Doctoral Programme in Biomedicine (DPBM)

MED12 MUTATIONS AND METABOLOMIC CHANGES IN UTERINE LEIOMYOMAS

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ACADEMIC DISSERTATION

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I Mäkinen N, **Heinonen H-R**, Moore S, Tomlinson IP, van der Spuy ZM & Aaltonen LA. *MED12* exon 2 mutations are common in uterine leiomyomas from South African patients. *Oncotarget* **2**: 966-969, 2011.

II **Heinonen H-R**, Sarvilinna NS, Sjöberg J, Kämpjärvi K, Pitkänen E, Vahteristo P, Mäkinen N & Aaltonen LA. *MED12* mutation frequency in unselected sporadic uterine leiomyomas. *Fertility and Sterility* **102**: 1137-1142, 2014.

III **Heinonen H-R**, Pasanen A, Heikinheimo O, Tanskanen T, Palin K, Tolvanen J, Vahteristo P, Sjöberg J, Pitkänen E, Bützow R, Mäkinen N & Aaltonen LA. Multiple clinical characteristics separate *MED12*-mutation-positive and -negative uterine leiomyomas. *Scientific Reports* **7**: 1015, 2017.

IV **Heinonen H-R**, Mehine M, Mäkinen N, Pasanen A, Pitkänen E, Karhu A, Sarvilinna NS, Sjöberg J, Heikinheimo O, Bützow R, Aaltonen LA & Kaasinen E. Global metabolomic profiling of uterine leiomyomas. *British Journal of Cancer* In press.

Publication I is included in the thesis of Netta Mäkinen (*MED12*: a novel player in uterine leiomyomas, Helsinki, 2014).

The publications are referred to in the text by their Roman numerals.

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ABBREVIATIONS

αKG	α-ketoglutaric acid
ANOVA	analysis of variance
BMI	body mass index
CDK	cyclin-dependent kinase
ECM	extracellular matrix
ESI	electrospray ionization
***FH*/FH**	*fumarate hydratase* gene/fumarate hydratase protein
HIF-1-α	hypoxia-inducible factor 1-α
HLRCC	hereditary leiomyomatosis and renal cell cancer
***HMGA2*/HMGA2**	*high mobility group AT-hook 2* gene/high mobility group AT-hook 2 protein
HUH	Helsinki University Hospital
MS	mass spectroscopy
***MED12*/MED12**	*mediator complex subunit 12* gene/mediator complex subunit 12 protein
NRF2	nuclear factor, erythroid 2 like 2 protein
P	p-value
PCR	polymerase chain reaction
PID	pelvic inflammatory disease
PPP	pentose phosphate pathway
QC	quality control
qPCR	quantitative polymerase chain reaction
RA	retinoic acid
***RAD51B*/RAD51B**	*RAD51 paralog B* gene/RAD51 paralog B protein
S-ado	N6-succinyladenosine
TCA	tricarboxylic acid
UPLC	ultra-performance liquid chromatography

ABSTRACT

Uterine leiomyomas are benign tumors that arise from the smooth muscle cells of the myometrium under the influence of estrogen and progesterone. Their incidence approaches 70% in Caucasian women and exceeds 80% in African American women by age 50. A quarter of these women suffer from symptoms that require medical attention. As leiomyomas impair the quality of life in a large proportion of women, these tumors pose a significant health and economic impact. Leiomyomas are heterogeneous in their number, size, location, growth rate, histological features, and clinical presentation, as well as in their molecular characteristics.

Multiple distinct genetic alterations lead to the development of these tumors, including chromosomal rearrangements affecting *high mobility group AT-hook 2* (*HMGA2*) and biallelic inactivation of *fumarate hydratase* (*FH*). Moreover, a recent Finnish exome sequencing study discovered that most leiomyomas harbor mutations in *mediator complex subunit 12* (*MED12*). This breakthrough finding pinpointed the main tumorigenic event underlying leiomyomas, but also instigated the demand for further characterization of these mutations. Furthermore, the downstream mechanisms of how these genetic aberrations drive leiomyoma growth remain elusive. This thesis aimed to study the occurrence and characteristics of *MED12*-mutation-positive and -negative uterine leiomyomas, and to elucidate the metabolomic profiles of leiomyomas harboring distinct genetic drivers.

To determine the frequency and clinical features of *MED12* mutations in uterine leiomyomas, we screened various sample series for these mutations by Sanger sequencing and assessed potential associations between clinical variables and mutation status. This work validated the occurrence of *MED12* mutations in uterine leiomyomas derived from South African women, and demonstrated that these mutations are the main genetic driver of leiomyomas regardless of ethnicity. Our results showed that when small leiomyomas are also examined, the mutation frequency is as high as 86%, and in the largest leiomyoma series thus far with carefully controlled size bias, the mutation frequency was 79%. We discovered that *MED12* mutations are associated with smaller size, subserosal location, conventional histology, and larger number of leiomyomas. In addition, the number of mutation-positive leiomyomas was inversely associated with parity, and the number of mutation-negative leiomyomas was associated with a history of pelvic inflammatory disease. These findings demonstrate the extremely high *MED12*-mutation frequency and their contribution to the observed heterogeneity of uterine leiomyomas.

Global metabolomic profiling of uterine leiomyoma and corresponding myometrium samples revealed that the three main genetic drivers result in differences in metabolic profiles; in particular, *FH*-deficient leiomyomas were distinguishable based on their unique profile. We observed shared metabolic changes for all leiomyomas and distinctive alterations for *FH*-deficient and *MED12*-mutation-positive leiomyomas. The dysregulated metabolic pathways identified in this study should be relevant for improving prevention and treatment of these lesions. Some of the uniquely dysregulated metabolites identified may be

utilized as biomarkers to distinguish between leiomyomas of the different molecular subtypes.

This thesis highlights the role of *MED12* mutations and metabolomic alterations in leiomyomas, further emphasizing the importance of molecular classification of leiomyomas in research, and possibly in clinical practice as well when targeted treatment options emerge.

REVIEW OF THE LITERATURE

1. Uterine leiomyomas

The uterus, an essential organ for the reproduction of eutherian mammals, consists of three distinct layers: the inner mucosal lining, the endometrium; the outer serosal layer, the serosa; and the thick muscular layer, the myometrium. The myometrium consists of smooth muscle cells surrounded by supporting stroma and vasculature, forming a three-layered structure of differentially organized muscle fibers designed to contract efficiently during labor. Occasionally, myometrial cells start to proliferate abnormally giving rise to benign smooth muscle tumors called uterine leiomyomas or fibroids. The pathogenic mechanism underlying this aberrant cell proliferation is multifactorial, and different leiomyomas may arise through diverse etiologies. In this thesis, the term leiomyoma refers to uterine leiomyoma unless otherwise specified.

1.1. Epidemiology

Leiomyomas occur in women during their reproductive years with the incidence increasing up to menopause. Prevalence estimates of leiomyomas range from 5 to 89% depending on the inclusion criteria, screening method, and the size, age, and race of the study population (Drayer and Catherino, 2015). According to a study with ultrasonographically confirmed cases, Caucasian women have an incidence of 40% by age 35, and nearly 70% by age 50, whereas African-American women have an incidence of 60% by age 35, and over 80% by age 50 (Baird *et al.*, 2003). Approximately 20 to 50% of affected women are symptomatic (Stovall, 2001). Because of the high incidence and morbidity of leiomyomas, they pose an enormous socio-economic burden; their estimated annual cost ranges from $6 to $34 billion in the USA (Cardozo *et al.*, 2012).

Leiomyomas affect different racial groups disproportionately. In addition to a higher incidence of leiomyomas in African-American women, these women display a younger age at diagnosis and at hysterectomy, larger and more numerous leiomyomas, as well as more severe symptoms than Caucasian women (Kjerulff *et al.*, 1996; Faerstein *et al.*, 2001a; Baird *et al.*, 2003; Huyck *et al.*, 2008; Laughlin *et al.*, 2009; Moorman *et al.*, 2013; Stewart *et al.*, 2013; Drayer and Catherino, 2015). Apart from African ancestry, leiomyomas seem to affect other racial groups as frequently as Caucasian women (Marshall *et al.*, 1997; Laughlin *et al.*, 2009; Zimmermann *et al.*, 2012). Although both genetic and environmental factors are likely to underlie this racial disparity, the fundamental mechanisms remain unclear (Catherino *et al.*, 2013).

The development of leiomyomas depends highly on female steroid hormones, as these tumors do not occur before puberty and typically regress after menopause (Ross *et al.*, 1986; Cramer and Patel, 1990; Moroni *et al.*, 2015). During pregnancy, leiomyomas may grow especially during the first trimester, but most leiomyomas eventually shrink and, after parturition, may become undetectable (Laughlin *et al.*, 2010; Ciavattini *et al.*, 2016). Many

of the risk factors for leiomyomas are related to exposure to estrogen and progesterone (Table 1). In addition, genetic, lifestyle, and environmental factors influence the risk of developing leiomyomas (Table 1). One of the main challenges in epidemiological leiomyoma research is obtaining leiomyoma-free controls due to their high prevalence in asymptomatic women and their clinical appearance during the late reproductive years. Further research is required to gain a better understanding of how these risk factors influence leiomyoma development.

1.2. Clinical course

Leiomyomas can be detected in a pelvic exam and the diagnosis can be confirmed by transvaginal sonography (Stewart *et al.*, 2016). Other imaging techniques, including transabdominal or saline-infusion sonography and magnetic resonance imaging, may be needed to accurately assess the size, number, location, or vascularity of leiomyomas (McLucas, 2008; Stewart *et al.*, 2016). Differential diagnoses for leiomyomas include leiomyosarcomas, adenomyosis or adenomyomas, ovarian masses, and endometrial polyps (McLucas, 2008).

The range and severity of symptoms from leiomyomas depend highly on the number, location, and size of the tumors (Stovall, 2001; Gupta *et al.*, 2008). Patients may have anything from a single tumor to innumerable leiomyomas of different sizes; an affected uterus contains on average 8 to 9 of these tumors, and they may commonly be 10 to 20 cm in diameter (Cramer and Patel, 1990; Stewart *et al.*, 2016). Leiomyomas can grow anywhere within the uterine wall, the cervix, or the uterine ligaments, and can protrude into the uterine cavity or outward into the peritoneal cavity while attached to the myometrium by a peduncle (Figure 1) (McLucas, 2008). Leiomyomas can be classified based on their location relative to the myometrial layers: submucosal leiomyomas underlie the endometrium, intramural lie within the myometrium, and subserosal underlie the serosa. A single uterus may harbor different types of leiomyomas at the same time, and the tumors may grow or regress at different rates in the same uterine environment (Peddada *et al.*, 2008).

The most frequent leiomyoma-related symptom is abnormal uterine bleeding usually presenting as heavy menstrual bleeding, which may also be prolonged or irregular, involve dysmenorrhea, and lead to iron deficiency anemia (Stovall, 2001; Gupta *et al.*, 2008). Abnormal bleeding is particularly associated with submucosal leiomyomas. Affected postmenopausal women may also suffer from bleeding symptoms, especially related to hormone-therapy use. Patients with leiomyomas frequently experience pain in the pelvis, abdomen, or back. The pain can be cyclic or non-cyclic, or dyspareunia. Necrotic or degenerative leiomyomas may lead to acute abdominal pain. The enlarged uterus or a large, leiomyoma may press the surrounding organs leading to pelvic or abdominal discomfort, urinary frequency or retention, hydronephrosis, or bowel disturbance. Leiomyomas may cause infertility by, for example, blocking the fallopian tubes or distorting the uterine cavity (Stovall, 2001). Women with leiomyomas have an increased risk of cesarean section, preterm birth, and other pregnancy complications (Stovall, 2001; Gupta *et al.*, 2008). The majority of women with symptomatic leiomyomas display multiple concurrent symptoms (Gupta *et al.*, 2008).

Table 1. Leiomyoma risk factors from epidemiological studies.

