women were reported to have been diagnosed with newly detected leiomyomas, whereas the proportion for white women was 43%. The estimated age-specific cumulative incidence of leiomyomas was >80% for black women aged 35 to 49 years, and nearly 70% for white women. Thus, most women in the United States will develop uterine leiomyoma tumours before menopause (Baird *et al.* 2003).

In recent years there have been several publications further exploring the ethnic disparity in uterine leiomyoma biology. A recent study involving use of admixture-based genome-wide scan methodology was carried out to further explore the inherited factors of uterine leiomyoma to explain the observed differences among African-American and European-American women (Wise *et al.* 2012). The study failed in its search for risk alleles that would be highly differentiated in frequency between the ethnic populations under study. The observed highly frequent somatic mutations in exon 2 of the *mediator complex subunit 12* (*MED12*) gene in Caucasians have been confirmed to have a major role in leiomyomas, regardless of African or Caucasian ancestry (Makinen *et al.* 2011a). In order to resolve the biological mechanism for this disparity, the role of vitamin D has been examined in relation to leiomyoma development in black and white women. As vitamin D is

claimed to have an effect on cell proliferation and extracellular matrix production in leiomyoma tissue in culture (Sharan *et al.* 2011), and treatment with calcitriol seems to limit leiomyoma growth *in vivo* (Sabry & Al-Hendy 2012), and adding the consideration that African-American women are vitamin D-deficient 10 times more commonly than white women (Nesby-O'Dell *et al.* 2002), this relationship is strongly justified to be thoroughly explored in humans. In The National Institute of Environmental Health Sciences (NIEHS) Uterine Fibroid Study, circulating levels of the vitamin D metabolite $25(OH)_2D_3$ and self-reported sun exposure in black and white women, who were also ultrasonographically screened for uterine leiomyomas were assessed (Baird *et al.* 2013). The report indicated a reduced risk of leiomyoma among women with sufficient vitamin D levels. The finding was similar among black and white women, adding that only 10% of black women had the required vitamin D level, the figure in white women being 50% (Baird *et al.* 2013). The association has been studied through polymorphisms in genes involved in vitamin D metabolism and skin pigmentation (Wise *et al.* 2014). The study reports on three of twelve polymorphisms having an association with uterine leiomyoma at a nominal significance level, thus offering support to the hypothesis that vitamin D deficiency is involved in leiomyoma development (Wise *et al.* 2014).

Ethnic disparity has mostly been investigated in American studies that differentiate their study populations among black and white women, usually grouping Hispanic and Asian women with white. Different incidence figures arising from Europe as a whole, and Scandinavia, encourage exploration of this area in more detail, firstly requiring more detailed ethnic grouping of populations under study. Interestingly, the first admixture mapping study on leiomyomas revealed an inverse association between European ancestry and leiomyoma risk (Wise *et al.* 2012), which is in line with the European studies discussed earlier in this review (Table 1).

2.2.5 Metabolic factors

Obesity

The association between obesity and uterine leiomyomas has been confirmed in many studies (Marshall *et al.* 1998b, Sato *et al.* 1998, Faerstein *et al.* 2001a, Wise *et al.* 2005a, Parazzini 2006, Takeda *et al.* 2008, Dandolu *et al.* 2010, Yang *et al.* 2014), although not all studies agree (Samadi *et al.* 1996, Chen *et al.* 2001). The

overall association is that uterine leiomyomas increase gradually along with increasing BMI (Marshall *et al.* 1998b, Faerstein *et al.* 2001a, Wise *et al.* 2005a, Yang *et al.* 2014). Some investigators have presented an inverse J-shaped pattern with a peak incidence associated with BMI categories of 20.4–23.9 kg/m^2 (Parazzini *et al.* 1996), 25–29 kg/m^2 (Lumbiganon *et al.* 1996) and 22.5–24.9 kg/m^2 in nulliparous women and 27.5–29.9 kg/m^2 in parous women (Wise *et al.* 2005a). Two studies have been able to prove that raised BMI is due to fat mass and not muscle mass by determining body fat percentages (Sato *et al.* 1998, Yang *et al.* 2014). Body fat distribution and its association with leiomyoma has been assessed by measuring waist-hip-ratios (WHRs) to differentiate central obesity from peripheral obesity. Two studies have shown central obesity to be associated with the risk of leiomyomas (Sato *et al.* 1998, Yang *et al.* 2014), while one study revealed no association (Wise *et al.* 2005a). Furthermore, a positive correlation has been reported between BMI and uterine weight, as every 1-point increase in BMI was associated with a 7.56 g increase in uterine weight (Dandolu *et al.* 2010), suggesting increasing body fat mass to have an increasing association with multiple and/or large leiomyomas.

Hypertension

There is strong evidence that hypertension and uterine leiomyomas are associated. The relationship has been shown in several studies and it has been shown to be bi-directional: hypertension increases the risk of leiomyomas (Faerstein *et al.* 2001b, Boynton-Jarrett *et al.* 2005, Settnes *et al.* 2005, Silver *et al.* 2005, Takeda *et al.* 2008, Templeman *et al.* 2009, Spies *et al.* 2010, Lambertino *et al.* 2011, Radin *et al.* 2012, Sivri *et al.* 2012) and leiomyomas increase the risk of hypertension (Luoto *et al.* 1995, Luoto *et al.* 2001, Haan *et al.* 2015). The NHS II study, which is a large prospective cohort study, reports every 10-mmHg increase in diastolic blood pressure to raise the risk of leiomyomas by 8% and 10% among non-users and users of antihypertensive medication, after appropriate confounding covariate adjustments (Boynton-Jarrett *et al.* 2005). Another large cohort study, on African-American women, indicates hypertension to be associated with hysterectomy-confirmed leiomyoma cases, but not with ultrasonographically or other-surgery-confirmed cases (Radin *et al.* 2012). Results with no association have also been published (Parazzini *et al.* 2004, Aksoy *et al.* 2014).

The data mostly relies on self-reported hypertension history, with no data on the time of diagnosis of either hypertension or uterine leiomyoma. Thus, a causal relationship has not yet been established. Light on this relationship has possibly been provided by a recent study on hypoxia-stimulated renal and vascular function-linked peptides (Wallace *et al.* 2014). The study showed that women with uterine leiomyomas not only have increased circulating peptides (soluble fms-like tyrosine kinase 1 (sFlt-1) and endothelin 1 (ET-1)), but also increased leiomyoma and myometrial secretion of ET-1. These results support a link between leiomyoma secretion of vasoactive factors and the development of hypertension.

Lipid metabolism

Lipid metabolism in women with uterine leiomyomas has been analysed in only a few studies (Sadlonova *et al.* 2008, Takeda *et al.* 2008, He *et al.* 2013, Aksoy *et al.* 2014) and the results are conflicting. All studies are of case-control type, with small sample sizes. Two studies revealed no difference in total cholesterol among women with and without leiomyomas (Sadlonova *et al.* 2008, He *et al.* 2013), whereas HDL-cholesterol and leiomyomas were shown to have an inverse association in two studies (He *et al.* 2013, Aksoy *et al.* 2014), but a contradictory result in another study (Sadlonova *et al.* 2008). This study also reported lower LDL-cholesterol levels among women with leiomyomas, whereas no association was reported in two other studies (He *et al.* 2013, Aksoy *et al.* 2014). Triglyceride levels were assessed in all four of these previous studies, three reporting no difference (Sadlonova *et al.* 2008, He *et al.* 2013, Aksoy *et al.* 2014), but one study reporting significantly higher serum triglyceride levels in the uterine leiomyoma group (Takeda *et al.* 2008).

Glucose metabolism

Published data on the association between glucose metabolism and uterine leiomyomas is very limited. The association has mainly been analysed by using self-reported diabetes diagnoses, but also fasting glucose levels, fasting insulin levels and short insulin tolerance test results (Faerstein *et al.* 2001b, Sadlonova *et al.* 2008, Baird *et al.* 2009, Templeman *et al.* 2009, He *et al.* 2013). These studies do not present a clear consensus on glucose metabolism alterations among women with uterine leiomyomas and controls. A large cohort study has shown a self-reported history of diabetes to be associated with a decreased risk of leiomyomas

(Templeman *et al.* 2009). The NIEHS Uterine Fibroid Study, on the other hand, reported either no association or inverse effects as regards leiomyomas, and IGF-I and insulin levels (Baird *et al.* 2009).

2.2.6 Endometriosis

The coexistence of uterine leiomyomas and endometriosis is suggested in only a few studies, but the results are encouraging. The first report arose from a large case-control study investigating several risk factors and the probability of having endometriosis. The study included women undergoing surgery for diagnostic laparoscopy, fertility-regulating surgery, or hysterectomy. Women with surgically confirmed endometriosis were observed to have a significantly higher frequency of leiomyomas that were identified during the surgical procedure (Hemmings *et al.* 2004). The association was observed among women having diagnostic laparoscopy and fertility-regulating surgery, but not with hysterectomy. The presence of uterine leiomyomas was not associated with the severity of endometriosis or with the presence of adhesions (Hemmings *et al.* 2004). It is noteworthy that leiomyomas were identified only during surgery, so this result only accounts for subserosal or multiple leiomyomas that significantly enlarge the uterus.

Thereafter, this finding has been confirmed in a few studies investigating the association between endometriosis and symptomatic uterine leiomyomas. In a series of women with symptomatic leiomyomas undergoing laparoscopic myomectomy or hysterectomy, 86% were found to have concomitant endometriosis (Huang *et al.* 2010), of which the majority were recognized as rAFS stage I–II endometriosis (1997, Huang *et al.* 2010). A retrospective cohort study reported a 21.1% prevalence of endometriosis and leiomyoma coexistence among women with symptomatic leiomyomas undergoing laparoscopic myomectomy (Maclaran *et al.* 2014). Other studies have presented prevalence estimates of 12% (1994), 12.7% (Isono *et al.* 2012) and 22.7% (Naphatthalung & Cheewadhanaraks 2012) for coexistence.

Women presenting with both conditions, when compared with women with symptomatic leiomyomas seem only to be younger (Huang *et al.* 2010, Isono *et al.* 2012, Naphatthalung & Cheewadhanaraks 2012) and to have reduced fertility (Huang *et al.* 2010, Isono *et al.* 2012, Maclaran *et al.* 2014). They present with moderate to severe pain (Huang *et al.* 2010, Naphatthalung & Cheewadhanaraks 2012), their leiomyomas are smaller in size at the time of operation (Huang *et al.*

2010, Isono *et al.* 2012) and are subserosal rather than intramural (Maclaran *et al.* 2014). The coexistence of leiomyomas and endometriosis has been observed more often among Asian women, while Afro-Caribbean women have a lower prevalence and whites show no difference between the coexistence and existence of leiomyomas alone (Maclaran *et al.* 2014). Of other uterine leiomyoma-documented risk factors, there were no differences in BMI among women having both conditions (Isono *et al.* 2012, Maclaran *et al.* 2014).

2.3 Uterine leiomyoma pathophysiology

2.3.1 Cellular origin of leiomyomas

Uterine leiomyoma tumours may present as single or multiple. Cytogenetic and X-chromosome-inactivation studies have investigated the cellular origin of leiomyoma tumours, aiming to reveal whether cells within one tumour arise from a single or multiple cells and whether multiple tumours develop independently or from a single primary tumour. The tumour cells within one tumour seem to originate from a single smooth muscle cell, sharing the same clonal origin. This has been studied by analysis of inactivation of the X- chromosome as demonstrated by human androgen receptor (*HUMARA* assay) or glucose-6-phosphate dehydrogenase (*G6PD*) isoform expression. In each leiomyoma tumour a monoclonal pattern of X-chromosome inactivation has been identified, identical to that of the individual tumour from which the cells were derived (Linder & Gartler 1965, Townsend *et al.* 1970, Mashal *et al.* 1994, Canevari *et al.* 2005, Zhang *et al.* 2006, Cai *et al.* 2007).

Whether different tumour nodules in multiple leiomyomas share a common origin is still awaiting clarification. Both random and same X-chromosome inactivation patterns have been reported, but the role of chance has to be considered, particularly when there are two tumours. A study of 55 cases with multiple leiomyomas revealed that most tumour nodules within a single uterus present with an identical allele inactivated in all nodules, providing evidence of a unicentric origin (Cai *et al.* 2007). Contradictory evidence in 14 and 24 cases has also been presented, suggesting multicentric origin (Canevari *et al.* 2005, Zhang *et al.* 2006). The common observation of chromosome reassembly resembling chromothripsis (a single genomic event resulting in focal losses and rearrangements in multiple

genomic regions) supports a clonal relationship in multiple leiomyomas (Mehine *et al.* 2013a).

Studies on telomere length provide further elucidation of the puzzle, offering support for monoclonal and multicentric origins of multiple leiomyoma tumours. The average length of telomere repeats has been observed to be the same within the same tumour, but the lengths differ significantly between multiple tumours (Rogalla *et al.* 1995, Bonatz *et al.* 1998).

Study of clonality of different cell types in uterine leiomyoma tumours may challenge the monoclonality theory of leiomyoma origin. However, a fairly recent study revealed that multiple cell types (smooth muscle cells, vascular smooth muscle cells, fibroblasts and leiomyoma-associated fibroblasts) originate from a single cell (Holdsworth-Carson *et al.* 2014). Another substantial aspect of this study is that monoclonality of leiomyoma cells implies that the parental cell must have multipotent stem cell properties in order to have the ability to differentiate into the multiple cell types listed above.

2.3.2 Stem cells

The human uterus is remarkable in its plasticity and regenerative capacity during menstrual cycles and over the course of pregnancy, during which it undergoes a 500- to 1000-fold increase in volume and a 25-fold increase in weight. The uterine myometrium is remodelled in each pregnancy and both cell hypertrophy and hyperplasia contribute to the dramatic growth (Cunningham *et al.* 2010). Myometrial hyperplasia dominates in early gestation (Shynlova *et al.* 2006), thus indicating potential stem-cell-like properties responsible for smooth muscle cell proliferation. Mature myometrial cells express much higher levels of oestrogen receptor α (ERα) and progesterone receptor (PR) than myometrial stem cells (Mas *et al.* 2012, Ono *et al.* 2012). It has been suggested that ERα and PR residing in the neighbouring mature myometrial cells mediate the oestrogen- and progesterone-dependent cell proliferation in a paracrine fashion. Paracrine factors, such as Wnt ligands belonging to the Wnt-β-catenin signalling pathway, are released by mature cells surrounding the stem cells (Tai *et al.* 2003). Oestrogen and progesterone may increase the secretion of Wnt ligands, which then activate the β-catenin-T-cell transcription factor (TCF) pathway, that then induces the production of transforming growth factor β (TGF-β) in mature cells, which again induces cell proliferation (Tanwar *et al.* 2009).