Factor	Reference(s)
Increased risk	
African-American origin	(Kjerulff *et al.*, 1996; Marshall *et al.*, 1997; Faerstein *et al.*, 2001a; Baird *et al.*, 2003; Laughlin *et al.*, 2009; Templeman *et al.*, 2009; Ott *et al.*, 2014)
Age up to menopause	(Ross *et al.*, 1986; Marshall *et al.*, 1997; Baird *et al.*, 2003; Ott *et al.*, 2014; Sommer *et al.*, 2015)
Alcohol	(Marshall *et al.*, 1997; Wise *et al.*, 2004b; Templeman *et al.*, 2009)
Carotid intima-media thickness	(Aksoy *et al.*, 2014)
Cosmetics and other chemicals	(Shen *et al.*, 2013)
Family history of leiomyomas	(Vikhlyaeva *et al.*, 1995; Lumbiganon *et al.*, 1996; Sato *et al.*, 2002; Templeman *et al.*, 2009)
Food additives	(Shen *et al.*, 2013)
Hormone therapy	(Templeman *et al.*, 2009; Sommer *et al.*, 2015; Uimari *et al.*, 2016)
Hypertension	(Faerstein *et al.*, 2001b; Boynton-Jarrett *et al.*, 2005; Takeda *et al.*, 2008; Templeman *et al.*, 2009)
Infertility	(Parazzini *et al.*, 1996; Marshall *et al.*, 1997; 1998; Templeman *et al.*, 2009)
Longer time since last birth	(Marshall *et al.*, 1997; 1998; Wise *et al.*, 2004a; Terry *et al.*, 2010)
Metabolic syndrome	(Uimari *et al.*, 2016)
Nulliparity	(Parazzini *et al.*, 1988; 1996; Wise *et al.*, 2004a; Parazzini, 2006)
Obesity/body mass index/ waist-to-hip ratio	(Ross *et al.*, 1986; Lumbiganon *et al.*, 1996; Marshall *et al.*, 1997; Sato *et al.*, 1998; Faerstein *et al.*, 2001a; Wise *et al.*, 2005; Parazzini, 2006; Takeda *et al.*, 2008; Templeman *et al.*, 2009; Sommer *et al.*, 2015; Uimari *et al.*, 2016)
Omega-3 (n-3) polyunsaturated fatty acids	(Wise *et al.*, 2014)
Pelvic inflammatory disease	(Faerstein *et al.*, 2001b)
Phthalates/plastic products	(Huang *et al.*, 2010; Shen *et al.*, 2013)
Polycystic ovary syndrome	(Wise *et al.*, 2007)
Prenatal diethylstilbestrol	(Baird and Newbold, 2005; Mahalingaiah *et al.*, 2014)
Psychological stress/depression	(Vines *et al.*, 2010; Boynton-Jarrett *et al.*, 2011; Wise *et al.*, 2015)
Red meat	(Chiaffarino *et al.*, 1999)
Soybean milk	(Shen *et al.*, 2013)
Thyroid disease	(Ott *et al.*, 2014)
Unfavorable serum lipid profile	(Aksoy *et al.*, 2014; Uimari *et al.*, 2016)
Vitamin D deficiency	(Baird *et al.*, 2013)
Decreased risk	
Breast feeding	(Lumbiganon *et al.*, 1996; Terry *et al.*, 2010)
Dairy	(Wise *et al.*, 2010)
Diabetes	(Wise *et al.*, 2007; Templeman *et al.*, 2009; Velez Edwards *et al.*, 2017)
Dioxin	(Eskenazi *et al.*, 2007)
Exercise	(Baird *et al.*, 2007)
Fruits and vegetables	(Chiaffarino *et al.*, 1999; Wise *et al.*, 2011)
Irregular menstrual cycle	(Terry *et al.*, 2010)
Later age at first birth	(Marshall *et al.*, 1997; 1998; Wise *et al.*, 2004a; Templeman *et al.*, 2009; Terry *et al.*, 2010)
Later age at last birth	(Ross *et al.*, 1986; Lumbiganon *et al.*, 1996; Terry *et al.*, 2010)
Later age at menarche	(Lumbiganon *et al.*, 1996; Marshall *et al.*, 1997; 1998; Faerstein *et al.*, 2001a; Wise *et al.*, 2004a; Terry *et al.*, 2010; Velez Edwards *et al.*, 2013)
Longer menstrual cycle length	(Terry *et al.*, 2010)
Menopause	(Ross *et al.*, 1986; Parazzini *et al.*, 1988; Parazzini, 2006; Templeman *et al.*, 2009; Sommer *et al.*, 2015)
Parity	(Ross *et al.*, 1986; Parazzini *et al.*, 1988; 1996; Lumbiganon *et al.*, 1996; Marshall *et al.*, 1998; Sato *et al.*, 2002; Wise *et al.*, 2004a; Parazzini, 2006; Terry *et al.*, 2010; Uimari *et al.*, 2016)
Progestin-only injectables	(Lumbiganon *et al.*, 1996; Wise *et al.*, 2004a)
Smoking	(Ross *et al.*, 1986; Parazzini *et al.*, 1988; 1996; Lumbiganon *et al.*, 1996; Templeman *et al.*, 2009)
Inconsistent risk effect	
Abortions	(Lumbiganon *et al.*, 1996; Parazzini *et al.*, 1996)
Oral contraceptives	(Ross *et al.*, 1986; Lumbiganon *et al.*, 1996; Parazzini *et al.*, 1996; Marshall *et al.*, 1997; 1998; Faerstein *et al.*, 2001a; Wise *et al.*, 2004a)
Vitamin A	(Martin *et al.*, 2011; Wise *et al.*, 2011)

The choice of treatment depends on the patient's age, symptoms, reproductive wishes, beliefs, and other medical conditions, as well as the location, size, and number of tumors. The selective progesterone receptor agonist ulipristal acetate, which was recently approved for intermittent use in Europe, has shown great promise as an effective treatment for leiomyomas. In most patients, but not all, this drug reduces the size of leiomyomas and controls the bleeding symptoms effectively. The effects of its long-term intermittent use are currently under investigation. Gonadotropin releasing hormone agonists also reduce the size of leiomyomas and induce amenorrhea, but due to severe side effects related to hormonal suppression, they are prescribed only preoperatively to facilitate less invasive surgery or to correct iron deficiency anemia. Other medical therapy options provide merely symptomatic relief. Hormonal contraceptives and levonorgestrel-releasing intrauterine devices alleviate the pattern and volume of menstrual bleeding. In addition, antifibrinolytics, such as tranexamic acid, and non-steroidal anti-inflammatory drugs reduce heavy bleeding. As medical therapies leave room for improvement, surgery is frequently used. Hysterectomy is currently the only definitive treatment; leiomyomas are the indication of one-third to half of all hysterectomies. Less invasive surgical options include myomectomy, uterine artery embolization, focused-ultrasound surgery, and radiofrequency ablation. Minimally invasive techniques, such as endometrial ablation and hysteroscopic electroresection of submucosal leiomyomas, are used to control heavy bleeding. (Stewart *et al.*, 2016).

The heterogeneity in the clinical presentation, growth patterns, and drug responses of leiomyomas suggests that complex biological mechanisms underlie the development and growth of these lesions.

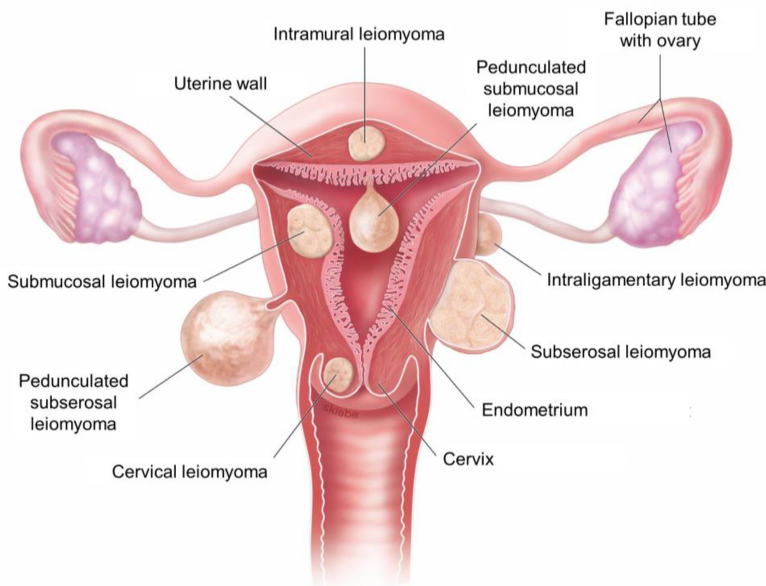

Figure 1. The female reproductive system and different types of leiomyomas classified according to their location relative to the myometrial layers. Modified July 24th, 2017, from informedhealth.org web site (https://www.informedhealth.org/uterine-fibroids.2622.en.html) with the permission of the copyright holder.

1.3. Histopathology

Leiomyomas are typically characterized as firm, whorled nodules with a white cut surface (Oliva *et al*, 2014). They are well circumscribed due to a surrounding pseudocapsule and compressed muscle fibers separating them from the adjacent myometrium. Leiomyomas consist mainly of four different cell types: smooth muscle cells, vascular smooth muscle cells, fibroblasts, and leiomyoma-associated fibroblasts (Holdsworth-Carson *et al.*, 2014). Histologically, the abnormally organized smooth muscle cells are characterized by obscure borders, eosinophilic cytoplasm, and cigar-shaped nuclei with small conspicuous nucleoli and little mitotic activity (Oliva *et al*, 2014). The cells are surrounded by abundant disorganized extracellular matrix (ECM) consisting of collagen, proteoglycans, and fibronectin (Malik *et al.*, 2010). Histological features of leiomyomas may vary in terms of infarct-type necrosis, cellularity, hyalinization, and calcification (Oliva *et al*, 2014).

Approximately 10% of leiomyomas display some features of malignant neoplasms, such as increased cellularity, mitotic activity, or nuclear atypia; these leiomyomas are classified as histopathological variants (Table 2) (Oliva *et al*, 2014). It is unclear whether leiomyomas precede their malignant counterpart, leiomyosarcoma, which is an extremely rare aggressive cancer type with poor prognosis. The majority of leiomyosarcomas are believed to arise *de novo*, and if malignant transformation does occur, it is very rare (Gupta *et al.*, 2008).

Table 2. Features of different subtypes of uterine smooth muscle tumors (Oliva *et al*, 2014).

Subtype	Characteristics
Histopathological leiomyoma variants	
Cellular leiomyoma	Higher cellularity when compared to the adjacent myometrium
Epithelioid leiomyoma	“Epithelial-like” rounded or polygonal cells.
Hydropic leiomyoma	Prominent zonal edema.
Leiomyoma with apoplectic change	Zones of hemorrhagic infarction surrounded by hypercellular areas.
Leiomyoma with bizarre nuclei	Groups of bizarrely shaped cells displaying nuclear atypia
Lipoleiomyoma	Adipocytes intermixed with the smooth muscle cells.
Mitotically active leiomyoma	Mitotic activity of >10 mitotic figures per 10 high-power fields
Myxoid leiomyoma	Myxoid acid-mucin stroma surrounding sparsely occurring cells.
Leiomyomas with unusual growth patterns	
Cotyledonoid dissecting leiomyoma	Irregular dissection of bland smooth muscle cells within the myometrium.
Diffuse leiomyomatosis	Innumerable, ill-defined, confluent, hypercellular tumors throughout the myometrium.
Intravenous leiomyomatosis	Worm-like growth of smooth muscle within vascular spaces
Metastasizing leiomyoma	Extrauterine; lungs are most frequently affected organ; related to a history of leiomyomas.
Intermediate/malignant smooth muscle tumors	
Smooth muscle tumor of uncertain malignant potential	Combination of features of leiomyoma and leiomyosarcoma.
Leiomyosarcoma	Malignant smooth muscle tumors, typically soft, fleshy, necrotic, and hemorrhagic with irregular margins that may display spindle cell, epithelioid, or myxoid morphology.

2. Tumorigenesis

Human tumors arise through a multistep process in which a single cell, or a group of cells, acquires genetic or epigenetic mutations that modify important cellular functions such as cell division, differentiation, or life span, leading to uncontrolled proliferation. Genes that commonly drive tumorigenesis are typically classified as oncogenes or tumor suppressor genes (Vogelstein and Kinzler, 2004). Oncogenes are activated by mutations leading to enhanced or renewed function that promotes an aberrant phenotype, whereas tumor suppressor genes are inactivated by mutations overcoming their normal inhibitory effect on cell proliferation. Genes that facilitate tumor development through genome integrity are called stability genes: their inactivation leads to an increased mutation rate and additional tumorigenic changes.

Carcinogenesis forms a continuum in which cells transform from benign to more malignant by accumulating mutations, which are either neutral passenger mutations or tumor-promoting driver mutations that provide a selective advantage to the cells (Vogelstein *et al.*, 2013; Vogelstein and Kinzler, 2015). During tumor progression, neoplastic cells acquire distinct tumor-promoting properties known as the hallmarks of cancer (Hanahan and Weinberg, 2011). The hallmarks include sustaining proliferative signaling; evading growth suppressors, apoptosis, and immune destruction; enabling replicative immortality; inducing angiogenesis; adapting cellular metabolism; and eventually activating invasion and metastasis. The acquisition of these traits is enabled by genomic instability and tumor-promoting inflammation. Benign tumors, which are typically more stable on the genetic level, may possess many of these hallmarks but lack the capability to spread. Accumulation of mutations during tumor evolution leads to intratumor heterogeneity, where different cell subpopulations that harbor unique genetic alterations may differ phenotypically and functionally. Contributing to the intratumor heterogeneity, a subpopulation of cancer cells possesses stem-like properties enabling them to self-renew and differentiate into various cell types (Hanahan and Weinberg, 2011; Kreso and Dick, 2014). These cancer stem cells may initiate and drive tumor growth. In addition to neoplastic cells, tumors contain stroma, vasculature, immune cells, and a variety of other cell types that form the tumor microenvironment (Whiteside, 2008; Hanahan and Weinberg, 2011). The microenvironment contributes to the tumor heterogeneity, supports tumor growth and invasion, and enables the tumor to escape host immunity.

Tumorigenesis is a sum of genetic, environmental, and lifestyle factors (Hemminki *et al.*, 2006). The majority of tumors occur sporadically, but 1 to 10% of cancers arise due to high-penetrance germline mutations that introduce a high risk for an individual to develop tumors (Nagy *et al.*, 2004; Hemminki *et al.*, 2006). Typically, hereditary tumor syndromes are inherited in an autosomal dominant manner and caused by heterozygous germline mutations in tumor suppressor genes (Nagy *et al.*, 2004). Syndromic tumors develop when somatic mutations inactivate the wild-type allele. Common low-penetrance variants may jointly increase the risk of developing tumors together with environmental factors (Antoniou *et al.*, 2002; Hemminki *et al.*, 2006; Zheng *et al.*, 2008). Somatic mutations arise spontaneously through DNA replication, repair, and recombination errors during cell division, or they can be caused by mutagens that can be endogenous like hormones, or exogenous, such as

tobacco, chemicals, and ultraviolet light. In addition, infectious agents, such as human papilloma virus, may initiate tumorigenesis. Tumor progression and growth requires proliferative signals provided by, for example, hormones and growth factors.

2.1. Leiomyomagenesis

Leiomyomas are monoclonal and multiple nodules within a single uterus generally have a unique origin, although some concurrent leiomyomas have displayed identical chromosomal changes indicating a shared origin (Linder and Gartler, 1965; Townsend *et al.*, 1970; Nilbert and Heim, 1990; Bonatz *et al.*, 1998; Mehine *et al.*, 2013; 2015; Holdsworth-Carson *et al.*, 2014). As leiomyomas are monoclonal but consist of multiple cell types, leiomyomas must originate from a single parental cell with multipotent stem cell properties, possibly from a transformed myometrial stem cell (Figure 2) (Mas *et al.*, 2012; Ono *et al.*, 2012). The triggers that may transform a myometrial stem cell into a leiomyoma stem cell are likely to be genetic or epigenetic changes potentially induced and promoted by a preceding condition or injury in the uterus, such as abnormal estrogen and progesterone signaling, ischemia, inflammation, or viral infections (Bullerdiek, 1999; Flake *et al.*, 2003; Wegienka, 2012). Leiomyomas contain these cells with stem-like properties that may have the potential to differentiate into all the cell types found in these tumors, possibly through estrogen and progesterone induced paracrine activation of Wnt/β-catenin signaling (Mas *et al.*, 2012; Ono *et al.*, 2012; 2013).

Estrogen and progesterone are essential promoters of leiomyomas. The molecular mechanism underlying this growth stimulation remains elusive, but it seems to involve altered activity of aromatase and 17β-hydroxysteroid dehydrogenase that lead to accumulation of estradiol and thereby upregulation of estrogen and progesterone receptors. The tumor promoting effect of estrogen may arise through induction of progesterone receptors and thus progesterone would be the main regulator of leiomyoma growth. Essentially, estrogen and progesterone upregulate various growth factors and activate signaling pathways that enhance cell proliferation and ECM formation. The promotion of leiomyoma development is likely to result from the interaction of these hormones and other factors. (Flake *et al.*, 2003; Borahay *et al.*, 2015; Stewart *et al.*, 2016).

In addition to steroid hormones, reduced levels of retinoic acid (RA) and vitamin D may play a role in leiomyoma development (Borahay *et al.*, 2015; Brakta *et al.*, 2015; Stewart *et al.*, 2016). Vitamin D3 regulates leiomyoma growth potentially through altering the levels of ECM degrading matrix metalloproteinases, vitamin D receptors, ECM proteins, as well as estrogen and progesterone receptors leading to induction of apoptosis and modulation of the ECM. RA inhibits proliferation, induces apoptosis, and reduces expression of ECM components in leiomyoma cells (Gilden *et al.*, 2012; Borahay *et al.*, 2015; Stewart *et al.*, 2016).

In comparison to the normal myometrium, leiomyomas display altered levels of several growth factors including transforming growth factor β, acidic and basic fibroblast growth factor, insulin-like growth factor, platelet-derived growth factor, epidermal growth factor, and vascular endothelial growth factor. These growth factors can activate signaling

pathways such as PI3K/AKT/mTOR, MAPK/ERK, and transforming growth factor β/Smad resulting in the promotion of cell proliferation and survival, as well as the synthesis of DNA, protein, and the ECM. (Borahay *et al.*, 2015).

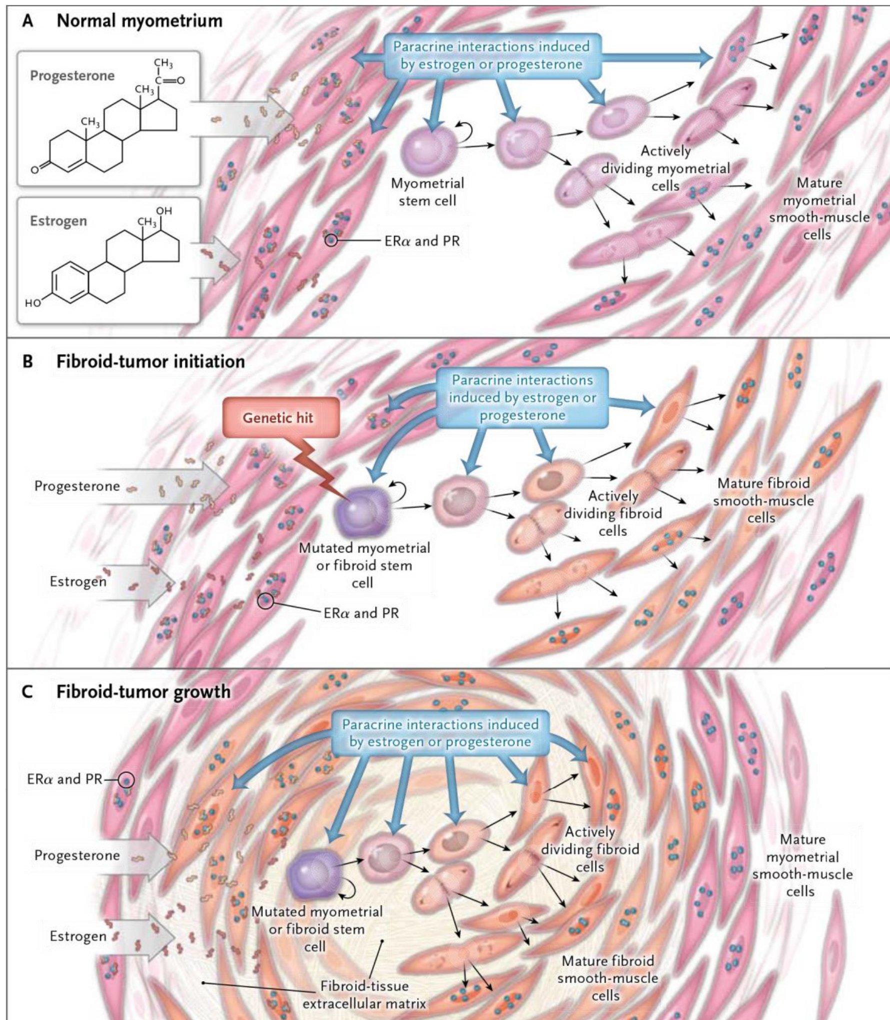

Figure 2. Schematic view of leiomyomagenesis. A) Myometrial stem cells account for the proliferation of the smooth muscle cells of the normal myometrium. These stem cells express lower levels of estrogen receptor (ERα) and progesterone receptor (PR) than the mature smooth muscle cells. Thus, estrogen and progesterone potentially induce stem cell self-renewal and proliferation in a paracrine fashion. B) Leiomyomas (fibroids) seem to originate from a myometrial stem cell that has been transformed into a leiomyoma stem cell possibly by a genetic hit. This leiomyoma stem cell has the potential to self-renew and proliferate until it differentiates into a mature leiomyoma cell. C) The uncontrolled proliferation of the self-renewing leiomyoma stem cell results in the formation and growth of a leiomyoma. Reproduced with permission from Bulun 2013, Copyright Massachusetts Medical Society.

2.2. Genetics of leiomyomas

Like most, if not all, human neoplasms, leiomyomas are a genetic disease. A genetic component is evident as leiomyomas are a manifestation of some genetic tumor predisposition syndromes (Hobert and Eng, 2009; Lehtonen, 2011; Hulsebos *et al.*, 2014). Racial disparity, familial aggregation, and twin studies suggest the presence of genetic susceptibility to leiomyomas as well (Vikhlyaeva *et al.*, 1995; Kjerulff *et al.*, 1996; Marshall *et al.*, 1997; Luoto *et al.*, 2000; Faerstein *et al.*, 2001a; Sato *et al.*, 2002; Baird *et al.*, 2003; Huyck *et al.*, 2008; Moorman *et al.*, 2013; Stewart *et al.*, 2013; Drayer and Catherino, 2015). Moreover, non-random somatic mutations have been observed in leiomyomas (Lehtonen *et al.*, 2004; Sandberg, 2005a; Mäkinen *et al.*, 2011). The genetic background of leiomyomas is diverse, suggesting that multiple different mechanisms lead to their development. Leiomyomas display epigenetic alterations involving DNA methylation, histone modification, and microRNAs, but their roles in leiomyomagenesis remain largely unclear (Yang *et al.*, 2016).

2.2.1. Genetic predisposition

Leiomyomas are associated with tumor predisposition syndromes including Cowden syndrome, Schwannomatosis, and hereditary leiomyomatosis and renal cell cancer (HLRCC), caused by heterozygous germline mutations in *phosphatase and tensin homolog* (*PTEN*)*, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily B, member 1* (*SMARCB1*)*,* and *fumarate hydratase* (*FH*), respectively (Hobert and Eng, 2009; Lehtonen, 2011; Hulsebos *et al.*, 2014). These high penetrance predisposition genes explain only a minor proportion of the estimated heritability of leiomyomas, however.

The most prominent of these syndromes, in regards to leiomyomas, is HLRCC, which is characterized by skin and uterine leiomyomas as well as increased risk for highly aggressive papillary type II renal cell cancer (Lehtonen, 2011). The risk of renal cell cancer is approximately 20%, whereas the penetrance for leiomyomas is nearly complete. HLRCC-related leiomyomas entail a more severe clinical phenotype as they emerge at an earlier age, are more numerous, and lead to symptoms more often than sporadic leiomyomas. These syndromic leiomyomas harbor atypical histological features including increased cellularity, multinucleated cells, and nuclear atypia with prominent orangeophilic nucleoli surrounded by a perinuclear halo, as well as hemangiopericytoma-like vessels (Oliva *et al*, 2014).

Genome-wide linkage, association, and admixture mapping studies have identified multiple low-penetrance predisposition loci for leiomyomas, but few have been validated by replication studies (Table 3) (Cha *et al.*, 2011; Eggert *et al.*, 2012; Wise *et al.*, 2012; 2016; Edwards *et al.*, 2013; Aissani *et al.*, 2015; Zhang *et al.*, 2015; Bondagji *et al.*, 2017). Pinpointing the causative variants within these chromosomal loci and understanding the mechanisms by which these variants increase leiomyoma risk requires further research. Challenges with genetic association studies are the same as with epidemiological studies: the high incidence of asymptomatic leiomyomas and their relatively late age of onset.

Table 3. Low-penetrance predisposition loci for leiomyomas.

Chromosome	Risk variant/locus	Candidate/nearby gene(s)	Population(s)	Reference(s)
1q42.2	rs7546784	*PCNXL2*	American	(Zhang *et al.*, 2015)
2q32.2	rs256552	*PMS1*	American	(Zhang *et al.*, 2015)
3p21.31	169 614-76 307 730	-	Caucasian	(Eggert *et al.*, 2012)
10p11.21	9 632 527-72 985 946	*COL13A1*	Caucasian	(Eggert *et al.*, 2012)
10q24.33	rs7913069	*SLK, OBFC1*	Japanese	(Cha *et al.*, 2011)
11p15.5	rs2280543	*ODF3, BETL1, RIC8A, SIRT3*	Japanese, Caucasian	(Cha *et al.*, 2011; Edwards *et al.*, 2013)
17q25.3	rs4247357	*FASN, CCDC57, SLC16A3*	Caucasian	(Eggert *et al.*, 2012)
22q13.1	rs12484776	*TNRC6B, ADSL*	Japanese, Caucasian, American, Saudi Arabian	(Cha *et al.*, 2011; Edwards *et al.*, 2013; Aissani *et al.*, 2015; Bondagji *et al.*, 2017)

2.2.2. Somatic chromosomal aberrations

According to cytogenetic studies, 40% of leiomyomas harbor chromosomal aberrations (Table 4) (Sandberg, 2005a; Mitelman *et al.* 2017). These chromosomally abnormal tumors tend to be larger in size, less frequently submucosal, and histologically more cellular and mitotically active than chromosomally normal leiomyomas (Sandberg, 2005a). Whether the chromosomal abnormalities are primary or secondary changes remains to be established, although some of them, such as translocations involving 12q15, occur clonally, suggesting that they are initiating driver events, whereas some, such as 7q22 deletions, may be subclonal and co-occur with other chromosomal abnormalities, suggesting that they are secondary events involved in tumor progression rather than initiation (Pandis *et al.*, 1990; Xing *et al.*, 1997).

Table 4. Recurrently altered chromosomal loci in leiomyomas (Mitelman *et al.* 2017).

Chromosome	Change(s)	Putative target gene(s)	Estimated proportion of leiomyomas (%)	Reference(s)
1p36	Translocation/Deletion	*AJAP1, NPHP4*	2	(van Rijk *et al.*, 2009)
1q43	Deletion	*FH*	<1	(Tomlinson *et al.*, 2002)
3q	Deletion	-	3	-
6p21	Translocation	*HMGA1*	3	(Nezhad *et al.*, 2010)
7q22	Deletion/Inversion/Translocation	*CUX1*	6	(Moon *et al.*, 2002)
10q22	Translocation/Deletion	*KAT6B*	2	(Moore *et al.*, 2004)
12q15	Translocation/Inversion/Trisomy	*HMGA2*	10	(Schoenmakers *et al.*, 1995)
14q24	Translocation	*RAD51B*	9	(Schoenmakers *et al.*, 1999)
19q	Deletion	-	1	-
22q	Deletion/Monosomy	*DEPDC5*	2	(Mehine *et al.*, 2015)
X	Microdeletions	*COL4A5/6, IRS4*	1	(Mehine *et al.*, 2013; 2016)

2.2.3. High mobility group AT-hook 2 (HMGA2)

The target gene of the most frequent rearrangements in leiomyomas is *high mobility group AT-hook 2* (*HMGA2*) on chromosome 12q15 (Schoenmakers *et al.*, 1995; Fusco and Fedele, 2007). Translocations involving 12q15 result in increased expression of *HMGA2,* potentially through acquisition of an active enhancer or promoter, or alternatively through loss of the 3'UTR of *HMGA2,* which contains binding sites for *let-7* microRNAs that directly inhibit *HMGA2* expression (Quade *et al.*, 2003; Klemke *et al.*, 2009; 2010). Upregulation of *HMGA2* may also derive from reduced expression of *let-7* in leiomyomas (Wang *et al.*, 2007; Peng *et al.*, 2008; Klemke *et al.*, 2009). Translocations and overexpression of *HMGA2*, as well as low *let-7* expression, associate with larger leiomyoma size, suggesting that *HMGA2* contributes to tumor growth (Sandberg, 2005a; Wang *et al.*, 2007; Peng *et al.*, 2008). In addition to leiomyomas, *HMGA2* rearrangements occur in most benign tumor types of mesenchymal origin, for example, in fibroadenomas of the breast (Fusco and Fedele, 2007). HMGA2, which indirectly regulates gene expression by binding to AT-rich DNA sequences and thereby alters chromatin structure, is normally expressed during embryonic development and is generally silenced in differentiated adult tissues. The tumor-driving mechanism of HMGA2 is incompletely understood, although it probably relates to its role in regulating gene expression and thereby in controlling crucial cellular functions, such as proliferation. The most frequent translocation partner of 12q15 is 14q24, affecting *RAD51 paralog B* (*RAD51B*), which encodes for a repair protein of DNA-double-strand breaks (Schoenmakers *et al.*, 1999). Whether RAD51B contributes to leiomyomagenesis is unclear.

2.2.4. Fumarate hydratase (FH)

In addition to HLRCC-related leiomyomas, *FH* mutations underlie 1% of sporadic leiomyomas (Lehtonen *et al.*, 2004). Both hereditary and sporadic tumors display point mutations or deletions in both alleles of *FH* resulting in FH deficiency (Lehtonen *et al.*, 2004; Lehtonen, 2011). FH is a tricarboxylic acid (TCA) cycle enzyme that catalyzes the conversion of fumarate into malate. FH deficiency leads to truncation of the TCA cycle and accumulation of fumarate (Pollard *et al.*, 2005). Accumulated fumarate spontaneously reacts with cysteine sulfhydryl groups to form S-(2-succinyl)cysteine, a reaction called succination (Kinch *et al.*, 2011). One of the proteins succinated by the accumulated fumarate is kelch-like ECH-associated protein 1 (KEAP1), which is a negative regulator of transcription factor Nuclear factor erythroid 2-related factor 2 (NRF2). NRF2, a master regulator of the antioxidant response, is normally activated under oxidative stress, but its continuous activation may drive tumorigenesis (Gorrini *et al.*, 2013). Additionally, fumarate accumulation leads to stabilization of hypoxia-inducible factor 1-α (HIF-1-α) through the inhibition of HIF prolyl hydroxylases that degrade HIF-1-α (Isaacs *et al.*, 2005; Pollard *et al.*, 2005). HIF-1-α is a transcription factor that under low-oxygen conditions activates target genes involved in vascularization, glycolysis, glucose transport, cell survival, and metastasis (Powis and Kirkpatrick, 2004).