Uterine leiomyoma cells have been observed to have smaller side populations (SPs; universal markers of somatic stem cells) than normal myometrium (Ono *et al.* 2012). They reside in quiescence, being arrested in the G0 phase of the cell cycle (Ono *et al.* 2007). Leiomyoma-derived SP cells present with very low levels of ERα, PR and smooth muscle cell markers when compared with leiomyoma-derived main population (MP) cells and whole leiomyoma tissue (Mas *et al.* 2012, Ono *et al.* 2012). However, after co-culture with myometrial cells, these markers were expressed naturally at the same levels as seen in leiomyoma-derived MP cells. These observations may indicate that leiomyoma SP cells represent immature cell populations that exist in an undifferentiated state within the leiomyoma and have the potential to differentiate into uterine leiomyoma cells within the environment of the normal uterine myometrium (Ono *et al.* 2012).

The Wnt-β-catenin pathway may have a role in uterine leiomyoma formation, as selective deletion of β-catenin in a mouse model decreases uterine size and disrupts the differentiation of smooth muscle stem cells observed in leiomyoma tissue (Arango *et al.* 2005). In stem cells, β-catenin action may be physiologically modified by MED12, which is a mediator complex subunit, regulating both global and gene-specific transcription (Conaway & Conaway 2011). Mediator is a transducer of Wnt-β-catenin signalling and MED12 binds directly to β-catenin and regulates canonical Wnt signalling (Kim *et al.* 2006). Interestingly, stem cells derived from leiomyoma tissue carry *MED12* mutations, but not the myometrium (Ono *et al.* 2012). Thus, in leiomyoma tissue the β-catenin action modification may be altered by this genetic hit. Lack or altered action of MED12 has also been linked to increased expression of TGF-β receptor, which leads to stimulation of cell proliferation and fibronectin expression (Arici & Sozen 2000). This in turn mediates stem cell self-renewal and proliferation through activating yet more signalling cascades, mothers against decapentaplegic homologue (SMAD) and mitogen-activated protein kinase (MAPK) family proteins (Levens *et al.* 2005). These observed interactions, starting from a genetic hit (*MED12* mutation) and involving Wnt-β-catenin activation, TGF-β pathways, oestrogen and progesterone, and stem cell renewal, may give rise to the monoclonal formation of uterine leiomyoma tumours (Bulun 2013).

2.3.3 Genetic features

Hereditary syndromes

Genetic predisposition to uterine leiomyoma development is supported by familial aggregation, varying incidences in different ethnic groups, and results arising from twin studies. Additional support is provided by the evidence of uterine leiomyoma identified as a characteristic phenotype in several hereditary syndromes.

Leiomyomas develop in hereditary leiomyomatosis and renal cell cancer syndrome (HLRCC; OMIM # 150800). It is an autosomal dominant tumour predisposition syndrome clinically characterized by multiple early-onset uterine leiomyomas, multiple cutaneous piloleiomyomas and an early-onset type II papillary renal cell cancer (Launonen *et al.* 2001). HLRCC-related uterine leiomyomas are highly penetrant among *FH* mutation carrier women. By the age of 35 years 72% were diagnosed with uterine leiomyoma, and by age 40 years the rate is above 75% (Alam *et al.* 2005), some studies showing even 100% penetrance (Wei *et al.* 2006). Women with HLRCC become symptomatic due to their uterine leiomyomas at a young age. Thereafter the high risk of hysterectomy (53% of all female *FH* mutation carriers by age 40) highlights the significant uterine disease in this tumour syndrome (Toro *et al.* 2003, Sanz-Ortega *et al.* 2013). Cutaneous piloleiomyomas, which originate from arrector pili muscles attached to hair follicles, are the most distinct characteristic (100% penetrance in men and 55% in women by age 35) (Alam *et al.* 2005) of the syndrome. They can be numerous, ranging from one to hundreds, and are localized on the trunk and limbs, causing pain in response to touch or temperature changes (Lehtonen 2011). Renal cell cancers (RCCs) are detected only in a subset of cases (20–25% of *FH* mutation-positive families) (Bayley *et al.* 2008). However, they are exceptionally aggressive in nature and the rate of distant metastasis in the very early stages is observed to be higher than for other hereditary renal cancer syndromes (Tomlinson *et al.* 2002, Vahteristo *et al.* 2010), thus creating a challenge for the detection and treatment of these lesions.

HLRCC is caused by heterozygous germline mutations in the *fumarate hydratase (FH)* gene at chromosome region 1q42. The gene encodes the enzyme fumarase, a component of the mitochondrial tricarboxylic acid cycle (TCAC), which is part of the aerobic respiration process in the cell's energy metabolism. Fumarase catalyzes the hydration of fumarate to malate. Most *FH* mutations are

either missense (~58%), nonsense (~11%), or frameshift mutations (~18%) scattered throughout the gene (Bayley *et al.* 2008, Lehtonen 2011). Frequently, HLRCC-related tumours display biallelic inactivation of *FH*, indicating that the gene functions as a tumour suppressor. Biallelic inactivation of *FH* results in elevated levels of fumarate and succinate (intermediate in the TCAC prior to fumarate) in the cell (Pollard *et al.* 2005b). This in turn seems to stabilize HIF1 (a key signalling molecule in the hypoxia pathway) aberrantly, leading to over-expression of hypoxia/angiogenesis pathway genes despite the presence of oxygen (therefore called the "pseudohypoxia" pathway) (Isaacs *et al.* 2005, Pollard *et al.* 2005b). Other molecular mechanisms studied in connection with HLRCC tumorigenesis involve activation of the antioxidant response pathway by Kelch-like ECH-associated protein-1 (KEAP1) succination (Adam *et al.* 2011), and overcoming apoptotic signalling by activation of the energy sensor AMP-activated protein kinase (AMPK) (Bardella *et al.* 2012).

Other hereditary syndromes associated with uterine leiomyomas are tuberous sclerosis complex (TSC), Birt-Hogg-Dube (BHD), Alport and Cowden syndromes. TSC is a multi-organ genetic disease caused by heterozygous germline mutation of the tumour suppressor genes *TSC1* or *TSC2*. The protein products, hamartin and tuberin, heterodimerize and negatively regulate activation of the mechanistic target of rapamycin (mTOR) protein kinase. The main clinical characteristics of the disease are hamartomas of the skin, brain and kidneys, and renal cell cancer in some patients (Curatolo *et al.* 2008). The most widely used animal model for uterine leiomyoma studies is the Eker rat, which has a mutation in one allele of *Tsc2*. The Eker rat develops uterine leiomyomas spontaneously at a frequency of ~65% (Eker *et al.* 1981, Walker *et al.* 2003). Spontaneous uterine leiomyomas arise in German shepherd dogs carrying a germline *Bhd* mutation and thus these serve as a canine model for BHD (Moe & Lium 1997, Lingaas *et al.* 2003). It is a condition involving lesions on the skin, face and neck, lung cysts and renal cancer (Toro 1993). Alport syndrome is mostly caused by mutations of the collagen type IV α5 chain gene (*COL4A5*), but also mutations of *COL4A5* and *COL4A6* genes at chromosome region Xq22. The characteristic findings are progressive renal disease, hearing loss and visual impairment, all as a consequence of structural deficiency of the basal membranes due to lacking collagen chains (Hudson *et al.* 2003). Some women with Alport syndrome develop diffuse leiomyomatosis or numerous leiomyomas outside the uterus (Kashtan 1999). Cowden syndrome, also known as multiple hamartoma syndrome, is caused by germline mutations in the tumour suppressor gene phosphatase and tensin homolog (*PTEN*) (Liaw *et al.* 1997). It is characterized by

typical mucocutaneous lesions, the growth of multiple hamartomas and increased risks of breast, thyroid, endometrial and renal cancers (Pilarski & Eng 2004), and in addition, the development of uterine leiomyomas (Hobert & Eng 2009).

Chromosomal changes

Somatic chromosomal (karyotypic, cytogenetic) aberrations have been detected in approximately 40–50% of uterine leiomyomas, such as rearrangements involving 12q15 and 6p21, and deletions of 7q (Sandberg 2005). These constitute 20%, <5% and 17% of karyotypically abnormal leiomyoma tumours respectively. Other less frequent aberrations are rearrangements involving 10q, trisomy 12 and deletions of 3q. Karyotypically abnormal leiomyomas have a tendency to be larger in size, more cellular and to have a higher mitotic index (Ligon & Morton 2000). Intramural and subserous leiomyomas have abnormal karyotypes more often than submucous leiomyomas (Brosens *et al.* 1998). The high mobility group AT-hook 2 (*HMGA2*) gene is located in region 12q13-15, and it serves as a driver gene for tumours carrying 12q15 rearrangements (Fusco & Fedele 2007). Proteins encoded by *HMGA2* are mainly expressed during embryonic development and are silenced in adult tissue. They function as DNA architectural factors in the nuclear scaffold and are critical for transcription regulation. Approximately 10% of all uterine leiomyomas display *HMGA2* over-expression (Sandberg 2005), making it the second most frequent driver gene in leiomyomas (Mehine *et al.* 2014). The second most common chromosomal aberration in leiomyomas is an interstitial deletion within chromosome 7. Despite numerous identified positional candidate genes at 7q22, such as *CUX1*, *ORC5L*, *PCOLCE* and *ZNHIT1*, their roles in leiomyoma development have not been confirmed (Quintana *et al.* 1998, Ligon *et al.* 2002, Mehine *et al.* 2013b, Schoenmakers *et al.* 2013). Rearrangements at 6p21 affect *HMGA1* and occasionally involve 14q23-24 and inversions (Kazmierczak *et al.* 1998, Sornberger *et al.* 1999).

MED12 mutations

Genome-wide DNA exome sequencing of samples from Finnish (Caucasian) women revealed that approximately 70% of uterine leiomyomas contain heterozygous somatic mutations affecting Mediator Complex Subunit 12 (*MED12)* exon 2 on the X chromosome (Makinen *et al.* 2011b). MED12 is part of the 26-

subunit mediator complex, which functions as a communicator of regulatory signals from DNA-bound transcription factors directly to the RNA polymerase II enzyme. Mediator is also crucial for genomic DNA organization into topological domains, i.e. fundamental gene loop structures that enable the coordinated regulation of cellular transcription (Allen & Taatjes 2015). 49% of the identified *MED12* mutations were missense mutations affecting codon 44 and 11% were insertion-deletion-type mutations (Makinen *et al.* 2011b). There is strong evidence that mutations in *MED12*, *FH* and *HMGA2*, and are mutually exclusive (Vanharanta *et al.* 2006, Markowski *et al.* 2012, Mehine *et al.* 2013b, Bertsch *et al.* 2014, Kampjarvi *et al.* 2016), suggesting that each mutation represents separate pathways for uterine leiomyoma development. The presence of *MED12* mutation is associated with smaller leiomyoma size, but no association has been observed with the patient's ethnicity or age at hysterectomy (Makinen *et al.* 2011a, Makinen *et al.* 2011b, McGuire *et al.* 2012).

Genome-wide association studies

A genome-wide association study (GWA study or GWAS) examines genetic variants at a genome-wide level with the aim of exploring associations between variants and disease traits. Uterine leiomyoma pathogenesis has been a subject of GWA studies. A recent study on Japanese women with clinically diagnosed leiomyomas resulted in identification of three genome-wide significant loci on chromosome regions 10q, 22q and 11p (Cha *et al.* 2011). The most significant association was for 10q24.33, mapped to the 5' region of *SLK*, which mediates apoptosis and actin stress fibre dissolution, and *OBFC1*, which takes part in DNA replication. At 22q13.1, the SNPs were mapped within a region encompassing *TNRC6B*, playing a role in RNA-mediated gene silencing. The third locus was at 11p15.5 near the telomeric end of the short arm of chromosome 11 (Cha *et al.* 2011). These loci were not identified in cohorts of white (Eggert *et al.* 2012), African-American (Wise *et al.* 2012) or European-American women (Edwards *et al.* 2013), suggesting divergent genetic variation in uterine leiomyomas in different ancestors. A novel leiomyoma risk allele for white women was identified in a genome-wide linkage and association study, showing evidence for *fatty acid synthase* (*FASN*) at chromosome region 17q25.3 as a candidate gene for uterine leiomyoma development. *FASN* encodes fatty acid synthesis (FAS), which is a multi-enzyme protein that catalyses de novo fatty acid synthesis. It has been suggested to serve as a metabolic oncogene, and indeed its up-regulation has been discovered in many

cancers (Flavin *et al.* 2010). Similarly to neoplasms, FAS levels were observed to be higher in uterine leiomyoma than in the surrounding myometrial tissue (Eggert *et al.* 2012).

Epigenetic changes

Epigenetics concerns heritable changes of chromatin structure and/or gene expression that do not involve changes in the underlying DNA sequence. According to a few recent studies epigenetics contributes to the pathogenesis of uterine leiomyoma. Results of genome-wide DNA methylation and mRNA profiling show altered gene expression of oestrogen receptor alpha (ERα) response genes and several genes that have consensus sequences of ER response elements (Hori *et al.* 2000, Asada *et al.* 2008, Maekawa *et al.* 2013). Another genome-wide study on African-American women revealed multiple tumour-suppressor genes showing differential promoter methylation with subsequent differences in mRNA expression in leiomyomas (Navarro *et al.* 2012). Before these results are replicated in other datasets with larger sample sizes, only careful conclusions can be drawn.

Histone modifications have been shown to be associated with diethylstilbestrol (DES) exposure in an Eker rat uterine leiomyoma model. After neonatal exposure these rats manifested permanent changes in myometrial gene expression throughout their adult lifetimes. Interestingly, several of the differentially expressed genes involved putative oestrogen-response elements (Greathouse *et al.* 2008).

Micro-RNAs (miRNAs) are deregulated in many biological pathways that have been associated with uterine leiomyoma development. Cell proliferation, apoptosis, cell adhesion, Wnt signalling, mitogen-activated protein kinase (MAPK) signalling, nuclear factor κB (NF-κB) activation and insulin signalling are deregulated in leiomyoma tissue compared with adjacent normal myometrium (Wang *et al.* 2007, Marsh *et al.* 2008, Zavadil *et al.* 2010, Georgieva *et al.* 2012). Interestingly, let-7, which is a target of deregulated miRNA, is up-regulated in leiomyomas and it targets HMGA2 protein, which in turn is encoded by *HMGA2* and it in turn is one of the leiomyoma driver genes, as discussed above. Down-regulation of miR-29b seems to have an association with excessive ECM formation, as restoration of miR-29b inhibited the accumulation of ECM in a leiomyoma xenograft model (Qiang *et al.* 2014). 17β-Estradiol and progesterone seem to regulate this interplay, as they down-regulate miR-29b and up-regulate mRNAs for multiple collagens in leiomyoma xenografts (Qiang *et al.* 2014).

2.3.4 Roles of oestrogen and progesterone

The widely accepted conception of the role of ovarian steroids in uterine leiomyoma growth stimulation is supported by observations that leiomyomas primarily occur in women during their reproductive years, and regress during menopause when ovarian steroid hormone production declines. This theory is further backed up by the actions of GnRH agonists, which disrupt ovarian oestrogen and progesterone production, resulting in leiomyoma shrinkage, which is then reversed when GnRH treatment is discontinued (West *et al.* 1987). Additionally, hormone replacement therapy (HRT) with oestrogen and progesterone has been shown to increase leiomyoma size in menopausal women (Palomba *et al.* 2001, Yang *et al.* 2002). Leiomyoma tissue is exposed to circulating oestrogen produced by ovarian steroidogenesis, but also to local conversion of androgens to oestrogens by aromatase activity in leiomyoma cells (Bulun *et al.* 1994). Cultured leiomyoma cells have been shown to produce oestrone after addition of androstenedione, and to further convert oestrone to estradiol by 17β-hydroxysteroid dehydrogenase (17β-HSD) (Sumitani *et al.* 2000). This was observed to result in similar cellular proliferation rates as after addition of estradiol, suggesting that uterine leiomyoma cells are capable of producing enough oestrogen to maintain their own growth. Proliferation was decreased when aromatase inhibitor was added to the cultured leiomyoma cells (Sumitani *et al.* 2000), offering further proof of aromatase being the key enzyme in mediation of *in situ* oestrogen production. Leiomyomas have been documented to have higher levels of aromatase and 17β-HSD type I when compared with surrounding myometrium, which is presumably related to the observed higher levels of oestrogens in leiomyoma tissue (Folkerd *et al.* 1984, Bulun *et al.* 1994, Sumitani *et al.* 2000, Shozu *et al.* 2004). Interestingly, aromatase transcripts are not found in the myometrium of leiomyoma-free uteri (Bulun *et al.* 1994). Further evidence of the pathological role of aromatase in leiomyoma formation is offered by a study reporting an increase in aromatase expression in leiomyomas of African-American women, who are well-documented to have larger and more numerous leiomyoma tumours (Bulun 2013).

The role of oestrogen in leiomyoma pathogenesis is characterized by its role as an up-regulator of the expression of several genes that take part in leiomyoma formation. These include the genes of growth factors, collagens, and most importantly, oestrogen and progesterone receptors (ERs, PRs) (Andersen *et al.* 1995, Li & McLachlan 2001, Maruo *et al.* 2004). The biologically active oestrogen oestradiol stimulates leiomyoma growth primarily through nuclear oestrogen

receptor α, but also β (ERα, ERβ), which then induce transcription of genes involved in cellular proliferation and ECM formation (Marsh & Bulun 2006). However, the principal function is up-regulation of progesterone receptor (PR) expression, thereby increasing leiomyoma responsiveness to progesterone signalling.

Progesterone and PR are essential for leiomyoma growth and development, as shown in clinical and experimental studies. The expression of two PR isoforms, PR-A and PR-B, is increased in leiomyoma tissue compared with neighbouring normal myometrium (Brandon *et al.* 1993, Englund *et al.* 1998, Nisolle *et al.* 1999). PR expression may be associated with genetic background, as PR mRNA levels have been observed to be higher in leiomyomas in Japanese women compared with African-American or Caucasian women (Ishikawa *et al.* 2009). Proliferation counts have been reported to peak in leiomyoma tissue during the luteal/secretory phase, when progesterone is dominant (Kawaguchi *et al.* 1989, Lamminen *et al.* 1992), again suggesting progesterone's key role in leiomyoma formation. This has been supported by clinical findings in postmenopausal women with combined HRT (oestrogen and progesterone), showing increased leiomyoma proliferative activity not observed with oestrogen alone (Lamminen *et al.* 1992). Light has been shed on the nature of oestrogen and progesterone interplay on leiomyoma formation by way of an *in vivo* human leiomyoma xenograft model that shows progesterone and PR to directly stimulate tumour growth, whereas the key action of oestrogen and ER was to maintain PR expression in leiomyoma tissue (Ishikawa *et al.* 2010). On the basis of these results, progesterone is suggested to be the primary hormone driving the growth of uterine leiomyomas. This model also showed that oestrogen and progesterone not only stimulated the cell proliferation rate, but also ECM formation. This was confirmed with co-treatment with the progesterone antagonist mifepristone (RU-486), as the ECM effect was abolished (Ishikawa *et al.* 2010).

Uterine leiomyoma growth may partly be explained by progesterone action through PR with induction of the anti-apoptotic protein Bcl-2. Progesterone induces PR binding to the progesterone response element (PRE) that lies upstream of the transcription site of Bcl-2. This leads to increased levels of Bcl-2, which then inhibit apoptosis and promote tumour growth (Yin *et al.* 2007).

2.3.5 ECM and its significance

The extracellular matrix (ECM) is a prominent component of uterine leiomyoma tissue. It is secreted in excessive amounts by fibroblasts, which are one of four key leiomyoma cell types. The ECM in leiomyomas is altered not only in content but also in structure, when compared with that in myometrium (Berto *et al.* 2003, Leppert *et al.* 2004). The altered secretion of ECM proteins such as interstitial collagens and glycosaminoglycans, along with cell proliferation, define leiomyoma-associated fibrosis. ECM collagen fibrils in leiomyomas are shorter, widely dispersed, disoriented and highly cross-linked, thus affecting stiffness. Furthermore, they modify mechanotransduction and biochemical cell signalling in leiomyomas (Catherino *et al.* 2004, Leppert *et al.* 2004). In fact, mechanical sensing is observed to be abnormal in leiomyoma cells. Mechanical stress-activated cellular signalling pathways involve mitogen-activated protein kinases (MAPKs) and the Rho signalling pathway, both of which are altered in leiomyoma tissue (Rogers *et al.* 2008).

Another characteristic feature of fibrosis is resistance to apoptosis. Mechanical stretch, i.e. as a consequence of excess ECM formation, modulates several cellular functions including apoptosis (Lehoux *et al.* 2006, Agha *et al.* 2011). In fact, uterine leiomyoma tissue has been documented to inhibit apoptosis, as Bcl-2 protein, an apoptosis-inhibiting gene product, has been observed to be increasingly expressed in leiomyoma tissue in comparison with normal myometrium. Furthermore, this was discovered to be associated with progesterone up-regulation (Maruo *et al.* 2000). IGF-I also contributes in inhibition of apoptosis in uterine leiomyomas, as the apoptosis-positive rate of leiomyoma cells treated with IGF-I was significantly decreased, while Bcl-2 protein expression was up-regulated (Gao *et al.* 2001). Another suggested mechanism for apoptosis inhibition in leiomyomas is alterations in the retinoid pathway (Catherino & Malik 2007, Zaitseva *et al.* 2008), and more specifically aldehyde dehydrogenase 1 (ALDH1) in leiomyoma fibroblasts (Zaitseva *et al.* 2007). The retinoic acid (RA) pathway controls a number of biological processes including apoptosis (Napoli 1996). Reduced intracellular levels of RA production lead to changes in cellular responses in leiomyoma cells and result in decreased apoptosis (Zaitseva *et al.* 2007).

Vitamin D plays a role in uterine leiomyoma ECM degradation. Previously it had been shown that $1,25(OH)_2D_3$ inhibits growth and induces apoptosis *in vitro* in human uterine leiomyoma cell culture (Blauer *et al.* 2009, Sharan *et al.* 2011). Additionally, $1,25(OH)_2D3$ treatment seems to shrink leiomyoma tumour size *in*

vivo in an animal model (Halder *et al.* 2012). Further studies have concerned the role of vitamin D3 on balance of the ECM degradation process. Matrix metalloproteinases (MMPs) contribute significantly to ECM degradation, synthesis and remodelling, which are in turn regulated by tissue inhibitors of matrix metalloproteinases (TIMPs) (Visse & Nagase 2003). Vitamin D3 has been observed to reduce the levels of MMP-2 and MMP-9 activity (Sharan *et al.* 2011, Halder *et al.* 2013), and it increased levels of vitamin D receptor (VDR) and TIMP-2 in a concentration-dependent manner. Additionally, uterine leiomyoma tissue has been documented to express low levels of VDR compared with adjacent normal myometrium (Halder *et al.* 2013). Accordingly, treatment with bioactive 1,25-dihydroxyvitamin D3 induced VDR in a concentration-dependent manner in human leiomyoma cells. It also reduced the protein expression of other ECM components such as ECM-associated collagen type 1, fibronectin and plasminogen activator-1. Moreover, vitamin D3 decreased the abnormal expression of structural smooth muscle fibres in human uterine leiomyoma cells (Halder *et al.* 2013).

2.4 Histopathology

Uterine leiomyoma by definition is a mesenchymal benign smooth-muscle tumour. Mesenchyme is a tissue type characterized by loosely associated cells that are surrounded by ECM. Uterine leiomyoma consist of spindle-shaped smooth-muscle cells arranged in disoriented fascicles, encircled by substantial ECM (Oliva E 2014).

Macroscopically, uterine leiomyomas are well circumscribed, but non-encapsulated. Their size varies widely and they are often multiple, spherical and firm. Some tumours however, if oedematous, highly cellular or epithelioid, are soft. Leiomyomas bulge from the surrounding myometrium and are easily enucleated. Their cut surface is usually white, but highly cellular and lipoleiomyomas can either focally or diffusely be tan to yellow in colour. Large tumours may be subject to infarction with haemorrhage, with these areas being dark red. Sharply demarcated yellow areas reflect necrosis. In oedematous or myxoid leiomyomas, cystic degeneration may be seen, and some become extensively calcified (Oliva E 2014).

Microscopically, uterine leiomyomas present as whorled patterns of smooth muscle cells separated by ECM. Most leiomyomas have well-demarcated borders. Muscle cells have indistinct borders and present with eosinophilic fibrillary cytoplasm. The nuclei are cigar-shaped with small nucleoli and mitoses are infrequent (Oliva E 2014). The ECM is structurally altered and abnormally formed,

compressing and stretching the muscle cells. It shows an accumulation of altered and increased collagen, fibronectin, and differing amounts of proteoglycan, which by definition is fibrosis (Malik *et al.* 2010, Leppert *et al.* 2014). This gives rise to clinicians' favoured term 'fibroids'.

Approximately 90% of uterine leiomyomas are conventional, but several variant types have been recognized with aberrant morphological features mimicking malignancy in one or more aspects. Cellular leiomyoma has significantly increased cellularity, with thick-walled vessels and cleft-like spaces. The borders are usually irregular and they merge with the surrounding myometrium, mimicking invasion. Leiomyomas with bizarre nuclei, formerly termed atypical leiomyomas, contain isolated atypical cells in the middle of an otherwise conventional leiomyoma. Rarely is this change extensive. Mitotically active leiomyomas have >10 mitoses per 10 high power fields (HPFs), but lack cytological atypia and tumour cell necrosis. They are usually submucosal and sometimes associated with hormonal therapy. These tumours might present with hypercellularity and focal bizarre nuclei, requiring attention to differentiate them from leiomyosarcoma. Hydropic leiomyomas are vascular and characterized by conspicuous zonal, watery oedema. Leiomyomas with apoplectic change are characterized by zones of haemorrhagic infarction surrounded by hypercellular areas. This is typically induced by progestational therapy. Lipoleiomyoma is characterized by single or multiple mature adipocytes within the otherwise conventional leiomyoma. Bone, cartilage, skeletal muscle, haematopoietic or lymphoid cells are other heterologous elements that can occasionally be found in leiomyomas. Epithelioid leiomyoma is composed of rounded or polygonal tumour cells with epithelial-like morphology, arranged in sheets, cords, trabeculae or nests. Myxoid leiomyoma is hypocellular, with smooth muscle cells separated by myxoid acid-mucin stroma. There is no cytological atypia and mitotic figures are infrequent. Cotyledonoid dissecting leiomyoma is characterized by irregular sections of bland smooth muscle cells within the myometrium. Intravenous leiomyomatosis is defined as the presence of benign smooth muscle within vascular spaces outside a uterine leiomyoma tumour. This is characterized by prominent vascularity and is commonly hydropic, but with infrequent mitotic figures. Diffuse leiomyomatosis is defined as multiple hypercellular tumour nodules that merge imperceptibly with each other and myometrial smooth muscle. Mitotic figures are infrequent. Metastasizing leiomyoma means conventional leiomyoma found in the lungs of a woman with a history of uterine leiomyomas and possibly hysterectomy in the past (Oliva E 2014). Hereditary leiomyomatosis and renal cell cancer (HLRCC)

syndrome-related uterine leiomyomas are often multiple and symptomatic at a young age. They have distinct morphology with increased cellularity, multinucleated cells, nuclei atypia and nuclei with large orangeophilic nucleoli surrounded by a perinucleolar halo (Garg *et al.* 2011, Sanz-Ortega *et al.* 2013).

Morphological evaluation for potential malignancy is required as part of histological uterine neoplasm diagnostics, even though uterine sarcomas are relatively rare (0.4 per 100,000 Nordic women and 3.6 per 100,000 white American women) (Brooks *et al.* 2004, Koivisto-Korander *et al.* 2012). The gross criteria for malignancy always include assessment of nuclear atypia, mitotic index and presence or absence of tumour cell necrosis (Toledo & Oliva 2008), but strict criteria vary according to different subsets of leiomyosarcoma. Smooth muscle neoplasms that lack cytological atypia and tumour cell necrosis, but are mitotically highly active (>20 mitoses per 10 HPFs) cannot reliably be distinguished as being benign or malignant (Toledo & Oliva 2008). These tumours should be diagnosed as smooth muscle tumours of uncertain malignant potential (STUMP) in order to avoid the drastic clinical implications of sarcoma (Oliva E 2014), but also to offer appropriate counselling and follow-up regarding the potential risk of recurrence as leiomyosarcoma (Dall'Asta *et al.* 2014).

According to current understanding, conventional uterine leiomyomas do not feature malignant potential. However, a continuum from benign leiomyomas to malignant leiomyosarcomas is suggested by studies showing similar X-chromosome inactivation patterns, identical *MED12* mutations and chromosomal aberrations in some leiomyomas and leiomyosarcomas in the same uterus (Zhang *et al.* 2006, Mittal *et al.* 2009, Matsubara *et al.* 2013). This supposedly applies to a small proportion of variant-type leiomyomas.

Immunohistochemistry

In uterine leiomyoma diagnostics, immunohistochemistry (IHC) has a role in differentiation of malignancy and diagnosis of leiomyoma variants. Endometrial stromal sarcomas and uterine leiomyosarcomas are the two most common uterine mesenchymal malignant tumours, therefore guiding uterine-lesion IHC to differentiation between uterine leiomyoma and these two sarcomas (Brooks *et al.* 2004, Abeler *et al.* 2009). The routine immunomarker panel used to distinguish endometrial stromal sarcomas from leiomyosarcomas and leiomyomas consists of oestrogen receptor (ER), progesterone receptor (PR), desmin, smooth muscle actin,

h-caldesmon and the cell surface enzyme CD10. Leiomyomas express ER, PR, desmin, smooth muscle actin and h-caldesmon. Low expression of CD10 distinguishes leiomyoma from endometrial stromal sarcomas, whereas leiomyosarcomas express lower levels of ER and PR (Hwang *et al.* 2015).