2.2.5. Mediator complex subunit 12 (MED12)

Owing to the recent evolution of next-generation sequencing techniques, exome sequencing of 18 leiomyomas and subsequent screening of additional 207 leiomyomas altogether derived from 80 Finnish patients revealed *mediator complex subunit 12* (*MED12*) mutations in 71% of the leiomyomas. All of the detected mutations clustered in exon 2 or in the preceding intron-exon boundary; this region is highly evolutionarily conserved. The majority of mutations were missense mutations affecting codon 44 (69%), yet missense mutations affecting codons 36 (7%) and 43 (2%) also occurred. Ten (6%) leiomyomas displayed an intronic point mutation eight base pairs upstream of exon 2 resulting in the addition of two amino acids to the protein product. The rest of the mutations (16%) were insertion-deletions and were predicted to result in an in-frame protein product. No additional mutations emerged when all *MED12* exons were sequenced in 20 mutation-negative and 10 mutation-positive leiomyomas. Mutations were heterozygous, but cDNA sequencing of 16 tumors showed that the tumors expressed mainly the mutated form of *MED12*. Sequencing of the corresponding normal myometrium samples confirmed the somatic origin of the mutations. *MED12*-mutation-positive leiomyomas were non-randomly distributed among the patients and were significantly smaller than mutation-negative tumors. Taken together, these data suggest that mutated *MED12* drives leiomyomagenesis. (Mäkinen *et al.*, 2011).

The 45 exons of *MED12* span 24 kilobases on chromosome Xq13. *MED12* is generally continuously expressed across human tissues (Philibert and Madan, 2007). The MED12 protein consists of 2177 amino acids comprising four domains and three conserved sequence motifs (Figure 3) (Philibert and Madan, 2007; Finn *et al.*, 2016). One of these conserved motifs, Med12-PQL, contains binding sites for β-catenin, the transcription factors Sox9 and Gli3, and the histone methyltransferase G9a (Zhou *et al.*, 2002; 2006; Kim *et al.*, 2006; Ding *et al.*, 2008). MED12 is a part of an evolutionarily conserved multiprotein complex called the Mediator (Allen and Taatjes, 2015). The Mediator consists of three core domains: the tail, middle, and head, and a detachable kinase module called the cyclin-dependent kinase (CDK) 8 module, which comprises MED12, mediator complex subunit 13 (MED13), Cyclin C, and CDK8 or alternatively CDK19 (Figure 3). The Mediator regulates basal transcription by acting as a bridge between RNA polymerase II and transcription factors. The attachment of the CDK8 module to the core Mediator controls the formation of the preinitiation complex and thereby initiation of transcription. The CDK8 module also indirectly regulates transcription by phosphorylating target molecules. Apart from its function as part of the CDK8 module, MED12 plays a role in multiple developmental pathways including Wnt and Sonic Hedgehog signaling (Zhou *et al.*, 2006; Rocha *et al.*, 2010). In addition, MED12 links G9a with RE1 silencing transcription factor (REST) to silence its neuronal target genes (Ding *et al.*, 2008). Germline mutations affecting the leucine-serine-rich and proline-glutamine-leucine-rich domains of MED12 cause a broad spectrum of X-linked intellectual disability syndromes (Graham and Schwartz, 2013). The role of *MED12* mutations in human tumors requires further elucidation.

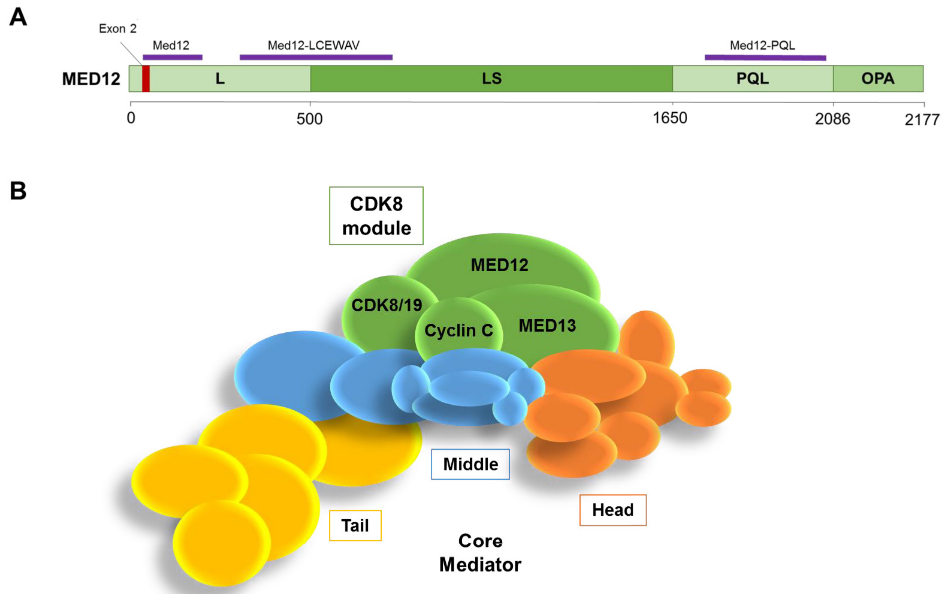

Figure 3. Schematic view of the MED12 protein and the Mediator. A) MED12 consists of 2177 amino acids comprising four domains: a leucine-rich (L) domain, a leucine-serine-rich (LS) domain, a proline-glutamine-leucine-rich (PQL) domain, and a glutamine-rich opposite paired (OPA) domain. Three sequence motifs, Med12, Med12-LCEWAV, and Med12-PQL, are conserved in eukaryotes. B) The Mediator consists of 30 subunits that form the core mediator and a detachable CDK8 module.

AIMS OF THE STUDY

1. To study the occurrence of *MED12* mutations in leiomyomas from women with African ancestry
2. To study the frequency of *MED12* mutations in an unselected sample set of leiomyomas
3. To study the clinical features of *MED12*-mutation-positive and -negative leiomyomas
4. To characterize the metabolomes of leiomyomas harboring distinct genetic drivers

MATERIALS AND METHODS

1. Ethics approval (I-IV)

The Human Research Ethics Committee, Cape Town, South Africa approved Study I. The Ministry of Social Affairs and Health and the Ethics Committee for gynecology and obstetrics, pediatrics, and psychiatry of the Hospital District of Helsinki and Uusimaa, Finland approved Studies I-IV. Patients from the B, My, My1000, My5000, My6000, and N series signed an informed consent (I-IV). The director of the health-care unit approved the acquisition of anonymous samples in the M series (IV).

2. Samples (I-IV)

For Study I, we obtained genomic DNA from 28 fresh frozen leiomyomas and 14 corresponding normal myometrial tissue samples derived from 18 women with African ancestry from the Department of Obstetrics and Gynecology, Faculty of Health Sciences, University of Cape Town/Groote Schuur Hospital, Cape Town, South Africa (Table 5). Information on the patients' age at diagnosis, total number of leiomyomas, and tumor size accompanied the samples.

Table 5. Summary of samples included in Studies I-IV.

Series	Patients	Leiomyomas	Time frame	Hospital	Clinical data available	Population (n)
Study I						
African	18	28	not available	Groote Schuur Hospital	Yes	Black African (6)/ Colored (12)
Study II						
Consecutive set						
My5000	14	103	2013	Jorvi hospital	Yes	Finnish (13)/ Romanian (1)
Unselected set						
My1000	11	54	2012	Women's hospital	Yes	Finnish (10)/ Swedish (1)
My5000	4	22	2013	Jorvi hospital	Yes	Finnish (4)
Study III						
My6000	244	763	2013-2015	Kätilöopisto	Yes	Finnish (219)/ non-Finnish (25)
Study IV						
B7 (HLRCC)	1	1	2002	Women's hospital	Yes	Finnish (1)
N7 (HLRCC)	1	1	2007	Turku University Hospital	Yes	Finnish (1)
M	6	9	2001-2002	Women's hospital	No	not available (6)
My	3	4	2003-2008	Women's hospital	Yes	Finnish (3)
My5000	1	1	2013	Jorvi hospital	Yes	Finnish (1)
My6000	5	9	2013-2015	Kätilöopisto	Yes	Finnish (5)

For Studies II-IV, leiomyoma and corresponding normal myometrial tissue samples were collected as fresh frozen from patients undergoing hysterectomy for any medical indication (Table 5). For Study II, we set out to collect all tumor nodules from unselected series of patients. The My1000 series constituted 54 leiomyomas from 11 patients operated on at the Women's Hospital, Helsinki University Hospital (HUH) between January and April 2012. The My5000 series included 125 leiomyomas from 18 patients operated on at Jorvi Hospital (part of HUH) between March and June 2013. In the My5000 series, some tumor samples that served as routine diagnostic specimens were lost to the study. For the My1000 and My5000 series, the uteri were carefully scrutinized and all visible tumor nodules were collected, with the exception of one patient (My5013) who had > 20 leiomyomas, of which 14 were obtained. For the My5000 series, the size of each tumor was recorded when possible; the smallest being 5 mm in diameter. The first 14 patients in the My5000 series, with a total of 103 leiomyomas, formed “the consecutive set”, whereas the My1000 series and the remaining samples from the My5000 series constituted “the unselected set” of 15 patients with a total of 76 leiomyomas. Clinical data were collected from the patients' medical records.

For Study III, we collected a large well-documented series (My6000) of 763 leiomyomas from 244 patients, who underwent hysterectomy at the Kätilöopisto Maternity Hospital (part of HUH) between October 2013 and November 2015 (Table 5). Pathologists dissected the uteri, harvested all feasible leiomyomas > 10 mm in diameter, and recorded the size, location, and any special characteristics of the tumors. A formalin-fixed paraffin-embedded tissue block was prepared from each tumor. Pathological evaluation was based on the diagnostic pathology reports and all leiomyomas that were suspected to be histopathological variants were re-evaluated from hematoxylin-eosin-stained tissue slides by an expert gynecological pathologist. Clinical data were collected from the patients' medical records and some of the patients completed a designed questionnaire.

In Study IV, we selected 25 leiomyoma samples, obtained from 17 patients, based on their genetic driver determined in Studies II-III or during our previous efforts: seven leiomyomas were *FH* deficient, seven harbored a mutation in *MED12*, two displayed *HMGA2* overexpression, and nine were negative for all three, “the triple wild-type leiomyomas” (Table 5) (Mäkinen *et al.*, 2011; Kämpjärvi *et al.*, 2016b; Mehine *et al.*, 2016). All tissue samples had been screened for *MED12* mutations using Sanger sequencing and for chromosomal alterations using whole-genome sequencing or single-nucleotide polymorphism array data. Tumor samples with a deletion affecting the *FH* locus and the corresponding normal myometrium samples had been screened for *FH* mutations using Sanger sequencing. For tumor samples with chromosomal alterations affecting the *HMGA2* locus, the relative quantification of *HMGA2* expression had been assessed using gene expression array data or *HMGA2* quantitative polymerase chain reaction (qPCR).

3. Mutation screening (I-IV)

The FastDNA® Kit (MP Biomedicals LLC, Solon, OH, USA) was used to extract genomic DNA from fresh frozen tissue. Oligonucleotide primers were designed using Primer3 with the GRCh37/hg19 genome serving as a reference (Untergasser *et al.*, 2012). Polymerase

chain reaction (PCR) followed a standard protocol, and ExoSAP-IT (USB Corporation, Cleveland, OH, USA) or A'SAP (ArcticZymes, Tromsø, Norway) was used to purify the PCR products. Sequencing was performed with the Big Dye Terminator v.3.1 Kit (Applied Biosystems, Foster City, CA, USA) on an ABI3730 Automatic DNA Sequencer (Thermo Scientific Inc., Waltham, MA, USA) at the Technology Center, Institute for Molecular Medicine Finland, Helsinki, Finland. Sequence graphs were analyzed manually and with Mutation Surveyor software (Softgenetics, State College, PA, USA).

4. *HMGA2* qPCR (IV)

Total RNA was extracted using NucleoZOL reagent (Macherey-Nagel GmbH & Co. KG, Germany). *HMGA2* qPCR was performed on a 7500 Fast Real-Time PCR System with Assay No. Hs04397751_m1 (Applied Biosystems), using 18S rRNA as an endogenous control. Gene expression levels were assessed using the comparative Ct method.

5. Gene expression data (IV)

Previously generated and analyzed gene expression data using GeneChip Human Exon 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) of 94 leiomyomas, including 10 with *FH* deficiency, 34 with a *MED12* mutation, 27 with an *HMGA2* rearrangement, and 23 triple wild-type leiomyomas, and of 60 corresponding myometrium samples were available for Study IV (Mehine *et al.*, 2016).

6. Metabolomic profiling (IV)

Metabolon Inc. (Durham, NC, USA) performed the metabolomic profiling. Samples were prepared using the automated MicroLab STAR® system (Hamilton Company, Reno, NV, USA). For quality control (QC), recovery standards were added prior to extraction. A pooled matrix of study samples served as a technical replicate, water samples served as process blanks, and QC standards added to every sample allowed instrument performance monitoring and aided chromatographic alignment.

The sample extract was dried then reconstituted in solvents compatible to each of the four independent platforms: two for analysis by two separate reverse phase ultra-performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) methods with positive ion mode electrospray ionization (ESI), one for analysis by reverse phase UPLC-MS/MS with negative ion mode ESI, and one for analysis by hydrophilic interaction chromatography UPLC-MS/MS with negative ion mode ESI. All methods utilized an ACQUITY UPLC (Waters, Milford, MA, USA) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated ESI-II source and Orbitrap mass analyzer operated at 35 000 mass resolution. The first aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05%

perfluoropentanoic acid and 0.1% formic acid. The second aliquot was analyzed using acidic positive ion conditions using chromatographical optimization for more hydrophobic compounds. In this method, the extract was gradient eluted from the same C18 column using methanol, acetonitrile, water, 0.05% perfluoropentanoic acid, and 0.01% formic acid. The third aliquot was analyzed using basic negative ion optimized conditions using a separate C18 column. The basic extracts were gradient eluted from the column using methanol and water with 6.5mM ammonium bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a hydrophilic interaction chromatography column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM ammonium formate at pH 10.8. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range covered 70 to 1000 m/z.