In uterine leiomyoma variant diagnostics CD10 can be used for identifying cellular leiomyomas, as it is expressed in up to 40% of highly cellular leiomyomas. The tumour suppressor p53 has a role in apoptosis, genomic stability and inhibition of angiogenesis. The p53 gene is the most frequently mutated gene in human malignancies, indicating crucial preventive functions in cancer formation (Surget *et al.* 2013). Leiomyomas with bizarre nuclei are positive for p53 (Sung *et al.* 2009). The protein p16 is another tumour suppressor and it plays a role in cell-cycle regulation. It has been found to be present in most leiomyomas with bizarre nuclei (Sung *et al.* 2009). In HLRCC-related uterine leiomyomas the vascular marker CD34 is highly expressed, indicating increased vascular density (Pollard *et al.* 2005a). Increased resistance to apoptosis has been detected as increased expression of Bcl-2, PCNA, (anti-apoptotic) Bcl-x and a decrease in (pro-apoptotic) Bak (Wortham *et al.* 2006).

3 Aims of the study

Uterine leiomyomas are the most common benign tumours in females and they cause significant morbidity. Even though the uterine leiomyoma study field has significantly advanced, particularly through next-generation genetic studies, leiomyoma treatment options are still limited and eventually many women end up requiring definitive treatment for their symptoms, i.e. hysterectomy. A comprehensive understanding of the underlying pathophysiology is lacking and studies aiming to identify different disease subtypes and to define typical characteristics and associated risk factors would enable implementation of tailored treatment plans for individual patients.

This study is focused on investigation of epidemiological and familial risk factors associated with uterine leiomyoma. The aim was to elaborate current knowledge of familial uterine leiomyomas and to investigate the associations between leiomyomas, cardiovascular diseases (CVDs) and the risk of endometriosis.

The specific aims of this study were:

1. To investigate the clinical characteristics of familial uterine leiomyoma.
2. To clarify the association between uterine leiomyomas and endometriosis.
3. To examine the clinical characteristics and to perform histopathological analysis of HLRCC uterine leiomyomas, in order to set up a management plan algorithm to improve detection of HLRCC patients.
4. To study the association between uterine leiomyomas and several known CVD risk factors.

4 Materials and methods

4.1 Subjects and materials

This study was approved by the Ethics Committee of Oulu University Hospital and the University of Oulu, the Ethics Committee of Northern Ostrobothnia Hospital District (Studies I, II, III and IV), Helsinki University Central Hospital (Study III) and the National Supervisory Authority for Welfare and Health in Finland (Study III).

4.1.1 Women with uterine leiomyomas (Studies I, II, IV)

A total of 192 uterine leiomyoma patients were recruited for Study I at Oulu University Hospital during 2001 (Table 2). The diagnoses were confirmed from hospital patient records based on WHO ICD disease codes for uterine leiomyomas (ICD-9: 218 and ICD-10: D25). Familial leiomyoma cases (27 women) were identified by self-reported knowledge on ≥2 first- or second-degree family members with diagnosed uterine leiomyomas. Women for whom this information was unavailable were not included in the study (88 women). The self-reported leiomyoma diagnoses among family members were validated by investigating hospital patient records in a random sample of 35 women from 10 families. Cases with a self-reported negative family history of uterine leiomyomas (77 women) were defined as non-familial.

Study II included a total 605 gynaecological surgical patients (aged 35 years or older) at Oulu University Hospital (Table 2). Cases were selected based on WHO ICD disease codes for uterine leiomyomas (ICD-9: 218 and ICD-10: D25), endometriosis (ICD-9: 617 and ICD-10: N80) or contraceptive management (ICD-9: V25 and ICD-10: Z30), and additionally with WHO ICD procedure codes for leiomyoma and endometriosis-related surgery, i.e. explorative laparoscopy (JAH01), laparoscopic excision of a peritoneal lesion (JAL), abdominal and laparoscopic hysterectomy (LCD) and sterilization (LGA). The hospital records of patients operated on for uterine leiomyoma or endometriosis (the study groups) or sterilization (the control group) were reviewed retrospectively. The diagnosis of uterine leiomyoma was based on preoperative transvaginal ultrasonographic examination, while the diagnosis of endometriosis was based on a pelvic view during surgery. Only patients aged 35 years or older at the time of surgery and those

with complete hospital records were included. The study subjects were divided into three groups according to age at surgery: 35 to 39 years, 40 to 44 years, and 45 years or older. The study design is clarified in Figure 2.

In Study IV the study population was derived from the prospective Northern Finland Birth Cohort 1966 (NFBC1966), which originally included 5,889 female children, all Caucasian (Table 2). 3,733 women attended the 46-year follow-up study, responded and returned the postal questionnaire, and 3,268 attended the clinical examination. Uterine leiomyoma cases were identified in the cohort through national outpatient and inpatient hospital discharge registers with data on disease diagnoses identified by WHO ICD codes. The national hospital discharge register includes ICD codes and dates for each hospital visit. Additionally, self-reported leiomyoma cases were identified through the postal questionnaire collected at the age of 46 via the question "Have you been diagnosed with uterine leiomyomas? If yes, at what age? If yes, was the diagnosis confirmed by gynaecological examination / ultrasonography / surgical operation (laparoscopy or laparotomy)?" Only cases with confirmation by either ultrasonography or surgical operation were recognized. Finally, the control group was formed from the rest of the cohort population (Figure 3).

In clinical health examinations measurements were taken of body weight, height, and waist and hip circumference. Body fat mass, fat percentage, muscle mass and visceral fat area were measured by using an InBody 720 bioelectrical impedance analyser (Biospace Co., Ltd., Seoul, Korea). Systolic and diastolic blood pressures were measured using an automated oscillometric blood pressure device and an appropriately sized cuff (Omron Digital Automatic Blood Pressure Monitor Model M10-IT, Japan).

4.1.2 Women with HLRCC (Study III)

All five Clinical Genetics Departments in Finland were contacted for Study III recruitment. A total of 20 women with HLRCC and known uterine leiomyoma participated in the study. The HLRCC diagnosis was confirmed from patient records on *FH* mutation testing results. The control group (77 women) arose from Study I (Table 2). It was formed from consecutive leiomyoma patients reviewed in Oulu University Hospital's Gynaecology Outpatient Clinic during the year 2001. Women were chosen for the control group based on their negative family history of uterine leiomyomas and renal cell cancer.

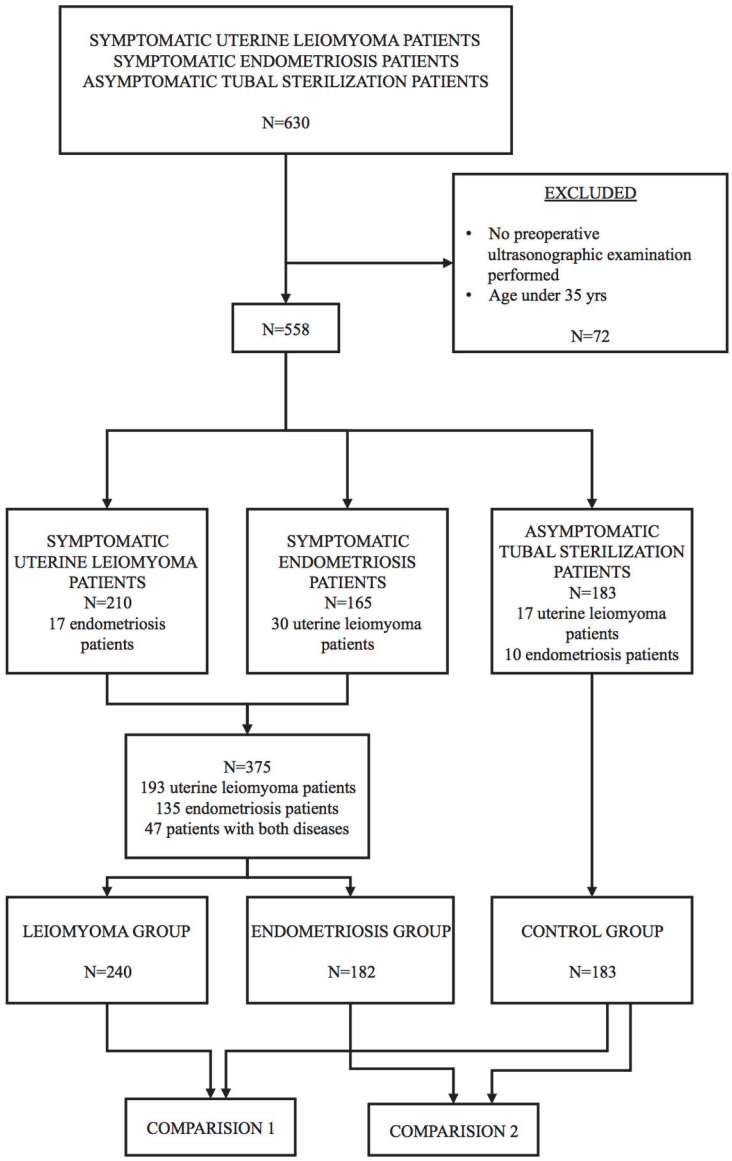

Fig. 2. Design of Study II.

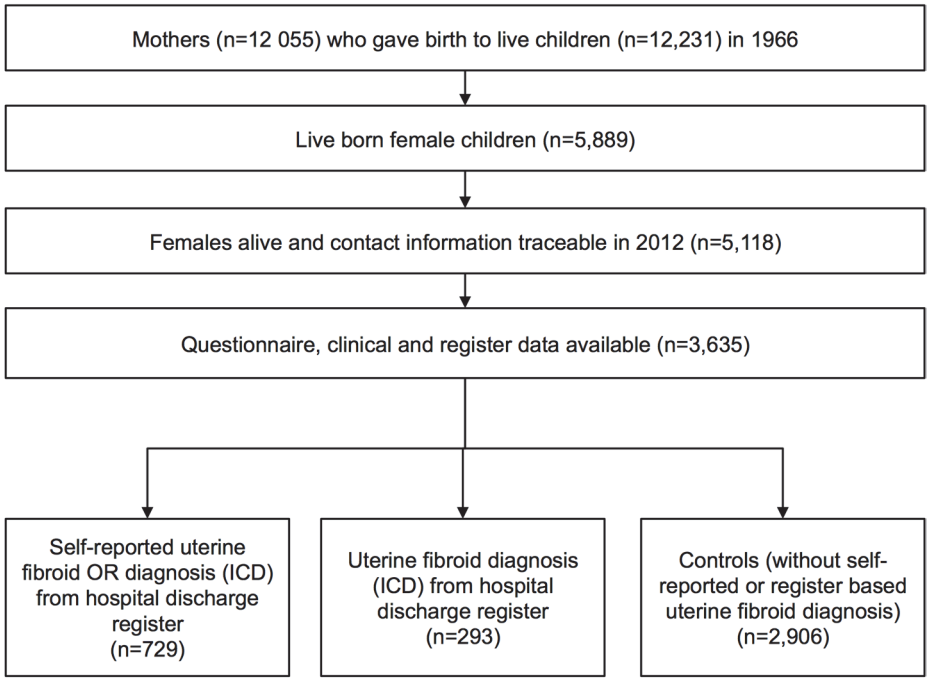

Fig. 3. Design of Study IV.

4.1.3 Uterine leiomyoma tissue samples (Study III)

For Study III, tissue samples were collected from those pathology departments in Finland where surgery took place for each patient. Haematoxylin/eosin (H&E)-stained microscope slides and formalin-fixed paraffin-embedded tissue blocks were obtained for the study. A total of 47 leiomyoma tissue samples from 20 HLRCC patients, and 24 tissue samples from 20 randomly selected control patients were included in the analyses.

4.2 Methods

4.2.1 Clinical characteristics analysis (Studies I, III)

Clinical characteristics were compared between the groups under study. The variables of interest were number of pregnancies and deliveries, infertility investigations, age at uterine leiomyoma diagnosis, leiomyoma-related symptoms, surgical treatment, age at surgery/hysterectomy, weight of the removed uterus, number of leiomyoma tumours and diameter of the largest tumour.

4.2.2 Association analysis (Study II)

To investigate the association between uterine leiomyoma and endometriosis, the prevalence of endometriosis was calculated among leiomyoma patients and compared with that of sterilization patients. The prevalence of leiomyoma was calculated among endometriosis patients and compared with that of sterilization patients. Further on, the prevalences were analysed in age groups of 35 to 39 years, 40 to 44 years, and 45 years or more to explore the prevalence trends in relation to aging.

4.2.3 FH mutation analysis (Study III)

Formalin-fixed paraffin-embedded (FFPE) uterine leiomyoma tissue blocks were obtained from pathology departments. Tissue-microarrays (TMAs) including all collected HLRCC-related and sporadic uterine leiomyoma samples were constructed prior to analysing the biallelic inactivation of *FH*. S-(2-succinyl) cysteine (2SC) IHC was used to assess the biallelic inactivation of *FH* (Kampjarvi *et al.* 2016). Samples displaying strong nuclear and cytoplasmic staining were scored as positive (+), indicating biallelic inactivation of *FH*, and samples showing no staining or only low cytoplasmic positivity in single cells were scored as negative (-).

4.2.4 Morphological and immunohistochemical analysis (Study III)

Haematoxylin/eosin (H&E)-stained uterine leiomyoma microscope slides were reviewed in 20 HLRCC cases, including 47 leiomyoma tumours in total and 20 sporadic cases, including 24 leiomyoma tumours in total. Histological evaluation

of cellularity, traditional nuclear atypia/multinucleate cells, prominent eosinophilic nucleoli with perinuclear halos, eosinophilic globules, hydropic degeneration, hyalinization and mitotic activity was carried out using the H&E slides. Eosinophilic nucleoli with perinuclear halos were considered to be present if features could be observed under ×20 objective scanning. Atypia/multinucleated cells, prominent eosinophilic nucleoli with perinuclear halos, eosinophilic globules, hydropic degeneration and hyalinization were reported as absent (0) or present (1). Cellularity was scored as normal (1) or high (2). Mitotic activity was calculated from 10 high-power fields (HPFs) of view in a hot-spot.

A set of routine IHC stainings was evaluated: CD34, Bcl-2 and p53. Microvessel density was defined as the number of CD34-positively stained vessels per HPF. Vessels were calculated from four HPFs in a hot-spot and average vessel density per HPF was reported. For Bcl-2, staining reactions were divided into four categories: 0, negative immunostaining; 1, weak immunostaining or <10% of cells showing positivity; 2, moderate immunostaining or 10–70% cells showing positivity; 3, strong immunostaining or >70% cells showing positivity. p53 results were categorised as 0, negative immunostaining (aberrant); 1, weak or moderate immunostaining (wild-type); 2, strong immunostaining (aberrant).

4.2.5 Anthropometric and cardiovascular measurements (Study IV)

All clinical health examinations for the NFBC 1966 46-year follow-up study took place and all measurements were taken at age 46 years. Body weight was measured with a digital scale. Height was measured twice (mean of the two measurements was used) by using a standard and calibrated stadiometer. Body mass index (BMI) was calculated as the ratio of weight to height squared (kg/m^2). Waist and hip circumferences were measured twice (mean of the two measurements was used) and the waist-hip ratio (WHR) was assessed as the ratio between circumferences of the waist (at the level midway between the lowest rib margin and the iliac crest) and the hip (at the widest trochanters). Body fat mass, fat percentage, muscle mass and visceral fat area were measured by using an InBody 720 bioelectrical impedance analyser. All measurements were done after an overnight (12 h) fasting period.

Systolic and diastolic blood pressures were measured three times with a 1-min interval after 15 min of rest on the right arm of seated participants using an automated oscillometric blood-pressure device and an appropriately sized cuff.

Finally, the mean of the two lowest systolic values and their diastolic values were used in the analyses.

4.2.6 Serum sampling and measurements of glucose and lipid metabolism and other biochemical markers (Study IV)

Serum samples were collected as part of the NFBC 1966 46-year follow-up study at the time of clinical health examinations. They were taken after an overnight fasting period.

For the two-hour oral glucose tolerance test (OGTT), both serum insulin and plasma glucose were measured at baseline and 30, 60 and 120 minutes after 75 g glucose intake. Glucose tolerance status was classified according to World Health Organization criteria: 1) normal glucose tolerance (NGT) was defined as having a fasting plasma glucose (FPG) level <6.1 mmol/l and a 2-hour glucose level <7.8 mmol/l, 2) impaired fasting glucose (IFG) as having an FPG level of 6.1–6.9 mmol/l and a 2-hour glucose level <7.8 mmol/l, 3) impaired glucose tolerance (IGT) as having an FPG level <7.0 mmol/l and a 2-hour glucose level of 7.8–11.0 mmol/l, and 4) screen-detected diabetes (scDM) as having an FPG level ≥7.0 mmol/l and/or a 2-hour glucose level ≥11.1 mmol/l. Exclusion criteria for the OGTT were medication for diabetes or a capillary blood glucose level >8.0 mmol/l just before the test. Previously known diabetes (prDM) was defined according to self-reported diagnoses and medication, hospital outpatient and inpatient registers and medication registers from the Social Insurance Institution of Finland.

Serum concentrations of total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, and triglycerides were determined using an enzymatic assay method. Serum samples for assay of testosterone (T) and sex hormone-binding globulin (SHBG) were collected.

4.2.7 Assessments of CVD risk, metabolic syndrome and fatty liver (Study IV)

Two cardiovascular disease risk assessment tools, the Framingham Risk Score and SCORE, were used to evaluate CVD risk. The Framingham Risk Score gives an estimate of the 10-year risk of developing coronary heart disease, cerebrovascular events, peripheral artery disease or heart failure. The risk-percentage result is based on the following factors: gender, age, smoking, total cholesterol, HDL-cholesterol, systolic blood pressure, requiring treatment for raised blood pressure, and diabetes.

When use of the Framingham risk assessment tool results in points ranging from -2 to ≥21, this refers to a risk percentage ranging from <1% to >30% (D'Agostino *et al.* 2008). SCORE gives an estimate of the 10-year risk of fatal cardiovascular disease on the basis of gender, age, smoking, total cholesterol and systolic blood pressure. Risk percentages range from <1% to ≥15% (Conroy *et al.* 2003).

Metabolic syndrome was assessed according to the International Diabetes Federation (IDF) Worldwide Definition (Alberti *et al.* 2006), which is a unified working diagnostic tool for metabolic syndrome. The tool, requiring "yes" or "no" responses, is based on central obesity measured by waist circumference (≥80 cm) and any two of the following: raised triglycerides (≥1.7 mmol/l or specific treatment for this lipid abnormality), reduced HDL (<1.29 mmol/l or specific treatment for this lipid abnormality), raised blood pressure (systolic ≥130 mmHg or diastolic ≥85 mmHg or treatment of previously diagnosed hypertension), raised fasting plasma glucose (≥5.6 mmol/l or previously diagnosed type 2 diabetes).

The fatty liver index (FLI) was assessed by using an algorithm based on BMI, central obesity measured by waist circumference, and triglyceride and gamma-glutamyl-transferase (GGT) levels (Bedogni *et al.* 2006). The FLI varies between 0 and 100, with cut-offs at 30 and 60; scores of <30 rule out fatty liver and a score of ≥60 is considered to be a strong predictor of fatty liver.

4.2.8 Statistical analysis

The statistical analyses were performed using IBM SPSS Statistics software (Studies I, II, III, IV) and the free software package R (Study IV).

The statistical significance of differences in patient phenotype characteristics between the studied groups (Studies I, II and III) were evaluated with Student's t-test for continuous variables and the Chi-square test or Fisher's test for categorial variables. Comparisons of disease prevalences in Study II were performed using the Chi-square test. Logistic regression analysis was used to explore associations between subfertility, uterine leiomyoma and endometriosis. Subfertility was determined as nulliparity (yes/no). Uterine leiomyoma and endometriosis were included in the statistical model as two independent variables. The study and control groups did not differ as regards patients' age and therefore no adjustments were applied in the analysis. The results were reported as odds ratios with 95% confidence intervals (CIs).

Table 2. Summary of patients, materials, study settings and main outcome measures of the studies.

Study	Subjects & materials	Study setting	Main outcome measures
I	27 subjects with familial UL 77 subjects with non-familial UL	Retrospective case-control study	UL-related symptoms Age at diagnosis Surgical treatment for UL Age at hysterectomy Pregnancies, deliveries Infertility Details of UL: uterine weight, tumour number and location of largest tumour
II	422 subjects with UL and/or endometriosis 183 controls	Retrospective case-control study	Age at surgery Pregnancies, deliveries BMI Diagnosis of UL Diagnosis of endometriosis
III	20 subjects with HLRCC and UL 77 controls with sporadic UL	Case-control study	Age at diagnosis Symptoms Details of UL: uterine weight, tumour number, diameter of largest tumour Age at surgery Pregnancies, deliveries Other tumours Histological morphology IHC: CD34, p53, Bcl-2

Study	Subjects & materials	Study setting	Main outcome measures
IV	729 subjects with UL 2906 controls	Cross-sectional population-based birth cohort study	Parity Menopausal status BMI Education Socioeconomic status Physical activity Cigarette smoking Alcohol usage Body size: waist and hip circumference, waist-hip ratio, fat percentage, fat mass, skeletal muscle mass, visceral fat area Glucose metabolism: OGTT (insulin and glucose levels), Lipid metabolism: total cholesterol, HDL, LDL, triglycerides Blood pressure (systolic and diastolic) Metabolic syndrome Cardiovascular risk scores: Framingham CVD risk score, SCORE Testosterone SHBG Fatty liver index

UL, uterine leiomyoma; BMI, body mass index; HLRCC, hereditary leiomyomatosis and renal cell cancer; IHC, immunohistochemistry; OGTT, oral glucose tolerance test; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CVD, cardiovascular disease; SCORE, Systematic Coronary Risk Evaluation; SHBG, sex hormone-binding globulin

To compare differences in morphology and protein expression in IHC between healthy women and subjects with HLRCC (Study III), the independent samples t-test was used for normally distributed data, and the Mann–Whitney U-test for skewed data. Pearson's Chi-Square and Fisher's Exact tests were used for categorial data. The observed differences among the studied groups were analysed to investigate their significance in the constructed screening algorithm for individuals for further mutation testing. Specificity, sensitivity, negative predictive value and likelihood ratios (with 95% CIs) were calculated by using contingency tables.

In Study IV the Chi-square test and the Mann–Whitney U-test were used to study associations between healthy women and subjects with uterine leiomyoma with cardiovascular risk factors. Log-transformation was used to normalize the skewness of the distributions of continuous variables in multivariate analyses. Logistic regression analysis was used to examine associations between healthy women and subjects with uterine leiomyoma with known cardiovascular risk factors. Parity, education, BMI and current use of exogenous hormones were used as potential confounding factors in the analyses. The statistical significance limit was set at (two-sided P-value) <0.05 in all studies.

5 Results and discussion

5.1 Clinical characteristics of familial and HLRCC uterine leiomyomas (Studies I, III)

The heritability of uterine leiomyoma has been implicated by the results of studies carried out to investigate leiomyoma frequency among monozygotic and dizygotic twin pairs (Snieder *et al.* 1998), different races (Meilahn *et al.* 1989, Kjerulff *et al.* 1993, Marshall *et al.* 1997), and first-degree relatives in families with multiple leiomyoma cases (Vikhlyaeva *et al.* 1995, Sato *et al.* 2002). However, little data is available on the natural history of familial leiomyoma, with only a few studies exploring clinical characteristics in leiomyoma cases with a heritable component. To clarify these issues further, fertility characteristics, leiomyoma-related symptomatology, treatment and tumour details were analysed in familial uterine leiomyoma cases and HLRCC leiomyoma cases in Studies I and III.

Study I concerned women with a positive family history, with a minimum of three leiomyoma cases being diagnosed at a relatively young age and the women more often being symptomatic. They required surgical treatment more often and more commonly had more than four leiomyoma tumours (Table 3). They did not differ from the sporadic leiomyoma cases as regards parity.

The results of Study III demonstrated a similar pattern: young age at diagnosis with a high frequency rate of symptoms. All women required surgical treatment at a mean age of less than 40 years. Most women had more than four leiomyoma tumours and they tended to be large in size (Table 3).

Previous studies have shown that women with a positive family history of uterine leiomyomas are more likely to have surgical treatment for their leiomyomas (Vikhlyaeva *et al.* 1995, Snieder *et al.* 1998). A similar result has been observed among black women (Meilahn *et al.* 1989). Additionally, the clinical picture seems to be more severe among black women, with younger age at diagnosis, greater uterine weight, more leiomyoma tumours, and the women more often being anaemic and experiencing pelvic pain (Kjerulff *et al.* 1996).

Clinical characteristics of uterine leiomyomas in women with HLRCC seem to follow the clinical picture of familial leiomyoma, with a deteriorating pattern. According to the results of previous studies, women with HLRCC are diagnosed at a young age (mean 30 and 31 years) (Toro *et al.* 2003, Alam *et al.* 2005), most being symptomatic (Toro *et al.* 2003, Alam *et al.* 2005, Sanz-Ortega *et al.* 2013).

Their tumours appear to be multiple and large in size (Toro *et al.* 2003). The severity of clinical characteristics is further underlined by the high frequencies of myomectomy and hysterectomy (Alam *et al.* 2005, Stewart *et al.* 2008).

Table 3. Clinical characteristics in cases of familial uterine leiomyoma and HLRCC .

Characteristics	Familial cases (N=27)	HLRCC cases (N=20)	Non-familial cases (N=77)	P
Pregnancies, mean (SD)	2.1 (1.5)	2.4 (1.5)	2.0 (1.5)	0.66/0.31
Deliveries, mean (SD)	1.7 (1.2)	2.0 (1.3)	1.7 (1.4)	0.77/0.33
Age at diagnosis (years), mean (SD)	41.1 (8.9)	33.8 (8.0)	45.4 (7.9)	0.02/<0.0001
Symptoms	88.9%	95.0%	6.5%	<0.001/<0.0001
Surgical treatment	85.2%	100.0%	68.0%	<0.001/0.004
Age at surgery (years) mean (SD)	45.4 (7.9)	37.3 (6.4)	48.3 (4.8)	0.12/<0.0001
Uterine weight (g), median (SD)	380.0 (321.0)	437.5 (315.2)	367.0 (328.1)	0.80/0.60
Number of leiomyoma tumours				0.01/ <0.0001
1	20.0%	5.6%	43.1%	
2–4	10.0%	5.6%	26.2%	
>4	70.0%	88.9%	30.8%	
Diameter of largest leiomyoma tumour (mm), median (SD)	50.0 (26.3)	65.0 (24.4)	50.0 (31.7)	0.77/0.08

HLRCC, hereditary leiomyomatosis and renal cell cancer; SD, standard deviation; g, gram; mm, millimetre

5.2 Histopathological features of HLRCC uterine leiomyomas (Study III)

HLRCC-related uterine leiomyomas have been found to have distinct tumour morphology: 1) increased cellularity, 2) nuclear atypia/multinucleate cells and 3) prominent eosinophilic nucleoli with perinuclear halos (Garg *et al.* 2011, Sanz-Ortega *et al.* 2013). In Study III the large dataset of HLRCC uterine leiomyomas was carefully analysed for its morphology and compared with sporadic leiomyomas (Table 4). HLRCC leiomyomas showed high frequencies of nuclear atypia and prominent eosinophilic nucleoli, and as a novel finding the absence of hyalinization as a distinct feature. The results did not confirm increased cell density as a typical characteristic of HLRCC-related leiomyomas (Table 4).

Table 4. Morphological features of HLRCC-related uterine leiomyomas.

Morphological feature	HLRCC leiomyomas (N=47)	Sporadic leiomyomas (N=24)	P
Cellularity			
Normal	42 (89.4%)	22 (91.7%)	
High	5 (10.6%)	2 (8.3%)	0.76
Mitotic activity, mean (SD)	0.72 (1.2)	0.54 (1.0)	0.53
Nuclear atypia/multinucleate cells	13 (27.7%)	0	0.003
Prominent eosinophilic nucleoli with perinuclear halos	17 (39.5%)	1 (4.3%)	0.003
Hyalinization	1 (2.1%)	7 (29.2%)	0.002
HydropicDegeneration and sclerosis	25 (53.2%)	12 (50.0%)	0.81
Eosinophilic globulus	21 (48.8%)	0	<0.0001
Necrosis	0	1 (4.2%)	0.34

HLRCC, hereditary leiomyomatosis and renal cell cancer; SD, standard deviation

The reproducibility of the proposed morphological criteria in identification of uterine leiomyomas in HLRCC has been assessed in a blinded control-cohort study setting and it was concluded that the criteria are largely irreproducible among pathologists and lack sufficient robustness to serve as a tool to select cases for further *FH* mutation testing (Alsolami *et al.* 2014). Therefore, other histological features are needed to distinguish these tumours from sporadic ones.

Limited data on the immunophenotypes of HLRCC-related uterine leiomyomas has been published. Vascular density was evaluated in a total of 47 HLRCC and 24 sporadic uterine leiomyoma tumours in Study III by vascular CD34 staining. The results showed a considerable difference between HLRCC and sporadic uterine leiomyomas, microvessel density being 111.0/HPF vs. 43.0/HPF (Figure 4). In a previous study on 14 HLRCC uterine leiomyomas microvessel density was observed to be higher in the leiomyomas than in the surrounding myometrium, whereas sporadic leiomyomas were less vascular compared with their surrounding myometrium (Pollard *et al.* 2005a).

The antiapoptotic protein Bcl-2 was investigated in Study III. There seems to be increased resistance to apoptosis in HLRCC uterine leiomyomas, as more than 45% of these tumours showed strong staining for Bcl-2 (>70% cells showing positivity) (Figure 4). All HLRCC and sporadic leiomyoma specimens displayed weak or moderate immunostaining (wild-type) for p53.