Raw data were extracted, peak-identified, and QC processed using Metabolon's hardware and software. Compound identification was based on retention time/index, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the Metabolon library spectrum containing >3300 purified standard compounds. Additional mass spectral entries were created for recurrent, structurally unnamed, biochemicals. The area under the curve was used to quantify the peaks.

7. Statistical analyses (I-IV)

In Study I, R software version 2.14.0 was used for statistical analyses (R Core Team, 2015). Pearson's χ^2 test was computed to assess the difference between the proportion of *MED12*-mutation-positive leiomyomas in Finnish and South African patients. Fisher's exact test was used to assess the difference between the frequency of large (> 5.5 cm in diameter, the mean tumor size in Finnish and South African patients) mutation-positive leiomyomas in South African and Finnish patients. A two-sided p-value (*P)* < 0.05 indicated statistical significance.

In Study II, R software version 3.0.2 was used for statistical analyses (R Core Team, 2015). We used the Wilcoxon rank-sum test to assess the associations between *MED12*-mutation status and continuous clinical variables, including tumor size, number of leiomyomas, age at hysterectomy and diagnosis, diagnosis-to-hysterectomy interval, body mass index (BMI), uterine weight, parity, menarche, and alcohol consumption. We used Fisher's exact test to assess the associations between *MED12*-mutation status and nominal clinical variables, including nulliparity, cesarean section, miscarriage, infertility, menopausal status, smoking, reported symptoms, prior treatments, abnormal Papanicolaou smear, and other gynecologic diagnoses. A false-discovery rate correction was applied (Benjamini and Hochberg, 1995). A two-sided $P < 0.05$ denoted statistical significance.

In Study III, R software version 3.2.3 was used for statistical analyses (R Core Team, 2015). To predict *MED12*-mutation status (a binary variable) by tumor characteristics (size, location, and histotype), we applied a generalized estimating equation method with an

exchangeable correlation structure to take into account the possibility that multiple leiomyomas within a uterus may not be independent. We used Poisson regression to model *MED12*-mutation-negative-leiomyoma counts, and negative binomial regression to model *MED12*-mutation-positive-leiomyoma counts to account for overdispersion in the corresponding Poisson model. In both of these count models, explanatory variables included parity, menopausal status, age at hysterectomy, infertility, smoking, BMI, family history of leiomyomas, oral contraceptive use, history of chlamydia and pelvic inflammatory disease (PID), hypertension, thyroid disorder, diabetes, and prior leiomyoma surgery. We applied the likelihood ratio test, the generalized Pearson statistic, and model deviance to compare negative binomial and Poisson regression models and assess goodness of fit. Spearman correlation matrix and variance inflation factors were computed to evaluate possible collinearity among explanatory variables. We applied a Bonferroni correction ($\alpha = 0.05/34$) to adjust for multiple comparisons; a two-sided $P < 0.00147$ indicated statistical significance.

In Study IV, Partek Genomics Suite™ version 6.6 (Partek Inc., St. Louis, MO, USA) was used for statistical analyses. Raw metabolomic data were rescaled to set the median of each compound to 1 and were $\log_2$ transformed. Missing values were imputed with the minimum observed value for each compound. Principal component and unsupervised hierarchical clustering analyses (cosine dissimilarity) were performed for all samples using all metabolites. The correlation between tumor size (largest diameter) and the individual metabolite levels were evaluated using Pearson's correlation coefficients. We used a two-way analysis of variance (ANOVA) to identify metabolites that were dysregulated between leiomyomas and the corresponding myometrium samples. We used a one-way ANOVA to identify metabolites that were dysregulated in each leiomyoma subtype (*FH*, *MED12*, *HMGA2*, and triple wild type) as compared with all myometrium samples. We used three-way ANOVA to compare leiomyomas of the *MED12* and *FH* subtypes against the rest of the leiomyoma and myometrium samples to identify the most uniquely dysregulated metabolites. We employed pathway enrichment analysis using the significantly dysregulated metabolites for each leiomyoma subtype to identify significantly dysregulated pathways. False discovery rate correction was applied (Benjamini and Hochberg, 1995). A q-value < 0.1 denoted statistical significance.

The gene expression data were analyzed previously by comparing leiomyomas of each subtype against all normal myometrium samples using one-way ANOVA followed by false discovery rate control (Mehine *et al.*, 2016). We employed comparison analysis in Ingenuity Pathway Analysis version 33559992 (Qiagen, Redwood City, CA, USA) to identify pathways that were significantly dysregulated in both the metabolomic and gene expression data ($P < 0.05$ in both) for leiomyomas of the *FH*, *MED12,* and *HMGA2* subtypes. Analysis was carried out with each set of significantly dysregulated metabolites (q-value < 0.1) and each set of significantly differentially expressed genes (q-value < 0.1, |fold change| > 1.5).

RESULTS

1. *MED12* mutations in leiomyomas (I-III)

1.1. MED12 mutations in different sample series (I-III)

We detected *MED12* exon 2 mutations in 14 (50%) out of 28 leiomyomas from 18 South African patients, including 6 Black African and 12 Colored women (Figure 4A). Nine (50%) patients had at least one mutation-positive tumor. All mutations were heterozygous and resulted in an in-frame transcript. Normal myometrium was available from 14 patients, and the somatic status of the mutations in leiomyomas from these patients (10/14, 71%) was confirmed.

We detected *MED12* exon 1 and 2 mutations in 73 (83%) out of 88 leiomyomas from 13 consecutive patients (Figure 4B), in 65 (86%) out of 76 leiomyomas from 15 unselected patients (Figure 4C), and in 599 (79%) out of 763 leiomyomas from the My6000 series of 244 patients (Figure 4D). Correspondingly, 10 (77%) out of 13 consecutive patients, 11 (73%) out of 15 unselected patients, and 177 (73%) out of 244 patients had at least one *MED12*-mutation-positive leiomyoma. All mutations were heterozygous and somatic, and resulted in an in-frame transcript. Of note, one patient with 15 *MED12*-mutation-negative leiomyomas in the consecutive series was excluded from the analysis due to a germline *FH* mutation, because the objective was to study the frequency of *MED12* mutations in sporadic leiomyomas.

1.2. MED12-mutation status and clinical characteristics (I-III)

The frequency of *MED12* mutations was significantly different in leiomyomas from South African women as compared to leiomyomas from Finnish women in the *MED12*-mutation discovery study ($P = 0.045$), but no significant difference was found when only the large tumors were taken into consideration ($P = 0.69$) (Mäkinen *et al.*, 2011). In the series of 244 patients, 25 were of non-Finnish origin; the mutation frequencies were similar in leiomyomas from Finns (535/679, 79%) and from non-Finns (64/84, 76%).

MED12-mutation-positive tumors were significantly smaller than mutation-negative tumors in the analysis of 108 leiomyomas in Study II ($P = 0.026$), as well as in the analysis of 748 leiomyomas in Study III ($P = 4.2\times10^{-9}$), in which positive mutation status was also associated with conventional histology ($P = 0.0013$), and subserous relative to intramural location ($P = 0.00082$).

In Study II, we explored the associations between *MED12*-mutation status and various clinical variables by comparing 21 patients having at least one mutation-positive leiomyoma with 7 patients having only mutation-negative leiomyomas. The patients with *MED12*-mutation-positive tumors had significantly more tumors than patients with only mutation-negative leiomyomas ($P = 0.010$). We found no significant associations ($P > 0.05$) between the *MED12*-mutation status and age at hysterectomy or diagnosis, BMI, diagnosis-to-

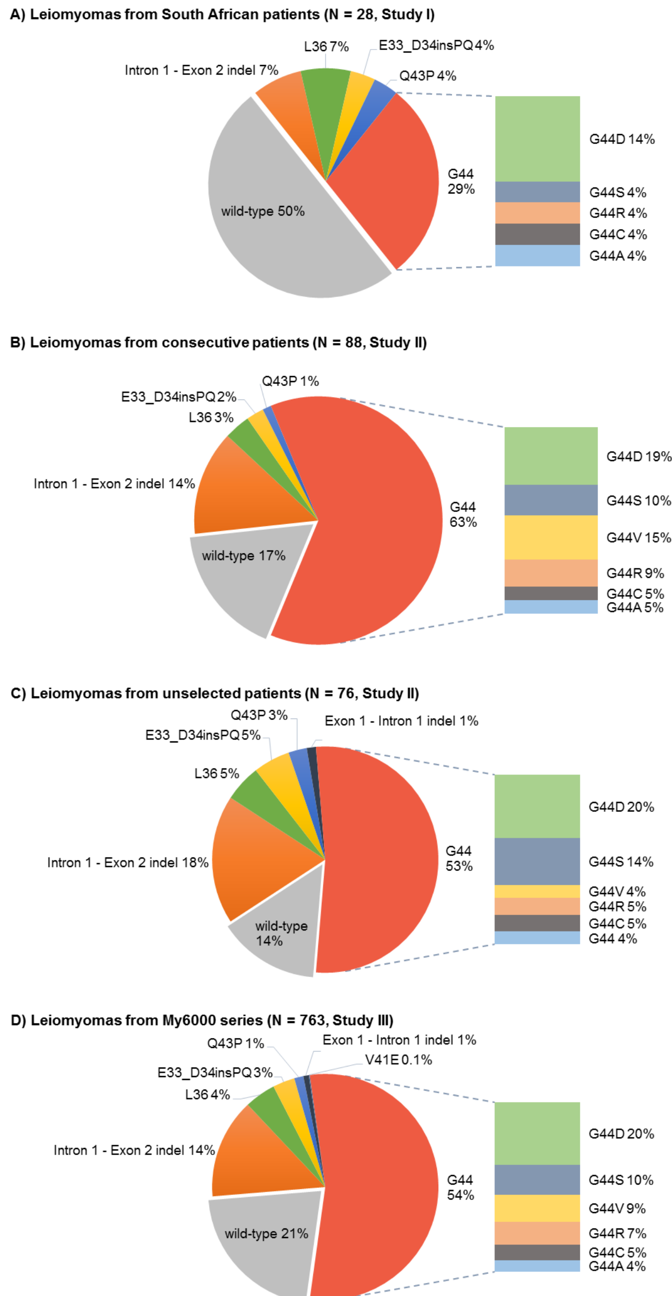

Figure 4. The distribution of different types of *MED12* mutations in the different sample series. The proportion of missense mutations affecting different codons, in-frame insertion and/or deletions (indel) affecting exon 1 - intron 1 and intron 1- exon 2, as well as wild-type lesions are shown separately. Codon 44 (G44) mutations are presented in more detail. Panel D is adapted from Study III under Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

hysterectomy interval, uterine weight, parity, cesarean section, miscarriage, infertility, menarche, menopausal status, alcohol consumption, smoking, reported symptoms, prior treatments, abnormal Papanicolaou smear, nor any other gynecologic diagnosis. A greater proportion of patients with mutation-positive tumors were nulliparous (12/29, 57%) than patients with only mutation-negative tumors (1/7, 14%), although the difference was non-significant ($P > 0.05$). The patient who was excluded from the analyses due to a germline *FH* mutation was diagnosed at the youngest age, had a history of a prior myomectomy, and had a notable family history of leiomyomas.

In the analysis of 244 patients in Study III, we observed a strong correlation between the number of *MED12*-mutation-positive tumors and the total number of leiomyomas (Spearman's correlation coefficient 0.87, $P = 1.3 \times 10^{-77}$), but no correlation between the number of mutation-negative tumors and the total number of leiomyomas. We used separate regression models to study the association between the number of *MED12*-mutation-positive and –negative leiomyomas and clinical factors that have previously been associated with the risk of developing leiomyomas; the number of mutation-positive tumors was inversely associated with parity ($P = 0.00017$) and the number of mutation-negative tumors was significantly associated with a history of PID ($P = 0.00024$). No associations were observed between the number of mutation-positive or -negative leiomyomas and age at hysterectomy, menopausal status, infertility, smoking, BMI, family history of leiomyomas, oral contraceptive use, history of chlamydia, hypertension, thyroid disorder, diabetes, nor prior leiomyoma surgery ($P > 0.00147$).

2. Metabolomic profile of leiomyomas (IV)

Metabolomic analysis identified a total of 588 named and 53 recurrent unnamed metabolites in 25 leiomyoma and 17 myometrium samples. The *FH*-deficient leiomyomas displayed a clearly distinct metabolomic profile according to the principal component and unsupervised hierarchical clustering analyses (Figure 5A-B). Tumor size correlated significantly with tryptophan betaine (r = 0.65, q-value = 0.082) and glucose (r = -0.64, q-value = 0.089) levels.

A total of 70 metabolites were significantly differentially abundant in all leiomyomas when compared to the corresponding myometrium samples. The most significantly dysregulated metabolite was homocarnosine (Figure 5C). Leiomyomas of the *FH*, *MED12, HMGA2,* and triple wild-type subtypes displayed 170, 104, 35, and 22 significantly differentially abundant metabolites, respectively (Table 6). The majority of the significantly dysregulated metabolites were elevated in leiomyomas of the *FH* and *HMGA2* subtypes, whereas the majority were reduced in the *MED12* and triple wild-type subtypes. In the analysis of uniquely dysregulated metabolites that represent candidate biomarkers, the most significant metabolites in leiomyomas of the *FH* subtype were fumarate, N6-succinyladenosine, and malate, and in leiomyomas of the *MED12* subtype histamine, phenylalanine, and homocarnosine.