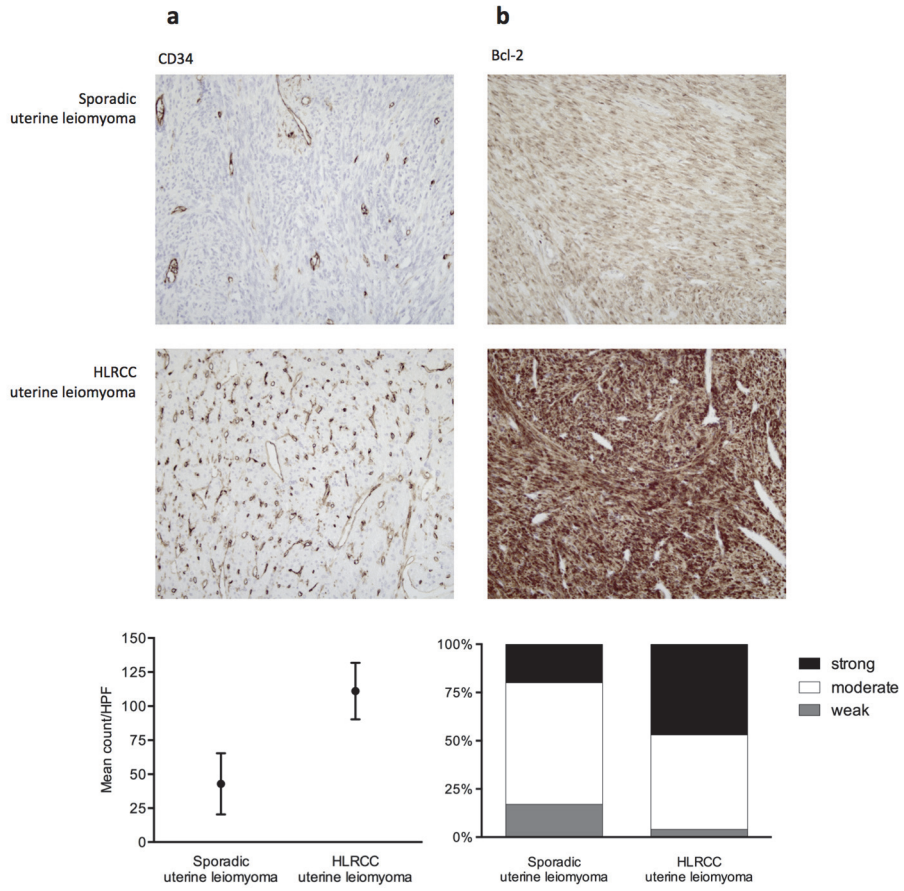

Fig. 4. Results of immunohistochemical staining of sporadic and HLRCC uterine leiomyomas. (a) Microvessel density expressed through CD34 antibody staining resulted in a higher mean count per high-power field (HPF) in HLRCC uterine leiomyomas when compared with sporadic uterine leiomyomas (111.0, SD 20.8 vs. 43.0, SD 22.5, P<0.0001). (b) Staining of the antiapoptotic protein Bcl-2 resulted in a differing proportion profile in HLRCC leiomyomas, with more frequent antiapoptotic cell positivity vs. sporadic leiomyomas (4.3%/48.9%/46.8% vs. 16.7%/62.5%/20.8%, P=0.04). Microscopic images are presented at ×20 magnification.

Significant differences in the expression levels of several other apoptotic proteins (Bcl-2, PCNA, Bcl-x and Bak) were reported in a previous study (Wortham *et al.* 2006). The results suggest stronger signals for survival and against apoptosis in HLRCC leiomyomas. A possible mechanism for reduced apoptosis may involve fumarate accumulation, which leads to activation of antioxidant response pathways where KEAP1 succination plays a role (Adam *et al.* 2011). Bcl-2 is a substrate of KEAP1 and it is dissociated from KEAP1 in tissues undergoing oxidative stress, resulting in an increase in Bcl-2:Bax heterodimers, further reducing apoptosis and enhancing cell survival (Tian *et al.* 2012).

A careful treatment plan should be applied for women diagnosed with HLRCC uterine leiomyomas. Study III showed that HLRCC-related leiomyomas have increased microvessel density. Uterine artery embolisation (Stewart 2015) may not reduce leiomyoma-related symptoms among women with HLRCC as successfully as in those with sporadic leiomyomas. Additionally, ulipristal acetate may also not be the choice of treatment as it induces apoptosis by decreasing Bcl-2 expression (Croxtall 2012), which was shown to be higher in HLRCC leiomyomas. However, these associations await verification before further conclusions and treatment guidelines can be presented.

An accurate screening method applicable to population-level routine clinical practice has been lacking as regards the diagnosis of HLRCC. Currently, patients are referred for genetic counselling on the basis of clinicians' alertness and awareness of the syndrome. Analysis of the large dataset in Study III enabled us to construct a screening algorithm to detect individuals for further mutation testing in order to improve diagnostic accuracy in cases of suspected HLRCC. The studied variables were assessed as regards specificity, sensitivity and evaluation of their significances and to set critical limits for the screening algorithm. The sensitivity of the first-step criteria (diagnosis at age <35 years, or multiple leiomyoma tumours, or surgery at age <40 years, or a family history of leiomyomas) was 100.0% (95% CI 80.0 to 100.0), with a 100.0% negative predictive value (95% CI 91.1 to 100.0). The positive likelihood ratio was 2.85 (95% CI 2.10 to 3.86). When microvessel density was set at a CD34 count of >70/HPF, the sensitivity was 100.0% (95% CI 90.6% to 100.0%), the negative predictive value 100.0% (95% CI 80.8 to 100.0) and the positive likelihood ratio 8.0 (95% CI 2.78 to 23.1). Strong Bcl-2 positivity gave 46.8% sensitivity (95% CI 32.4% to 61.8%), a negative predictive value of 43.2% (95% CI 28.7% to 58.9%) and a positive likelihood ratio of 2.25 (95% CI 0.97 to 5.19). The suggested management plan was based on these results (Figure 5) with the aim of detecting those women who are likely to carry the *FH* mutation.

According to the results of Study III, it is suggested that patients requiring surgical treatment for their uterine leiomyomas and who fulfil the clinical characteristics should undergo IHC analyses. If the IHC results give further support for HLRCC, referral for genetic counselling and *FH* mutation testing is advised in order to identify other affected family members. This would permit earlier diagnoses of uterine and renal tumours, thus improving the prognosis of the impact these tumours have on health.

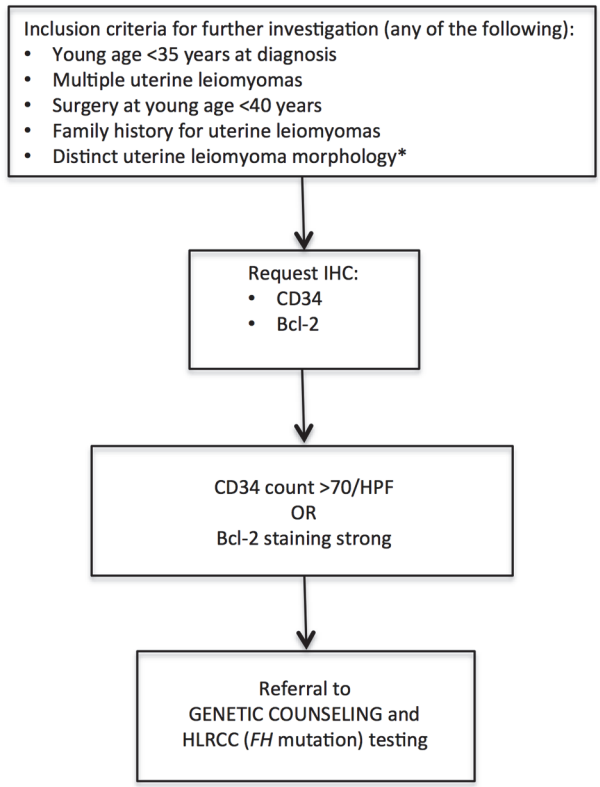

Fig. 5. Suggested management plan for diagnosing HLRCC among women with uterine leiomyoma requiring surgical treatment. *Increased cellularity, nuclear atypia/multinucleate cells, prominent eosinophilic nuceoli with perinuclear halo, or absence of hyalinization.

5.3 Uterine leiomyoma and CVD risk (Study IV)

The first indications of possible underlying atherosclerotic mechanisms in uterine leiomyoma development arose from studies performed in the 1970s, when leiomyoma tissue and atherosclerotic plaque were recognised to have similarities in growth behaviour, as they both become fibrotic and calcified (Moss & Benditt 1975). Further suggestions of atherosclerotic mechanisms arose from the observation of lipid accumulation in myometrial smooth muscle cells in women with pregnancy-related hypertension (Haust *et al.* 1977). Vascular endothelial and myometrial smooth muscle cells seem to react similarly to injury and promote monoclonal expansion of smooth muscle cells in the uterine wall (Cramer *et al.* 1995). A monoclonal theory of origin is another shared similarity of these two phenomena (Benditt & Benditt 1973, Mashal *et al.* 1994, Hashimoto *et al.* 1995, Andreassi *et al.* 2000).

Comprehensive metabolic and cardiovascular risk profiles and their association with uterine leiomyomas were explored in Study IV using the Northern Finland Birth Cohort 1966 (NFBC 1966). The study involved the use of extensive clinical health examination data collected from women at the age of 46 years linked with national hospital discharge register data.

A total of 729 uterine leiomyoma cases were identified in the NFBC1966 by 2012 for this study, of which 293 cases were identified through WHO ICD disease codes for uterine leiomyomas. The rest of the cohort population formed the control group (n=2,906) (Figure 3). Figure 6 presents the overall ICD-code-based uterine leiomyoma incidence in the cohort. This includes all women regardless of their participation in postal questionnaires or clinical examinations. The cumulative incidence was 7.7%. The number of newly detected cases started to increase after the age of 41 and by the age of 46 there was a total of 395 ICD-code-identified leiomyoma cases in the cohort. The mean age at leiomyoma diagnosis was 37.3 years (median 40.0, SD 7.1, range 13–47) and for the ICD-code-confirmed leiomyoma cases, 40.2 years (median 42 years, SD 5.3, range 23–46).

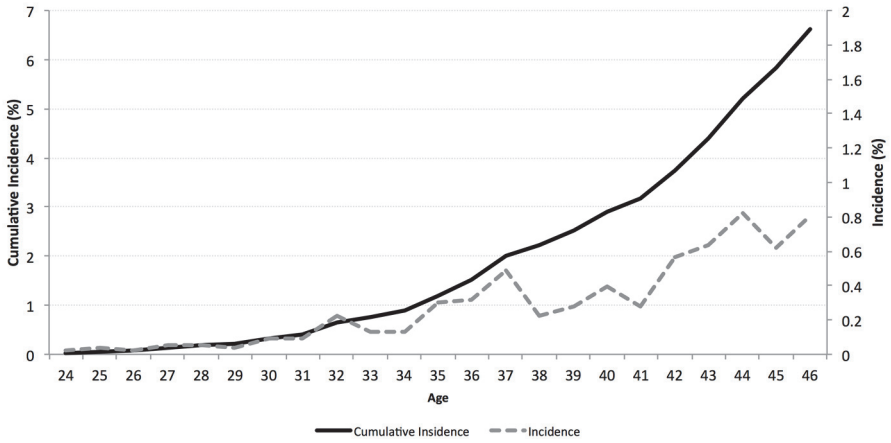

Fig. 6. Uterine leiomyoma incidence in the NFBC1966 in Study IV. Cases were identified through the national hospital discharge register based on WHO ICD codes for uterine leiomyomas (ICD-9: 218 and ICD-10: D25, IVD-8 converted to ICD-9). NFBC1966, Northern Finland Birth Cohort 1966; WHO, World Health Organization; ICD, International Classification of Diseases.

Women with uterine leiomyomas had significantly lower parity than women without leiomyomas (mean 1.8 SD 1.7 vs. 2.2 SD 1.8, P<0.001). There were significant differences in body size, as women with leiomyomas were more frequently overweight or obese (35.4% and 21.8% vs. 31.6% and 21.4%, P=0.04). There were no differences in lifetime use of exogenous hormones, but current use differed as regards hormone replacement therapy in all cases of leiomyoma (3.8% vs. 2.4%, P=0.04). Women with leiomyomas had a lower lifetime education level (basic-only 3.8% and tertiary 39.8% vs. 2.0% and 41.6%, P=0.012), but they did not differ in their socioeconomic status when compared with women without leiomyomas. These results defined the adjustment models for parity, education level, BMI and current use of exogenous hormones as applied in the logistic regression analyses for several known cardiovascular disease risk factors.

Table 5. Association between CVD risk factors and uterine leiomyoma at age 46 years, adjusted odds ratio model from the logistic regression analysis.