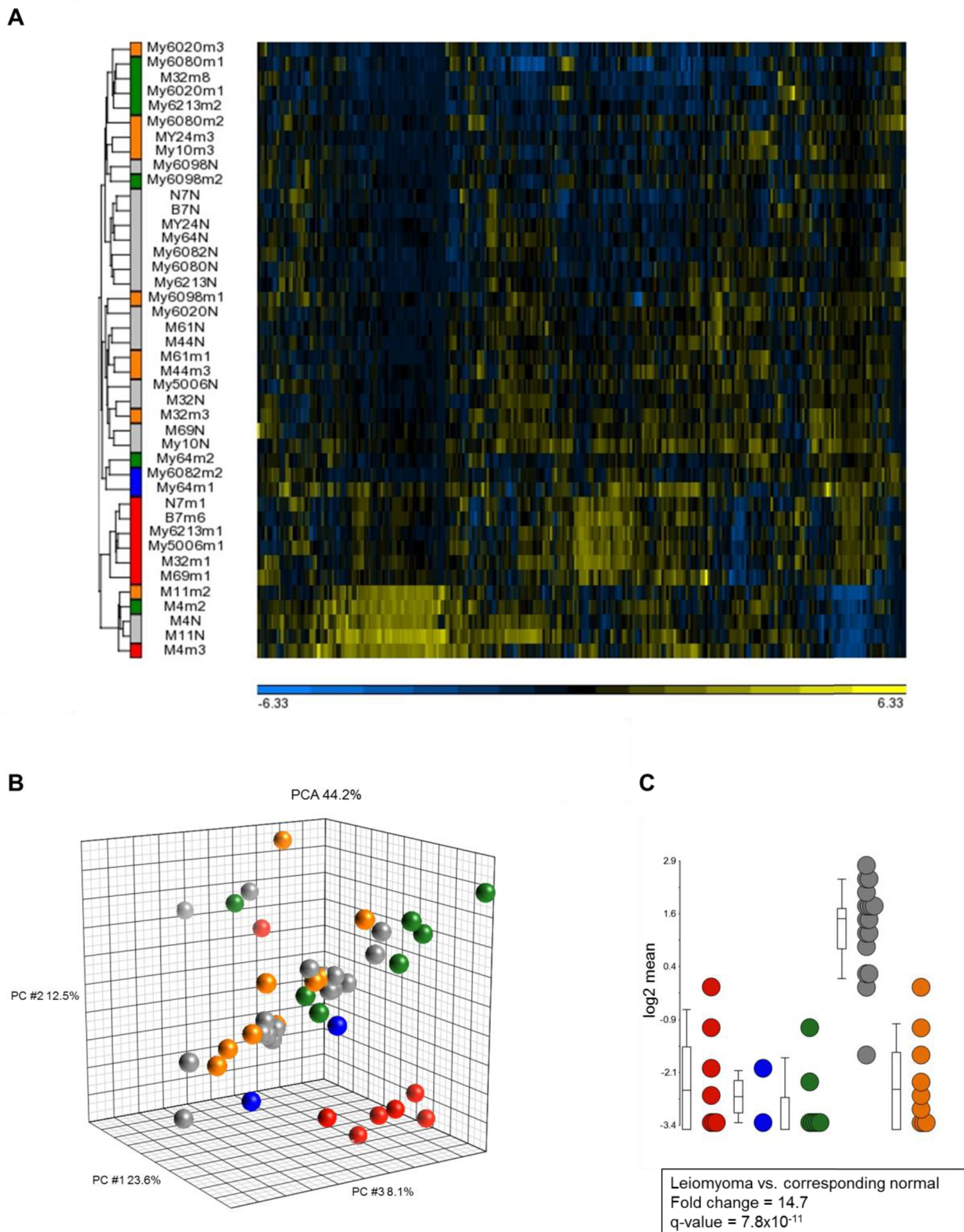

Figure 5. Metabolomic profile of leiomyomas. *FH*-deficient leiomyomas clustered together in A) the unsupervised hierarchical clustering analysis and B) the principal component analysis (PCA). C) Homocarnosine was the most significantly dysregulated metabolite when all leiomyomas were compared with the corresponding myometrium samples. Red denotes leiomyomas with *FH* deficiency, blue with *HMGA2* overexpression, and green with a *MED12* mutation, grey denotes normal myometrium samples and orange triple wild-type leiomyomas.

Table 6. The ten most significantly dysregulated metabolites in leiomyomas of *FH, MED12, HMGA2,* and triple wild type subtypes.

Metabolite	Pathway	Q-value	Fold change
FH			
N6-succinyladenosine	Purine metabolism, adenine containing	$8.3x10^{-11}$	193.7
Fumarate	Tricarboxylic acid cycle	$8.3x10^{-11}$	12.0
Argininosuccinate	Urea cycle; arginine and proline metabolism	$9.6x10^{-11}$	38.5
Malate	Tricarboxylic acid cycle	$1.5x10^{-9}$	10.5
3-methyl-2-oxobutyrate	Leucine, isoleucine, and valine metabolism	$4.4x10^{-9}$	8.3
X - 24728	Unnamed	$6.1x10^{-9}$	231.1
Maleate	Fatty acid, dicarboxylate	$2.2x10^{-8}$	18.3
4-methyl-2-oxopentanoate	Leucine, isoleucine, and valine metabolism	$7.2x10^{-7}$	6.5
X - 15150	Unnamed	$1.4x10^{-6}$	-8.8
β-citrylglutamate	Glutamate metabolism	$1.4x10^{-6}$	-5.8
MED12			
Homocarnosine	Dipeptide derivative	$5.5x10^{-6}$	-18.8
Phenylalanine	Phenylalanine metabolism	$5.5x10^{-6}$	-2.2
Histamine	Histidine metabolism	$1.3x10^{-5}$	-35.9
Biliverdin	Hemoglobin and porphyrin metabolism	$1.3x10^{-5}$	-5.1
Tyrosine	Tyrosine metabolism	$1.3x10^{-5}$	-2.0
X - 21796	Unnamed	$1.4x10^{-5}$	-7.5
X - 15150	Unnamed	$2.2x10^{-5}$	-7.4
X - 12206	Unnamed	$5.3x10^{-5}$	-4.7
Leucine	Leucine, isoleucine, and valine metabolism	0.00051	-1.5
N-acetyl-aspartyl-glutamate	Glutamate metabolism	0.00068	9.1
HMGA2			
3-hydroxydecanoate	Fatty acid, monohydroxy	0.00038	9.4
Behenoyl dihydrosphingomyelin (d18:0/22:0)*	Sphingolipid metabolism	0.00039	-5.6
X - 24243	Unnamed	0.00039	3.5
Palmitoyl dihydrosphingomyelin (d18:0/16:0)*	Sphingolipid metabolism	0.00091	-4.1
1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)*	Plasmalogen	0.0028	-1.8
Linoleoyl-arachidonoyl-glycerol (18:2/20:4) [2]*	Diacylglycerol	0.0029	5.8
Sphingomyelin (d18:0/18:0, d19:0/17:0)*	Sphingolipid metabolism	0.0041	-5.5
Oleoyl-linoleoyl-glycerol (18:1/18:2) [2]	Diacylglycerol	0.0041	2.8
Heme	Hemoglobin and porphyrin metabolism	0.0044	-12.6
Isobutyrylcarnitine (C4)	Leucine, isoleucine, and valine metabolism	0.0052	-8.1
Triple wild type			
Homocarnosine	Dipeptide derivative	$3.8x10^{-5}$	-12.7
Phenylpyruvate	Phenylalanine metabolism	0.00022	-2.7
Heme	Hemoglobin and porphyrin metabolism	0.0015	-3.9
Biliverdin	Hemoglobin and porphyrin metabolism	0.0015	-3.2
X - 21796	Unnamed	0.0043	-3.4
Threonate	Ascorbate and aldarate metabolism	0.0051	-3.0
Retinol (Vitamin A)	Vitamin A metabolism	0.0054	-1.8
Dehydroascorbate	Ascorbate and aldarate metabolism	0.0086	-2.2
Isobutyrylcarnitine (C4)	Leucine, isoleucine, and valine metabolism	0.012	-3.4
Histamine	Histidine metabolism	0.012	-2.7

Pathway enrichment analysis using the metabolomic data predicted multiple pathways to be significantly dysregulated in each leiomyoma subtype (Table 7). Ingenuity pathway analysis comparing the metabolomic and gene expression data revealed six significantly dysregulated pathways in the *FH* subtype: Pentose Phosphate Pathway, Pentose Phosphate Pathway (Oxidative Branch), tRNA Splicing, Gluconeogenesis I, IL-10 Signaling, and Salvage Pathways of Pyrimidine Ribonucleotides; two significantly dysregulated pathways in the *MED12* subtype: Antioxidant Action of Vitamin C and Type II Diabetes Mellitus Signaling; and two significantly dysregulated pathways in the *HMGA2* subtype: Putrescine Degradation III and Sphingosine-1-phosphate Signaling.

Table 7. Significantly enriched pathways predicted from metabolomic data for leiomyomas of *FH, MED12, HMGA2,* and triple wild type subtypes.

Pathway	Enrichment score	Enrichment p-value	Metabolites significantly elevated | reduced	Total number of metabolites in pathway	Proportion of significantly dysregulated metabolites in pathway (%)
FH					
Plasmalogen	5.4	0.0047	6 | 1	10	70
Glycogen metabolism	5.3	0.0048	4 | 0	4	100
Tricarboxylic acid cycle	5.1	0.0059	5 | 0	6	83
Sphingolipid metabolism	4.6	0.0096	7 | 10	38	45
Pentose phosphate pathway	4.0	0.018	0 | 3	3	100
Diacylglycerol	3.3	0.036	8 | 0	16	50
MED12					
Sphingolipid metabolism	22.6	1.5×10^{-10}	0 | 23	38	61
Phosphatidylserine	5.5	0.0042	0 | 3	3	100
Ascorbate (vitamin C) and aldarate metabolism	4.2	0.015	0 | 3	4	75
Dipeptide	3.2	0.043	0 | 4	9	44
Methionine, cysteine, S-adenosylmethionine, and taurine metabolism	3.1	0.044	1 | 4	13	38
HMGA2					
Plasmalogen	9.5	7.5×10^{-5}	4 | 1	10	50
Sphingolipid metabolism	7.6	0.00053	1 | 7	38	21
Diacylglycerol	4.8	0.0085	4 | 0	16	25
Fatty acid, monohydroxy	3.6	0.026	2 | 0	5	40
Triple wild type					
Hemoglobin and porphyrin metabolism	5.0	0.0065	0 | 2	4	50
Ascorbate (vitamin C) and aldarate metabolism	5.0	0.0065	0 | 2	4	50
Vitamin A metabolism	3.4	0.034	0 | 1	1	100
Dipeptide derivative	3.4	0.034	0 | 1	1	100

DISCUSSION

1. *MED12* mutations in tumorigenesis

1.1. MED12 mutations in leiomyomas and other neoplasms

The breakthrough discovery of *MED12* mutations in the majority of leiomyomas suggested that MED12 plays an important role in leiomyomagenesis (Mäkinen *et al.*, 2011). Since the initial finding, this thesis and multiple other studies with various sample sizes encompassing patients of diverse racial and ethnic origins have validated the occurrence of these mutations in leiomyomas (Table 8). The reported frequencies of *MED12* mutations have ranged from 31 to 92%. This large variance in mutation frequencies is probably attributable to small sample sizes and bias introduced by tumor size rather than the ethnicity of the patients. This thesis demonstrated that when the smaller leiomyomas are also examined, the mutation frequency is as high as 86%, and in the largest leiomyoma series thus far with carefully controlled size bias, the mutation frequency was 79%. Recently, we detected that a small proportion of leiomyomas, including one leiomyoma from the South African series, harbor mutations at the end of *MED12* exon 1 (Kämpjärvi *et al.*, 2014). Exon 1 mutations were not covered in *MED12*-mutation-screening studies by others; however, these mutations explain only a small proportion of the variance in the mutation frequencies. Despite the large variance in the frequencies of *MED12* mutations, their spectrum has been consistent across all mutation-screening studies, with the vast majority of the mutations affecting the highly conserved codon 44.

As also demonstrated in this thesis, *MED12*-hotspot mutations occur in leiomyoma variants, but with notably lower frequencies than in conventional leiomyomas (Table 9). Moreover, up to 30% of malignant leiomyosarcomas harbor these mutations. Leiomyoma variants and leiomyosarcomas are typically driven by other genetic aberrations than *MED12* mutations, including *FH, HMGA2*, *tumor protein P53* (*TP53*), *RB transcriptional corepressor 1* (*RB1*), and *ATRX, chromatin remodeler* (*ATRX*) mutations (Sandberg, 2005b; Zhang *et al.*, 2014; Liegl-Atzwanger *et al.*, 2016; Mäkinen *et al.*, 2016; 2017). In addition, most leiomyosarcomas display aneuploidy and structural chromosomal alterations (Sandberg, 2005b). The presence of *MED12* mutations in a subset of leiomyosarcomas indicates that a benign leiomyoma, or more plausibly a leiomyoma variant, may gain additional mutations transforming it into a malignant leiomyosarcoma. Further affirming the possibility of malignant transformation, leiomyosarcomas with morphologically benign areas, which often resemble leiomyomas with bizarre nuclei or high cellularity, display identical *MED12* mutations in both, the benign and malignant, components (Mittal *et al.*, 2009; Matsubara *et al.*, 2013). Considering the large difference in the incidences of these two tumor types, malignant transformation would occur on rare occasions. In leiomyosarcomas, *MED12* mutations may represent merely passenger mutations or secondary mutations providing a growth advantage to malignant cells. Furthermore, the role of mutated *MED12* in malignancy seems unlikely since the majority of leiomyosarcomas display reduced protein expression of MED12 (Perot *et al.*, 2012; Markowski *et al.*, 2013; Bertsch *et al.*, 2014).

Table 8. Summary of the studies reporting *MED12* mutations in leiomyomas.