CVD risk factor variable	All UL cases (N=729)		ICD-code-confirmed UL cases (N=293)	
	Adjusted odds ratio (95% CI)	P	Adjusted odds ratio (95% CI)	P
Waist circumference, cm	1.02 (1.00,1.04)	0.02	1.03 (1.01,1.06)	0.01
Hip circumference, cm	0.99 (0.97,1.01)	0.48	1.01 (0.98,1.05)	0.36
Waist-hip ratio	1.32 (1.10,1.57)	0.003	1.31 (1.02,1.68)	0.04
Body composition				
Fat percentage, %	1.01 (0.99,1.04)	0.23	1.03 (1.00,1.07)	0.06
Fat mass, kg	0.99 (0.96,1.02)	0.39	1.02 (0.98,1.07)	0.32
Skeletal muscle mass, kg	1.00 (0.97,1.03)	0.79	1.00 (0.95,1.04)	0.82
Visceral fat area, cm^2	1.00 (1.00,1.01)	0.29	1.01 (1.00,1.02)	0.07
Insulin levels in OGTT, mU/l				
Fasting	1.00 (0.99,1.02)	0.71	1.00 (0.99,1.02)	0.71
30 min*	1.02 (0.99,1.04)	0.18	1.01 (0.98,1.04)	0.43
60 min*	1.01 (1.00,1.03)	0.11	1.02 (1.00,1.05)	0.07
120 min*	1.01 (0.99,1.03)	0.41	1.01 (0.99,1.04)	0.33
Insulin AUC	1.001 (1.000,1.002)	0.21	1.001 (0.999,1.003)	0.17
Glucose levels in OGTT, mmol/l				
Fasting	1.04 (0.88,1.21)	0.65	1.00 (0.78,1.24)	0.99
30 min*	1.05 (0.98,1.12)	0.17	1.05 (0.98,1.12)	0.17
60 min*	1.02 (0.97,1.07)	0.39	1.02 (0.97,1.07)	0.39
120 min*	1.03 (0.96,1.10)	0.38	1.03 (0.96,1.10)	0.38
Glucose AUC	1.018 (0.983,1.055)	0.32	1.024 (0.972,1.077)	0.37
Glucose tolerance status, %				
NGT <6.1 mmol/l	reference		reference	
IFG 6.1–6.9 mmol/l	1.14 (0.69,1.83)	0.60	1.81 (0.98,3.14)	0.045
IGT ≥7.0 mmol/l	1.22 (0.86,1.71)	0.25	1.22 (0.73,1.95)	0.42
ScDM	1.03 (0.52,1.90)	0.93	0.65 (0.19,1.68)	0.42
PrevDM	1.01 (0.54,1.76)	0.99	0.67 (0.22,1.60)	0.41
Serum lipids, mmol/l				
Total cholesterol	1.10 (1.00,1.22)	0.08	1.21 (1.05,1.41)	0.01
HDL	0.83 (0.64,1.06)	0.14	0.81 (0.55,1.16)	0.26
LDL	1.13 (1.02,1.26)	0.02	1.22 (1.05,1.42)	0.01
Triglycerides	1.27 (1.09,1.49)	0.003	1.37 (1.11,1.68)	0.004

CVD risk factor variable	All UL cases (N=729)		ICD-code-confirmed UL cases (N=293)	
	Adjusted odds ratio (95% CI)	P	Adjusted odds ratio (95% CI)	P
Blood pressure, mmHg				
Systolic mean**	1.01 (0.95,1.08)	0.71	1.03 (0.94,1.13)	0.48
Diastolic mean**	0.97 (0.88,1.06)	0.50	1.00 (0.87,1.14)	0.95
>140/90, %	0.92 (0.72,1.18)	0.53	1.00 (0.69,1.42)	1.00
>140/90 medicated, %	1.11 (0.88,1.40)	0.39	1.07 (0.75,1.50)	0.71
Metabolic syndrome (IDF), %	1.22 (0.98,1.51)	0.08	1.48 (1.09,2.01)	0.01
Cardiovascular risk scores				
Framingham CVD risk score	1.00 (0.96,1.04)	0.91	0.99 (0.94,1.05)	0.79
SCORE, %	0.95 (0.49,1.77)	0.86	1.16 (0.46,2.68)	0.74
Serum total testosterone, nmol/l	0.87 (0.68,1.08)	0.25	0.60 (0.40,0.89)	0.01
Serum SHBG, nmol/l	1.00 (0.99,1.00)	0.03	1.00 (1.00,1.00)	0.52
Fatty liver index	0.99 (0.98,1.01)	0.21	0.99 (0.97,1.01)	0.36

*Odds ratios (with 95% CIs) were calculated per 10 unit change.

**Odds ratios (with 95% CIs) were calculated per 10mmHg change.

UL, uterine leiomyoma; CVD, cardiovascular disease; CI, confidence interval; cm, centimetre; kg, kilogram; cm^2, square centimetre; OGTT, oral glucose tolerance test; mU/l, milliunits per litre; min, minute; AUC, area under the curve; mmol/l, millimoles per litre; NGT, normal glucose tolerance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; ScDM, screen-detected diabetes mellitus; PrevDM, previously known diabetes mellitus; HDL, high-density lipoprotein; LDL, low-density lipoprotein; mmHg, millimetres of mercury; IDF, International Diabetes Federation; SCORE, Systematic Coronary Risk Evaluation; nmol/l, nanomoles per litre; SHBG, sex hormone-binding globulin

Anthropometrics

Body fat distribution was investigated in Study IV by analysing the associations between middle body measurements and body composition, and uterine leiomyomas. The risk of prevalent uterine leiomyoma rose significantly for every 1 cm increase in waist circumference (OR=1.02, 95% CI 1.00 to 1.04 P=0.02) (Table 5). Also, every unit increase in WHR was associated with leiomyomas (OR=1.32, 95% CI 1.10 to 1.57 P=0.003) (Table 5). Other adiposity traits (visceral fat, gynaecoid-pattern fat accumulation), examined through body composition using bioelectrical impedance analysis, did not show associations. The results for ICD-code-confirmed cases were congruent (Table 5).

In previous studies body size has been determined by calculating BMI and the results are fairly consistent: data arising from the NHS II study, with 2,967 identified leiomyoma cases, showed an increased risk of leiomyomas with increasing adult BMI (Marshall *et al.* 1998b). Furthermore, central obesity as measured by WHR has been associated with an increased risk of leiomyomas (Sato, et al., 1998, Wise, et al., 2005).

Glucose metabolism

In the present study we analysed the association between uterine leiomyomas and glucose metabolism with a large set of glucose metabolism tests and indices, again at the same age for all cohort participants. The 2-hour OGTT results suggested a positive association between glucose metabolism and uterine leiomyoma risk. This was shown in insulin levels at 60 minutes when adjusting for parity and education (all uterine leiomyoma cases: OR=1.02, 95% CI 1.00 to 1.04, P=0.03) and in glucose levels at 30 minutes (ICD-code-confirmed cases: OR=1.13, 95% CI 1.03 to 1.24, P=0.01) (Table 5). When adjusting for BMI and current use of exogenous hormones, the association was no longer present. Women with ICD-code-confirmed leiomyomas showed an association with impaired fasting glycaemia (IFG), as their glucose tolerance status revealed IFG in the full adjustment model (OR=1.81, 95% CI 0.98 to 3.14, P=0.045) (Table 5). This reflects constant elevation of fasting plasma glucose levels. It can progress to more severe forms of glucose intolerance and further on to diabetes, and is thus considered as a pre-diabetic state (Nichols *et al.* 2007). Additionally, the ICD-code-confirmed leiomyoma cases showed an association with IDF-defined metabolic syndrome, with clustering of several metabolic traits and inferring adverse cardiovascular events, which is the main adverse outcome of metabolic syndrome (Mottillo *et al.* 2010, DeFronzo & Abdul-Ghani 2011).

A differing result has been reported in connection with a large cohort named The Californian Teachers Study. Women with a history of diabetes were concluded to have a lower risk of surgically treated uterine leiomyomas (Templeman *et al.* 2009). This study differs from Study IV as regards several factors that have an impact on the results. Women recruited for the study had a wide age range, they were 22 to 80 years old, and they represented multiple ethnicities. The study was aimed at exploring risk factors of leiomyomas and the role of diabetes at baseline was analysed in connection with symptomatic leiomyomas over a follow-up period. As the women were not screened for uterine leiomyomas at baseline, the study

result may indicate that diabetes can cause growth-rate reduction in leiomyomas and therefore these women were at a lower risk of surgical treatment. Study IV shows an association between leiomyomas and early signs of impaired glucose metabolism in a cross-sectional design, and hence the results from Study IV and those from The Californian Teachers Study are not fully in contradiction.

Lipid metabolism

Lipid metabolism in women with uterine leiomyomas has not been thoroughly investigated and thus final conclusions cannot be drawn. In the present study lipid levels were assessed at the same age for all cohort participants, showing a positive association between LDL and triglycerides and risk of leiomyomas. For every 1 mmol/l increase in LDL and triglycerides the risk of prevalent leiomyomas rose significantly (OR=1.13, 95% CI 1.02 to 1.26, P=0.02 and OR=1.27, 95% CI 1.09 to 1.49, P=0.003) (Table 5). The associations were stronger for hospital-discharge-defined leiomyoma cases (OR=1.22, 95% CI 1.05 to 1.42, P=0.01 and OR=1.37, 95% CI 1.11 to 1.68, P=0.004). Additionally, in these cases, every 1 mmol/l increase in total cholesterol was associated with leiomyomas (OR=1.21 95% CI 1.05 to 1.41 P=0.01). These associations were not altered when the model was additionally adjusted for polycystic ovary syndrome.

Metabolic syndrome, cardiovascular risk scores and blood pressure

International Diabetes Federation-defined metabolic syndrome was significantly associated with hospital-discharge-based uterine leiomyoma diagnosis, independent of parity, education, BMI and current use of exogenous hormones (OR=1.48, 95% CI 1.09 to 2.01, P=0.01) (Table 5). CVD risk assessment scoring was performed by using two widely used tools; the Framingham CVD risk score and SCORE. The analysis did not show an association with leiomyomas according to either of the CVD risk assessment scores, in any of the adjusted models. Blood pressure was not associated with uterine leiomyomas. One reason for this may be the relatively young age of the cohort (Table 5).

There is evidence in previously published studies that hypertension and uterine leiomyomas are associated. Such a relationship has been shown in two studies (Boynton-Jarrett *et al.* 2005, Radin *et al.* 2012), but a suggestion of no association has also been published (Parazzini *et al.* 2004). The NHS II study, which is the

largest study on leiomyomas to date, offers strong evidence of an association and it was reported that every 10 mmHg increase in diastolic blood pressure increased the risk of leiomyomas by 8% among non-users and by 10% among users of antihypertensive medication (Boynton-Jarrett *et al.* 2005).

Liver function, chronic inflammation and sex hormones

The fatty liver index (FLI) was not associated with uterine leiomyomas in the analysis. After adjusting for parity and education, serum hs-CRP at 1–3 mg/l was associated with hospital-discharge-based leiomyoma diagnosis (OR=1.35, 95% CI 1.01 to 1.80, P=0.04), but this association became non-significant after adjusting additionally for BMI and current use of exogenous hormones (Table 5). With the full adjustment model, no association was observed as regards SHBG (Table 5). However, every 1 nmol/l increase in serum total testosterone was associated with ICD-code-confirmed cases (OR=0.60 95% CI 0.40 to 0.89, P=0.01) (Table 5).

Possible mechanisms

Obesity is associated with different grades of insulin resistance, which is a substantial underlying key factor in the development of cardio-metabolic disorders. Central obesity in particular raises the risk of development of metabolic complications, with mounting evidence that not only visceral adipose tissue, but also subcutaneous adipose tissue has a significant impact on the process (Patel & Abate 2013). In fact, fat distribution in obese premenopausal women is more often characterised by excess subcutaneous fat, but this changes during menopause transition to visceral fat accumulation (Toth *et al.* 2000). In the first phase of insulin resistance, hyperinsulinaemia increases hepatic synthesis and activity of insulin-like growth factors, such as IGF-I. Insulin resistance seems to play a role in uterine leiomyoma development, as IGF-I may act to promote leiomyoma growth in an autocrine/paracrine fashion. Levels of IGF-I receptors are increased in leiomyoma tissue compared with myometrium (Chandrasekhar *et al.* 1992) and the levels of IGF-I peptide, IGF-I mRNA and IGF-II mRNA are also elevated (Vollenhoven *et al.* 1993, van der Ven *et al.* 1994, Englund *et al.* 2000). A recent study involving an experimental mouse model concerned induced insulin resistance. Administration of oestrogen and progesterone promoted uterine smooth muscle growth and insulin resistance had an enhancing effect on this steroid hormone stimulation (Hou *et al.* 2015). The authors suggest that this might imply an effect of insulin resistance in

the development of uterine leiomyomas. Again, an association study concerning leiomyoma tumour size and extended candidate chromosomal regions resulted in identification of a sole significant variant, in *SORCS2* (sortrilin-related VPS10 domain-containing receptor 2) (Aissani *et al.* 2015), which is also a strong candidate gene as regards circulating IGF-I and IGFBP-3 (Kaplan *et al.* 2011).

Interestingly, subunits of the Mediator complex kinase module are associated with metabolic syndrome and obesity (MED13) and negative regulation of lipid metabolism (CDK8) (Schiano *et al.* 2014). In the mouse heart MED13 controls metabolic homeostasis and energy expenditure, which has been verified by cardiac-specific deletion of *MED13*, which increases susceptibility to metabolic syndrome and severe obesity (Grueter *et al.* 2012). *In vitro* and *in vivo* data show that CDK8 promotes degradation of nuclear SREBP-1c and results in triglyceride accumulation in hepatocytes. CDK8 and CycC regulate the lipogenic pathway in *Drosophila* and mammalian hepatocytes, depending on protein quantity. Additionally, *CDK8* knockdown in mouse liver *in vivo* has been reported to result in a fatty liver-like phenotype and a dramatic elevation of triglycerides in plasma, offering yet another finding similar to that among women presenting with clinically relevant uterine leiomyomas (Zhao *et al.* 2012). Even though it is believed that the MED12-MED13 complex and the CDK8-CycC complex have distinct functions in regulating developmental patterns (Carrera *et al.* 2008, Gobert *et al.* 2010), our results concerning women with leiomyomas, obesity and alterations in lipid metabolism, triglycerides in particular, justify study of the function of the Mediator kinase module as a whole as regards uterine leiomyoma biology.

The frequencies of self-reported hysterectomy among the uterine leiomyoma group and the control group were 41.7% vs. 3.8%, respectively. This may in part explain the observed associations between increased leiomyoma prevalence and CVD risk factors, as hysterectomy has been documented to have an association with a significantly increased later-life CVD risk (Atsma *et al.* 2006, Ingelsson *et al.* 2011). Among premenopausal women it is likely that the immediate surgically induced ovarian failure via circulation disruption and the sudden fall in oestrogen and testosterone levels after bilateral oophorectomy are the underlying reasons for the association between hysterectomy and increased CVD risk. This may again be explained by a mechanism involving increased total cholesterol and LDL levels (Zhang *et al.* 2005, Appiah *et al.* 2015). However, the crucial role of hysterectomy alone in regard to CVD has been questioned by the results of a study indicating an association between pre-existing CVD risk factors and CVDs, and hysterectomy

with ovarian conservation (Laughlin-Tommaso *et al.* 2016). This finding suggests that hysterectomy has a role in CVD.

5.4 Association between uterine leiomyoma and endometriosis (Study II)

An association between uterine leiomyoma and endometriosis has been suggested in previous publications. The prevalence of uterine leiomyoma was reported to be higher in a cohort study of surgically treated women with endometriosis (Hemmings *et al.* 2004), and endometriosis prevalence has been shown to be higher in a small case-control study among women with symptomatic leiomyoma (Huang *et al.* 2010).

Endometriosis prevalence and uterine leiomyoma prevalence were investigated in Study II among women with symptomatic leiomyoma or endometriosis, and the data compared with prevalences among asymptomatic women undergoing tubal sterilization. The overall endometriosis prevalence in the uterine leiomyoma group was 19.6% (47/240) and in the control group 5.5% (10/183) ($P<0.0001$). Further analysis in different age groups revealed that the prevalence of endometriosis was increased particularly in the age groups of 35–39 years and 40–44 years: 37.0% vs. 6.8% ($P<0.0001$) and 34.0% vs. 4.6% ($P<0.0001$) (Figure 7). Among women aged 45 years and older the prevalence of endometriosis decreased in the leiomyoma group to 12.3%. There were no endometriosis diagnoses in the control group in this age group.

The overall uterine leiomyoma prevalence in the endometriosis group was 25.8% (47/182) and in the control group 9.3% (17/183) ($P=0.01$). Further analysis in the age groups showed that leiomyoma prevalence increased with advancing age in both groups (Figure 8). However, when comparing the leiomyoma prevalences within each age group, the difference was significant only between women aged 40 to 44 years (35 to 39 years: 14.1% vs. 5.7%, $P=0.07$; 40 to 44 years: 25.0% vs. 11.6%, $P=0.03$; 45 years and older: 46.5% vs. 22.2%, $P=0.18$).