Population	Exon 1 mutations	Exon 2 mutations	Average tumor size (cm)	Patients with mutation-positive tumors	Total number of patients	Reference(s)
Finnish	1% (4/225)	71% (159/225)	3.7	75% (60/80)	80	(Mäkinen *et al.*, 2011; Kämpjärvi *et al.*, 2014)
Finnish	0% (0/71)	61% (43/71)	NA	NA	NA	(Mäkinen *et al.*, 2013; 2017; Kämpjärvi *et al.*, 2016b)
Finnish/Romanian	0% (0/88)	83% (73/88)	3.5	77% (10/13)	13	Study II
Finnish/Swedish	1% (1/76)	85% (64/76)	2.8	73% (11/15)	15	Study II
Finland	1% (6/763)	79% (599/763)	3.8	73% (177/244)	244	Study III
Finns	1% (6/679)	79% (535/679)	3.7	73% (160/219)	219	
non-Finns	0% (0/84)	76% (64/84)	4.7	68% (17/25)	25	
Russian	NA	73% (11/15)	NA	73% (11/15)	15	(Osinovskaya *et al.*, 2013)
Russian	NA	52% (63/122)	NA	NA	76	(Osinovskaya *et al.*, 2015)
German	NA	59% (47/80)	5.3	NA	50	(Markowski *et al.*, 2012)
German	NA	48% (10/21)	NA	48% (10/21)	21	(Markowski *et al.*, 2013)
German	NA	92% (11/12)	NA	100% (2/2)	2	(Rieker *et al.*, 2013)
German	NA	70% (179/256)	NA	NA	NA	(Markowski *et al.*, 2014a)
Dutch	NA	58% (11/19)	6.5	56% (9/16)	16	(de Graaff *et al.*, 2013)
French	NA	67% (6/9)	NA	67% (6/9)	9	(Perot *et al.*, 2012)
American/ Austrian/French	NA	61% (11/18)	5.0	58% (8/14)	12	(Liegl-Atzwanger *et al.*, 2016)
Mixed American	NA	68% (100/148)	5.5	68% (100/148)	148	(McGuire *et al.*, 2012)
Black	NA	78% (18/23)	6.1	78% (18/23)	23	
White	NA	66% (79/120)	5.4	66% (79/120)	120	
Mixed American	NA	75% (133/178)	11.9	NA	134	(Bertsch *et al.*, 2014)
Asian	NA	67% (4/6)	NA	NA	4	
Black	NA	79% (64/81)	NA	NA	64	
Hispanic	NA	81% (13/16)	NA	NA	13	
White	NA	72% (53/74)	NA	NA	52	
Mixed American	NA	64% (92/143)	NA	62% (84/135)	135	(Halder *et al.*, 2015)
Black	NA	NA	NA	66% (45/68)	68	
Hispanic	NA	NA	NA	60% (26/43)	24	
White	NA	NA	NA	54% (13/24)	43	
American	NA	69% (9/13)	NA	NA	NA	(Ravegnini *et al.*, 2013)
American	NA	54% (15/28)	NA	54% (15/28)	28	(Schwetye *et al.*, 2014)
American/Chinese	NA	75% (30/40)	NA	75% (30/40)	40	(Zhang *et al.*, 2014)
Chinese	NA	52% (95/181)	NA	52% (95/181)	181	(Wang *et al.*, 2015)
Japanese	NA	80% (36/45)	NA	NA	NA	(Matsubara *et al.*, 2013)
Korean	NA	52% (35/67)	6.1	52% (35/67)	67	(Je *et al.*, 2012)
Iranian	NA	48% (11/23)	NA	48% (11/23)	23	(Shahbazi *et al.*, 2015)
Iranian	NA	31% (32/103)	5.3	NA	58	(Sadeghi *et al.*, 2016)
South African	4% (1/28)	50% (14/28)	8.0	56% (10/18)	18	Study I (Kämpjärvi *et al.*, 2014)

Table 9. Frequency of *MED12* mutations in uterine smooth muscle tumors.

Histopathological subtype	Frequency	Reference(s)
Cellular leiomyoma	33% (2/6)	(Matsubara *et al.*, 2013)
	9% (6/69)	(Mäkinen *et al.*, 2013; 2017)
	14% (3/22)	(Zhang *et al.*, 2014)
	0% (0/1)	(Liegl-Atzwanger *et al.*, 2016)
	44% (7/16)	Study III
Epithelioid leiomyoma	100% (1/1)	Study III
Intravenous leiomyomatosis	0% (0/9)	(Buza *et al.*, 2014)
Leiomyoma with bizarre nuclei	25% (1/4)	(Matsubara *et al.*, 2013)
	16% (3/19)	(Mäkinen *et al.*, 2013; 2017)
	10% (4/42)	(Zhang *et al.*, 2014)
	10% (1/10)	(Liegl-Atzwanger *et al.*, 2016)
	50% (1/2)	Study III
Lipoleiomyoma	0% (0/4)	(Matsubara *et al.*, 2013)
	0% (0/3)	Study III
Mitotically active leiomyoma	38% (10/26)	(Mäkinen *et al.*, 2013)
	86% (6/7)	(Zhang *et al.*, 2014)
	75% (3/4)	Study III
Smooth muscle tumor of uncertain malignant potential	11% (1/9)	(Perot *et al.*, 2012)
	8% (1/12)	(Schwetye *et al.*, 2014)
	11% (2/18)	(Zhang *et al.*, 2014)
Uterine leiomyosarcoma	0% (0/5)	(Je *et al.*, 2012)
	7% (3/41)	(Kämpjärvi *et al.*, 2012)
	20% (2/10)	(Perot *et al.*, 2012)
	14% (1/7)	(de Graaff *et al.*, 2013)
	7% (1/14)	(Markowski *et al.*, 2013)
	17% (2/12)	(Matsubara *et al.*, 2013)
	20% (3/15)	(Ravegnini *et al.*, 2013)
	9% (3/32)	(Bertsch *et al.*, 2014)
	30% (6/20)	(Schwetye *et al.*, 2014)
	11% (4/38)	(Zhang *et al.*, 2014)
	4% (1/24)	(Wang *et al.*, 2015)
	25% (2/8)	(Liegl-Atzwanger *et al.*, 2016)
	22% (11/51)	(Mäkinen *et al.*, 2016; 2017)
	5% (7/153)	(Yatsenko *et al.*, 2017)

MED12-hotspot mutations occur also in extrauterine smooth muscle tumors: disseminated peritoneal leiomyomatosis (7/7, 100%), extrauterine leiomyomas (15/192, 8%), one extrauterine leiomyosarcoma (1/136, 1%), and genital leiomyomas in dogs (2/10, 20%) (Kämpjärvi *et al.*, 2012; Perot *et al.*, 2012; de Graaff *et al.*, 2013; Markowski *et al.*, 2013; Matsubara *et al.*, 2013; Ravegnini *et al.*, 2013; Rieker *et al.*, 2013; Schwetye *et al.*, 2014). In other benign tumor types, *MED12*-hotspot mutations have been observed with notable frequencies in fibroadenomas (47 to 86%) and phyllodes tumors (45 to 80%) of the breast (Lim *et al.*, 2014; Cani *et al.*, 2015; Mishima *et al.*, 2015; Nagasawa *et al.*, 2015; Tan *et al.*, 2015; Yoshida *et al.*, 2015). In malignant neoplasms, hotspot mutations are frequent in uterine adenosarcomas (11%) and chronic lymphocytic leukemia (5%), but only few mutations have been found in other cancer types (Je *et al.*, 2012; Kämpjärvi *et al.*, 2012; 2015; Yuan *et al.*, 2017). Mutations slightly further down exon 2 of *MED12,* affecting amino acids Q48 and D54, have emerged in 3 out of 14 (21%) endometrial stromal sarcomas, but the role of these mutations in pathogenesis is unclear (Yuan *et al.*, 2017). Mutations affecting other parts of the *MED12* gene are relatively frequent (4 to 5%) in

prostate cancer, adrenocortical carcinomas, endometrioid endometrial carcinomas, and renal cell cancer; these mutations are likely to promote tumorigenesis through distinct mechanisms in comparison to leiomyoma-hotspot mutations (Barbieri *et al.*, 2012; Arai *et al.*, 2014; Assié *et al.*, 2014; Kämpjärvi *et al.*, 2016a; Uterine Corpus Endometrioid Carcinoma, TCGA, USA, import from ICGC: COSU419).

In summary, *MED12* mutations are frequent in benign female-steroid-hormone-dependent tumors of mesenchymal-stem-cell origin, suggesting that MED12 plays a role in the benign tumorigenesis of the mesenchymal cell lineage under hormonal control. Although *MED12* mutations occur also in malignant neoplasms, their role and timing in tumor evolution and malignancy remain obscure.

1.2. MED12 mutations and clinical features

Our results confirmed the prior notion that smaller tumor size is associated with positive *MED12*-mutation status, a finding supported by multiple reports, although a few studies with small sample sizes failed to detect a significant association (Mäkinen *et al.*, 2011; Je *et al.*, 2012; Markowski *et al.*, 2012; de Graaff *et al.*, 2013; Bertsch *et al.*, 2014; Osinovskaya *et al.*, 2015; Ye *et al.*, 2015). In addition, this thesis showed that patients with *MED12*-mutation-positive leiomyomas tend to have multiple concurrent lesions, which is supported by findings of other studies (McGuire *et al.*, 2012; Markowski *et al.*, 2014a; Osinovskaya *et al.*, 2015). The multiplicity of tumors may contribute to the smaller size of *MED12*-mutation-positive leiomyomas, as multiple tumors are likely to manifest clinically earlier than solitary tumors. Otherwise, other genetic drivers, such as *HMGA2,* may induce more potent growth-promoting changes than *MED12* mutations (Markowski *et al.*, 2012). Patients having multiple synchronous leiomyomas, typically harboring non-identical *MED12* mutations, may be explained by genetic predisposition, epigenetic alterations, or exposure to external factors, such as steroid hormones, rendering the myometrium prone to *MED12* mutations. Approximately a quarter of the patients have only mutation-negative leiomyomas; these patients are likely to harbor leiomyomas driven by, for example, *HMGA2* aberrations (Markowski *et al.,* 2014a). Comparing the genetic, epigenetic, and clinical features between patients having numerous *MED12*-mutation-positive leiomyomas and patients having solely mutation-negative lesions could enlighten the mechanisms underlying the predisposition for mutation-positive tumors.

This thesis revealed that subserosal leiomyomas tend to be *MED12*-mutation positive. Two other studies found no significant association between the mutation status and leiomyoma location, possibly due to smaller sample sets (Je *et al.*, 2012; Osinovskaya *et al.*, 2015). Nonetheless, our finding together with the previous notion that submucosal leiomyomas display significantly fewer clonal cytogenetic changes compared to other types suggests that differences in the structurally and functionally distinct myometrial layers influence the molecular mechanisms leading to leiomyoma development (Brosens *et al.*, 1998; Noe *et al.*, 1999). As the range of symptoms caused by leiomyomas depends on the number, location, and size of the tumors, the established differences in these features between *MED12*-mutation-positive and -negative leiomyomas suggest that these tumors are likely to manifest in different ways as well.

This thesis discovered that the number of *MED12*-mutation-positive leiomyomas decreases with increasing parity. Another study found no difference between the parity of patients with only one leiomyoma with *HMGA2* translocation and patients with at least one *MED12*-mutation-positive tumor (Markowski *et al.*, 2014a). Increasing parity and more recent pregnancies are known to reduce the risk of leiomyomas, but the underlying mechanism is unclear (Ross *et al.*, 1986; Parazzini *et al.*, 1988; 1996; Lumbiganon *et al.*, 1996; Marshall *et al.*, 1998; Sato *et al.*, 2002; Wise *et al.*, 2004a; Parazzini, 2006; Terry *et al.*, 2010; Uimari *et al.*, 2016). Possibly, post-pregnancy remodeling of the uterus induces leiomyoma regression and eliminates smaller lesions, which are typically *MED12*-mutation positive (Baird and Dunson, 2003; Laughlin *et al.*, 2010). Alternatively, the hormonal changes during and after pregnancy may affect mutation-positive and -negative leiomyomas differently, as many, but not all, leiomyomas shrink during pregnancy (Laughlin *et al.*, 2010).

Our analysis revealed a significant association between increasing number of *MED12*-mutation-negative tumors and a history of PID, suggesting that either inflammatory stimuli or a tumorigenic infectious agent may induce the development of mutation-negative leiomyomas. This view is supported by epidemiological studies: reproductive tract infections increase the risk of leiomyomas, whereas a history of PID showed a trend towards an inverse association with the number of tumors (Faerstein *et al.*, 2001b; Moore *et al.*, 2015). If inflammation or infectious agents give rise to some leiomyomas, these tumors could perhaps be prevented with an appropriate treatment or a vaccine.

We found one patient to be a carrier of a germline *FH* mutation. This patient had 15 *MED12*-mutation-negative leiomyomas, was young at the time of diagnosis (30 years old), had a history of a prior myomectomy, and had a prominent family history of leiomyomas. This patient case highlights the clinical features of HLRCC-related leiomyomas, which include early age of onset, familial aggregation, and multiplicity of tumors that, when encountered in the clinic, should raise the suspicion of an underlying hereditary syndrome so that appropriate counseling and follow-up can be arranged (Lehtonen, 2011; Tolvanen *et al.*, 2012). To facilitate screening for *FH* deficiency in patients presenting with these clinical features, establishing robust non-invasive biomarkers is vital.

Taken together, these differences in the characteristics and predisposing factors of *MED12*-mutation-positive and –negative leiomyomas highlight the diversity of the molecular mechanisms leading to the development of these tumors. The genetic defects underlying leiomyomas may be triggered and affected by different factors, such as inflammation and hormones, and may partly account for the heterogeneity in the clinical presentation, growth pattern, and drug responses of leiomyomas.

1.3. Tumorigenic mechanism of MED12 mutations

In addition to the high frequency of *MED12* mutations, the presence of these mutations in leiomyoma stem cells suggests that *MED12* mutations drive leiomyomagenesis (Ono *et al.*, 2012). Recent studies on leiomyomas have shown that *MED12* mutations, *FH* inactivation, and *HMGA2* aberrations are mutually exclusive genetic events that result in distinct gene expression profiles (Markowski *et al.*, 2012; Bertsch *et al.*, 2014; Kämpjärvi *et al.*, 2016b;

Mehine *et al.*, 2016; Mäkinen *et al.*, 2017). Furthermore, a *MED12* mutation alone seems to be sufficient for tumor formation, as no other concurrent recurrent small-scale mutations have been detected in *MED12*-mutation-positive leiomyomas, and many of the chromosomal aberrations that co-occur with *MED12* mutations, such as 7q deletions, typically exist as subclonal changes indicating that they represent secondary events with a tumor-promoting role (Markowski *et al.*, 2012; Mäkinen *et al.*, 2014b).

The mutation pattern strongly suggests that *MED12* mutations are of a gain-of-function type and that mutated *MED12* acts as an oncogene. Functional studies revealed that *MED12*-hotspot mutations disrupt the binding interface between MED12 and Cyclin C resulting in diminished CDK8-kinase activity (Kämpjärvi *et al.*, 2014; Turunen *et al.*, 2014). Leiomyomas harbor no recurrent mutations in any of the other components of the CDK8 module further highlighting the specific functional effect of *MED12* mutations (Mäkinen *et al.*, 2014a). These mutations may promote tumorigenesis through altered Wnt/β-catenin signaling, although the data are contradictory: our recent gene expression data indicated inhibition of the Wnt/β-catenin pathway in leiomyomas, whereas many studies have suggested that the activation of this pathway plays a role in leiomyomagenesis (Mäkinen *et al.*, 2011; Markowski *et al.*, 2012; Perot *et al.*, 2012; de Graaff *et al.*, 2013; Ono *et al.*, 2013; 2014; Mehine *et al.*, 2016; Al-Hendy *et al.*, 2017). *MED12*-mutation-positive tumors show dysregulation of extracellular matrix genes implying that *MED12* may also affect extracellular matrix remodeling (Lim *et al.*, 2014; Mehine *et al.*, 2016; Al-Hendy *et al.*, 2017). In addition, *MED12* mutations may contribute to tumorigenesis by altering the cells' sensitivity to steroid hormones, as MED12 regulates steroid-hormone receptors and *MED12*-mutation-positive breast fibroadenomas display activation of estrogen signaling (Prenzel *et al.*, 2012; Lim *et al.*, 2014; Al-Hendy *et al.*, 2017). Intriguingly, a non-coding transcript of *RAD51B*, the preferred translocation partner of *HMGA2,* is the most significantly dysregulated gene in *MED12*-mutation-positive leiomyomas (Mehine *et al.*, 2016). The tumorigenic mechanism of *MED12* mutations requires further studies, which have been hampered due to the challenges encountered in establishing a leiomyoma cell line carrying a *MED12* mutation (Markowski *et al.*, 2014b). On a positive note, a recently engineered mouse model, which conditionally expresses the most frequent hotspot mutation in uterine mesenchymal cells and consequently develops leiomyoma-like lesions in the uterus, provides a platform for studying *MED12*-driven tumorigenesis (Mittal *et al.*, 2015).

Aside from *MED12* mutations, next-generation sequencing studies have identified no other genes that harbor recurrent somatic point mutations in leiomyomas (Mäkinen *et al.*, 2011; 2014b; McGuire *et al.*, 2012; Mehine *et al.*, 2013). On the contrary, whole-genome sequencing revealed interconnected complex chromosomal rearrangements in mostly *MED12*-mutation-negative leiomyomas (Mehine *et al.*, 2013). These rearrangements resembled a milder form of an event previously observed in cancers called chromotripsis, in which chromosomes shatter into pieces and randomly reassemble (Stephens *et al.*, 2011). Some of these complex chromosomal rearrangements had formed aberrations characteristic for leiomyomas, such as *HMGA2-RAD51B* translocations (Mehine *et al.*, 2013). In addition, a whole-genome sequencing and subsequent validation study showed that multiple separate concurrent leiomyomas may have a common clonal origin (Mehine *et al.*, 2013; 2015).

Taken together, next-generation sequencing technologies have revolutionized our understanding of leiomyomas: instead of a single homogeneous group of tumors, they comprise multiple distinct molecular subtypes that seem to arise and behave differently, and perhaps should be managed with different treatments. The established molecular subtypes include leiomyomas driven by *MED12* mutations*, HMGA2* overexpression*,* and *FH* deficiency. Together these drivers account for the great majority of leiomyomas, leaving only a fraction of them without an identified initiator. Putative driver genes affected by a sole chromosomal alteration in leiomyomas include *high mobility group AT-hook 1* (*HMGA1*)*, collagen type IV α 5 chain* and *6 chain* (*COL4A5* and *COL4A6*)*,* and *lysine acetyltransferase 6B* (*KAT6B*), but due to the rarity of these aberrations, more samples are required to establish their potential initiating role (Moore *et al.*, 2004; Nezhad *et al.*, 2010; Mehine *et al.*, 2013). Although the main drivers of leiomyomagenesis have now been identified, their tumor-driving molecular mechanisms remain largely elusive.

2. Metabolomic alterations in leiomyomas

As a first study of its kind, we explored the global metabolomic profiles of leiomyomas representing the three main molecular subtypes classified according to their mutually exclusive driver changes: *MED12* mutations, *FH* inactivation, and *HMGA2* aberrations (Markowski *et al.*, 2012; Bertsch *et al.*, 2014; Kämpjärvi *et al.*, 2016b; Mäkinen *et al.*, 2017). We discovered that these genetic triggers lead to differences in the metabolomic profiles of leiomyomas: the *FH*-deficient leiomyomas represented a metabolically distinct subtype that can be distinguished from other leiomyomas and the myometrium samples based on their metabolomic profile. We identified multiple dysregulated metabolites in the different molecular subtypes of leiomyomas, which may contribute to their development in multiple ways, such as by providing energy and building blocks, acting as antioxidants, or by regulating signaling pathways involved in proliferation and differentiation. Moreover, some of the dysregulated metabolites may prove to be useful biomarkers or targets for prevention and treatment of leiomyomas.

All leiomyomas displayed significant reduction of the antioxidant homocarnosine, the important cofactor heme, and its degradation product biliverdin indicating that alterations in the metabolic pathways of these compounds are characteristic for all leiomyomas (Kohen *et al.*, 1988). How these alterations arise and affect leiomyoma development is unclear, and thus their further scrutiny could uncover similarities in the tumor-driving mechanisms of the different genetic drivers that could be targeted to treat all leiomyomas.

2.1. Metabolomic alterations in MED12-mutation-positive leiomyomas

In *MED12*-mutation-positive leiomyomas, the significant changes in the metabolite levels were mainly decreases, possibly reflecting limited supply of building blocks that may contribute to the smaller size of these leiomyomas. Among the reduced metabolites were various amino acids, their derivatives, and dipeptides, which may indicate enhanced protein synthesis in these leiomyomas. In addition, dysregulated methionine, cysteine, S-adenosylmethionine, and taurine metabolism may reflect adapted methylation of proteins

and DNA, as S-adenosylmethionine is a coenzyme in methyl-group transfer reactions (Teperino *et al.*, 2010). *MED12*-mutation-positive leiomyomas displayed also reduced levels of sphingolipids and phosphatidylserines, which are components of the cell membrane and play a role in various signaling pathways (Ogretmen and Hannun, 2004; Vance and Steenbergen, 2005).

One of the reduced metabolites in *MED12*-mutation-positive leiomyomas was RA precursor retinol (vitamin A). Prior studies have suggested that RA inhibits proliferation, induces apoptosis, and reduces expression of ECM components in leiomyoma cells (Broaddus *et al.*, 2004; Gilden *et al.*, 2012; Borahay *et al.*, 2015; Stewart *et al.*, 2016). In epidemiological studies, higher serum concentration of vitamin A was associated with increased risk of leiomyomas, whereas dietary vitamin A intake was associated with reduced risk (Martin *et al.*, 2011; Wise *et al.*, 2011). Based on gene expression data, RA receptor activation is one of the most significantly dysregulated pathways in leiomyomas (Mehine *et al.*, 2016). Importantly, drugs increasing RA levels have been well tolerated in clinical studies (Debruyne *et al.*, 1998; Camerini *et al.*, 2001). Hence, targeting RA metabolism should be further explored as a putative therapy and prevention mechanism for leiomyomas, and in particular, for *MED12*-mutation-positive leiomyomas.

MED12-mutation-positive leiomyomas displayed reduction of metabolites of ascorbate (vitamin C) together with the predicted activation of the antioxidant action of vitamin C pathway. In addition to its antioxidant activity, vitamin C contributes to other important cellular processes including collagen synthesis, DNA methylation, and nuclear factor κB mediated signaling (Cárcamo *et al.*, 2004; Du *et al.*, 2012; Young *et al.*, 2015). Epidemiological studies have found no association between vitamin C and leiomyoma risk (Martin *et al.*, 2011; Wise *et al.*, 2011). This appears to be the first time vitamin C has been linked to leiomyomagenesis, and thus its significance requires further clarification.

2.2. Metabolomic alterations in FH-deficient leiomyomas

FH-deficient leiomyomas displayed dysregulation of a large number of metabolites, with the majority of them elevated when compared to the normal myometrium, suggesting severe deregulation in cellular metabolism. These leiomyomas had elevated levels of fumarate and other TCA cycle intermediates including malate, succinate, α-ketoglutaric acid (αKG), and homocitrate. Accumulation of fumarate and succinate in *FH*-deficient cells and tissues has been reported previously, but the results regarding the levels of malate, αKG, and citrate have been contradictory (Pollard *et al.*, 2005; Frezza *et al.*, 2011; Tong *et al.*, 2011; Adam *et al.*, 2013; Yang *et al.*, 2013; Zheng *et al.*, 2013). In addition, these TCA cycle metabolites accumulate in physiological fluids of patients with a rare autosomal recessive disorder, congenital fumarase deficiency (Allegri *et al*, 2010; Tregoning *et al*, 2013). One suggested route for the production of citrate, acetyl coenzyme A, and the remaining four-carbon TCA cycle intermediates in *FH*-deficient cells is glutamine-dependent reductive carboxylation of αKG, but contradicting data also exists (Frezza *et al.*, 2011; Mullen *et al.*, 2012; Adam *et al.*, 2013). Studies on *FH*-deficient leiomyoma cells are required to determine whether this rerouting occurs in leiomyomas.

Accumulated fumarate is considered to drive tumorigenesis by activating NRF2, which results in the activation of the NRF2-mediated Oxidative Stress Response pathway and the redirection of glucose and glutamine into anabolic pathways such as the pentose phosphate pathway (PPP) (Adam *et al.*, 2011; Kinch *et al.*, 2011; Mitsuishi *et al.*, 2012; Gorrini *et al.*, 2013; Mehine *et al.*, 2016). In this work, *FH*-deficient leiomyomas displayed several metabolic alterations known to be affected by NRF2 including enhanced PPP flux and altered heme metabolism, changes which have been observed previously in *FH*-deficient cells (Frezza *et al.*, 2011; Yang *et al.*, 2013). The enhanced PPP generates NADPH, which is needed for reductive carboxylation of glutamine-derived αKG, synthesis of fatty acids, and protection against oxidative stress; processes important for proliferative cells. *FH*-deficient cells seem to boost the removal of accumulated TCA cycle metabolites through enhanced heme metabolism, which excretes TCA-cycle-derived carbon as bilirubin (Frezza *et al.*, 2011). Inhibition of this process is synthetic lethal with *FH* deficiency, highlighting it as a potential treatment target.

FH-deficient leiomyomas displayed uniquely high levels of argininosuccinate and N6-succinyladenosine (S-ado). Argininosuccinate is normally produced in the urea cycle by argininosuccinate lyase (ASL) and is further converted into fumarate and arginine, but to detoxify the accumulating fumarate, the enzyme function is reversed (Adam *et al.*, 2013; Zheng *et al.*, 2013). *FH*-deficient kidney cancers employ exogenous arginine to produce argininosuccinate; blocking this mechanism reduces proliferation of *FH*-deficient cells, suggesting that the arginine pathway is essential for the growth of *FH*-deficient tumors. Furthermore, *FH*-deficient mice secrete argininosuccinate into their urine, as do *FH*-deficient cell lines into their growth media (Zheng *et al.*, 2013). Argininosuccinate may therefore emerge as a robust metabolic biomarker, and arginine metabolism as a treatment target, for HLRCC-associated tumors. S-ado accumulation in *FH*-deficient cells likely derives from inhibition of adenylosuccinate lyase (ADSL), which catalyzes two reactions in purine *de novo* synthesis both producing fumarate as a side product (Jurecka *et al.*, 2015). S-ado is formed through dephosphorylation of adenylosuccinate, elevated levels of which have been observed in *FH*-deficient kidney specimens from mice and in physiological fluids of congenital fumarase deficiency patients, further supporting our results (Allegri *et al*, 2010; Adam *et al*, 2013; Tregoning *et al*, 2013). The potential tumorigenic role of S-ado and its utility as a biomarker require further exploration.

The two leiomyomas overexpressing *HMGA2* displayed mainly an accumulation of metabolites. Although the number of these tumors was too small to derive any conclusions, they displayed some similarities with *FH*-deficient leiomyomas including the accumulation of plasmalogens and diacylglycerols. These lipids are components of cell membranes that affect the membrane properties and facilitate various signaling processes (Gómez-Fernández and Corbalán-García, 2007; Braverman and Moser, 2012). In addition, both of these leiomyoma subtypes showed accumulation of polyamines. Elevated levels of polyamines have been implicated in the induction of cell proliferation and the reduction of apoptosis (Gerner and Meyskens, 2004). Furthermore, targeting their metabolism may prove an effective therapy or, more importantly, a prevention mechanism for these leiomyomas, as a prior study demonstrated that treatment with difluoromethylornithine, a well-tolerated inhibitor of a central enzyme in polyamine synthesis, reduces leiomyoma cell

growth (Broaddus *et al.*, 2004; Gerner and Meyskens, 2004). These results require confirmation in a larger sample set, but provide new insight into the molecular mechanism underlying these tumors and potential targets for limiting their growth.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This thesis is based on the groundbreaking finding of *MED12* mutations in leiomyomas; this work validates and elucidates their role and clinical features. In view of all the data, *MED12* mutations are oncogenic and drive the growth of the vast majority of leiomyomas. Furthermore, *MED12*-mutation-positive lesions represent a molecularly and clinically distinct subgroup of leiomyomas, suggesting that the molecular background of these tumors should be taken into consideration in basic research, drug development, and when targeted treatments emerge, in clinical practice as well. The emergence of animal and cell models for *MED12*-mutation-positive tumors is critical for advancing our understanding of the molecular basis for the tumorigenic potential of these mutations and for developing targeted therapies. The high frequency of *MED12*-hotspot mutations in female-steroid-hormone-dependent tumors is striking; further research into how *MED12* mutations relate to steroid hormones is required.

The main genetic drivers of leiomyomagenesis have now been identified; this thesis reveals the metabolomic heterogeneity of leiomyomas and contributes to our understanding of the pathophysiology underlying their molecular subtypes. Additional studies are required for a deeper understanding of how these mutations arise and drive tumorigenesis, however. In addition, as the genetic driver still remains unidentified for a small subset of conventional leiomyomas and in particular for a large proportion of leiomyoma variants, large sample sets are warranted to obtain sufficient numbers of tumors harboring these rarer events.

Further work addressing the clinical features of all molecular subtypes of leiomyomas is required to assess which subtypes may be induced by inflammation or infection, and whether the different subtypes respond differently to treatments, such as ulipristal acetate, and other hormonal changes. The molecular classification of leiomyomas has raised a demand for biomarkers for research and clinical purposes. On the other hand, studying the molecular changes shared by all leiomyoma subtypes may result in the discovery of a therapy effective against all of them.

The genetic and environmental predisposition to the different molecular subtypes of leiomyomas requires clarification. Understanding the mechanisms that lead to the emergence and growth of leiomyomas is crucial in order to identify means to prevent the development of these lesions. The most effective approach to reduce the morbidity and costs of these extremely common tumors would be primary prevention.

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