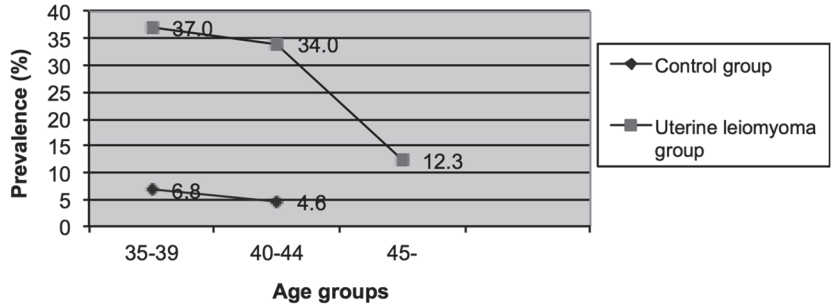

Fig. 7. Endometriosis prevalence among women with uterine leiomyoma and controls.

There was a total of 47 women with both diseases in this study. Independent associations between uterine leiomyomas, endometriosis and subfertility were explored. When subfertility was defined as nulliparity, both leiomyoma and endometriosis groups showed increased risks of subfertility (OR 3.84, 95% CI 2.25–6.54; OR 6.78, 95% CI 3.98–11.56).

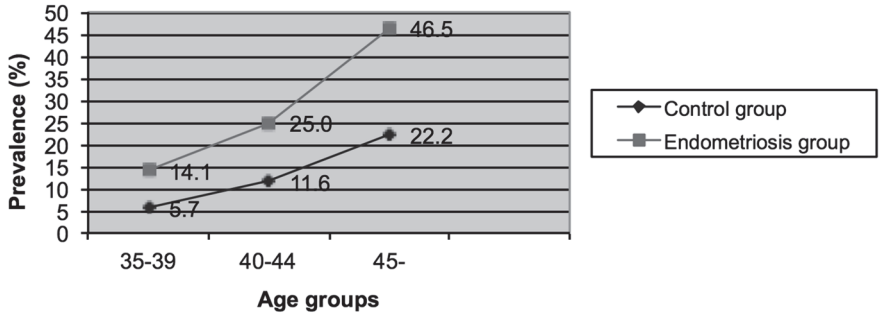

Fig. 8. Uterine leiomyoma prevalence among women with endometriosis and controls.

The results of Study II offer confirmation of an association between symptomatic uterine leiomyoma and endometriosis among Finnish women. These two common gynaecological diseases share some biological similarities that could contribute to the association. A monoclonal origin of endometriosis has been shown in connection with endometrial cysts, where the methylation pattern for X

chromosome inactivation has been similar within most epithelial cells (Jimbo *et al.* 1997, Tamura *et al.* 1998, Wu *et al.* 2003). This suggests that endometriotic lesions may carry neoplastic potential and that they are derived from the same cell population, which is similar to uterine leiomyoma tumour initiation. Another shared similarity is the high regenerative capacity: bilayered endometrium in menstrual cycles and after parturition, and myometrium over the course of pregnancy (Spencer *et al.* 2005, Jabbour *et al.* 2006). This has led to the investigation of stem cell/progenitor populations in endometrium and also in disorders of endometrial proliferation such as endometriosis. Endometrial epithelial progenitor cells of the basal layer have been proposed to play a role in endometriosis, as basal-layer epithelial cells have been found in endometriotic lesions (Valentijn *et al.* 2013) and basal-layer fragments have been identified more often in menstrual blood of women with endometriosis, vs. controls (Leyendecker *et al.* 2002). Ectopic endometriotic epithelial and stromal colony-forming units (CFUs) have also been observed after serial cloning (two to three times) (Chan *et al.* 2011), suggesting their potential role in pathogenesis. Additionally, ectopic mesenchymal stem cells (MSCs) have shown greater migration and invasion than eutopic MSCs, with increased angiogenesis and invasion into surrounding tissue in a mouse model (Kao *et al.* 2011).

Endometriosis and uterine leiomyomas both have a significant heritable component in their development. The involvement of genetic factors in endometriosis is supported by numerous studies (Rahmioglu *et al.* 2012) and its heritability is estimated at approximately 50% (Treloar *et al.* 1999, Saha *et al.* 2015). To date, four GWASs have been conducted, identifying ten genomic regions harbouring genome-wide significant common risk variants for endometriosis (Rahmioglu *et al.* 2014, Zondervan *et al.* 2016). Interestingly, two of these regions (harbouring *WNT4* and *GREB1*) were then shown to have an association with leiomyomas (Gallagher *et al.* 2015). *WNT4* is a key gene in the Wnt/β-catenin pathway (Bernard *et al.* 2008). It encodes a protein crucial for development of the female reproductive tract (Vainio *et al.* 1999). WNT4 has been shown to be expressed in normal peritoneum, suggesting that endometriosis can arise through metaplasia via developmental phases involved in embryonic development of the female reproductive tract (Gaetje *et al.* 2007). Additionally, evidence has been presented of shared genetic origins between endometriosis and fat distribution, pointing at Wnt signalling (Rahmioglu *et al.* 2015). *GREB1* (growth regulation by oestrogen in breast cancer 1) encodes for an early response component in the oestrogen receptor-regulated pathway and it is involved in oestrogen-induced

growth of breast cancer cells (Rae *et al.* 2005). Its role in the development of endometriosis and uterine leiomyoma remains to be uncovered. Both endometriosis and uterine leiomyoma are oestrogen-dependent and they appear to produce oestrogen locally through aromatase expression and activity (Bulun *et al.* 2005).

5.5 Limitations and strengths of the studies

The investigated population in Study I was recruited at the Gynaecology Outpatient Clinic, Oulu University Hospital, and this may bring in limitations with an impact on the study results. Women reviewed in speciality care units may represent a patient population with more severe symptoms and thus the findings would be applicable to symptomatic uterine leiomyoma type only. Another limitation in this study is that recognised clustering of leiomyoma cases within a family might encourage women to seek medical advice earlier after minor symptoms, resulting in more diagnosed cases. The study populations were not screened for uterine leiomyomas, and this may leave leiomyoma cases unrecognised among controls, thus weakening the observed differences between the studied groups. Family size also has an impact on the detection of inherited traits for diseases, and therefore complicates the distinction between true cases and controls. The strength of Study I is the accurate clinical data on leiomyomas under analysis. In addition, the self-reported positive family history of leiomyomas was validated in a set of familial study subjects.

For Study III, patients were recruited on the basis of personal knowledge of prior uterine leiomyoma diagnosis. On recruitment, appropriate information was given on leiomyoma tissue collection, including the requirement of prior surgical treatment and an available tissue sample for the study. The selected recruitment method might exclude women with either asymptomatic or only mildly symptomatic leiomyomas and those who did not require surgical treatment. Therefore, only women with the most severe clinical characteristics might have been included in this study, enhancing the differences in clinical characteristics in comparison with the women with sporadic leiomyomas.

To our knowledge this is the largest dataset to date concerning HLRCC-related uterine leiomyoma tissue for comprehensive histological analysis. However, it should be taken into consideration that the size is fairly small for statistical analyses and replication studies with bigger datasets are needed for verification of the observed results.

The asymptomatic nature of both uterine leiomyomas and endometriosis sets limitations in studying their prevalence at a population level. For Study II the subjects were selected among women attending a gynaecological speciality care unit, and therefore are not representative of the general population. Additionally, both leiomyoma and endometriosis require clinical procedures (pelvic ultrasonographic imaging and laparoscopy for a pelvic view) for reliable diagnosis and therefore control group selection is limited to those having undergone both these procedures. To decrease possible confounding related to other disease pathologies, women undergoing sterilization through laparoscopy were selected as the control group for Study II. The disadvantage of this control group selection concerns the opposing procedure indications, disease vs. family planning, and thus leads to insurmountable differing fertility characteristics between the groups under comparison. Both uterine leiomyomas and endometriosis are associated with impaired fertility, and therefore the prevalences are most likely to be lower among women seeking sterilization than among the general population, thus increasing the observed prevalence differences in Study II. Another challenge in studies of uterine leiomyomas and endometriosis is the age of the patients, as the mean ages at diagnosis differ from each other. This might have caused under-diagnosis of both diseases. The strength of Study II was that all subjects had gone through both clinical procedures, albeit with differing indications. The screening enabled us to study the prevalence of both diseases in the same study population, and thus the association was tested regardless of symptoms related to either disease.

The strengths of Study IV are the large population-based cohort with accurate data on medical diagnoses at speciality care units, a great number of clinical examinations and extensive questionnaire data. It was possible to analyse all CVD risk factors simultaneously in the same study population and during the same time period. However, there may have been some limitations in case ascertainment. There was likely to have been under-ascertainment of cases and misclassification of some cases as controls due to the asymptomatic nature of uterine leiomyomas. The incidence of uterine leiomyomas in this study was 20.1% (729/3635) when considering all cases, and 8.1% (293/3635) when considering ICD-code-identified cases, whereas the overall ICD-code-based incidence in the cohort, when including all women regardless of their participation in the clinical examinations, was 7.7%. Indeed, there is a discrepancy when comparing this figure with the reported cumulative incidences. A screening study revealed a 34% prevalence of ultrasonographically detected leiomyomas among white women with no previous leiomyoma diagnosis (Baird *et al.* 2003). When applying this figure to the NFBC66

population, it can be estimated that there may be nearly 1000 undetected leiomyoma cases among the controls. This would have diluted the effects of reported associations to roughly half. There are no comparable figures for the Finnish population, but an ultrasonographic screening study revealed a uterine leiomyoma prevalence of 7.8% among Swedish women aged 33 to 40 years (Borgfeldt & Andolf 2000), which indeed is more in proportion to our findings. Age of the cohort at the time of clinical examinations was not ideal for cardiovascular risk assessment, as age is the strongest risk factor for CVD and the risk starts to rise significantly after the age of 60 years (Tuomilehto 2004). The data analysed were cross-sectional and therefore cause and effect for the associations observed cannot be distinguished.

6 Conclusions and future directions

The current study provides novel information on the clinical characteristics of familial uterine leiomyomas and on the immunophenotype of HLRCC-related leiomyomas. This study also offers significant confirmation of the association between uterine leiomyomas and endometriosis, and between leiomyomas and several CVD risk factors.

Uterine leiomyomas are known to be the most common benign tumours in females (Baird *et al.* 2003). Recent studies have elucidated the genetic background of leiomyoma development (Mehine *et al.* 2013b) and this has enabled a presentation of molecular classification of uterine leiomyomas (Mehine *et al.* 2014). Thus it can be hypothesised that the natural history differs among molecularly different leiomyomas. The results in Study I offer confirmation of this hypothesis, as familial leiomyomas have more severe clinical characteristics. Additionally, Study III provides further proof of this hypothesis, as women with HLRCC present with multiple uterine leiomyomas at a younger age and require surgical treatment more often. According to the results of Study III, HLRCC-related uterine leiomyomas also share a distinct immunophenotype: higher microvessel density and inhibition of apoptosis, when compared with sporadic leiomyomas. Together with the clinical picture, the results of Study III suggest that this information can be used to improve the identification of female individuals and their families carrying the *FH* mutation responsible for HLRCC. Future studies are required to validate the results and to clarify the functional mechanisms of HLRCC-related uterine leiomyoma pathogenesis.

The suggestion of coexistence of uterine leiomyomas and endometriosis receives further support from Study II. It showed an association between the prevalence of symptomatic endometriosis and symptomatic uterine leiomyomas in women aged 35 years or more. To date, only a few studies have been aimed at exploring this association, and so far only a little is known about the effect of the combination of these diseases on female reproductive health. Therefore, future studies should be directed at investigating the shared pathogenic pathways and also the clinical significance of uterine leiomyomas and endometriosis to enable better understanding of their coexistence and whether, for example, endometriosis is associated with all, or only one subclass of the leiomyoma molecular classification system.

Study IV provides evidence for the previously presented hypothesis on the association between uterine leiomyomas and CVDs. Study IV revealed

unfavourable alterations in several well-documented CVD risk factors in women diagnosed with uterine leiomyomas. Increased serum total cholesterol, LDL and triglyceride levels were associated with an increased risk of leiomyoma diagnosis. Additionally, central obesity, impaired glucose tolerance and metabolic syndrome were associated with leiomyoma risk. The observed associations may suggest that there are shared predisposing factors underlying both uterine leiomyoma and adverse metabolic and cardiac disease risks, or that metabolic factors have a role in biological mechanisms underlying leiomyoma development. Future studies should be designed in a prospective setting to further investigate the underlying biological mechanisms in leiomyoma pathogenesis, as the causality cannot be determined by way of a cross-sectional study such as Study IV. Genes encoding mediator complex have been associated with both uterine leiomyomas and metabolic syndrome (Makinen *et al.* 2011b, Schiano *et al.* 2014). Exploring the biological pathways involving mediator subunits would be one way of investigating the common biology of these traits.

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Original publications

This thesis is based on the following publications, which are referenced throughout the text by their Roman numerals:

I Uimari O, Suomalainen-Konig S, Sakkinen N, Santala M, Nieminen P & Ryynanen M (2005) Natural history of familial myomas. Eur J Obs & Gyn and Reprod Biol 125(2): 255–258.

II Uimari O, Jarvela IY & Ryynanen M (2011) Do symptomatic endometriosis and uterine fibroids appear together? J Hum Reprod Sci 4(1): 29–33.

III Uimari O, Ahtikoski A, Kampjarvi K, Butzow R, Jarvela IY, Ryynanen M, Aaltonen LA, Vahteristo P & Kuismin O (2016) Clinical characteristics and histological features of uterine leiomyomas in Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC) syndrome. Manuscript.

IV Uimari O, Auvinen J, Jokelainen J, Puukka K, Ruokonen A, Jarvelin MR, Piltonen T, Keinanen-Kiukaanniemi S, Zondervan K, Jarvela IY, Ryynanen M & Martikainen H (2016) Uterine fibroids and cardiovascular risk. Hum Reprod 31(12):2689-2703.

Original publications are not included in the electronic version of this dissertation.

1390. Alahuhta, Ilkka (2016) The microenvironment is essential for OTSCC progression

1391. Hagnäs, Maria (2016) Health behavior of young adult men and the association with body composition and physical fitness during military service

1392. Korhonen, Vesa (2016) Integrating near-infrared spectroscopy to synchronous multimodal neuroimaging : applications and novel findings

1393. Keinänen, Tuija (2016) Infra-slow fluctuations in simultaneous EEG-fMRI

1394. Eranti, Antti (2016) The role of electrocardiographic abnormalities, obesity, and diabetes in risk stratification for sudden cardiac death in the general population

1395. Kubin, Minna (2016) Glucocorticoid receptors in inflammatory skin diseases : the effect of systemic and topical glucocorticoid treatment on the expression of GRα and GRβ

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