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# Prediction of the Structure and Mutations Instability of the Med12 Exon2 Gene in Uterine Fibroids in Senegalese Women

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**Abstract:** Uterine fibroids are benign proliferations of slow evolution. They are associated with significant morbidity and constitute a real public health problem. Despite the large-scale medical and financial burden posed by uterine fibroids, the functional roles of the various factors and genes involved in their etiology and growth remain unclear. This shows a great need to undertake a study that would evaluate the molecular features of uterine fibroids. It is in this context that our study is based on 50 Senegalese women with uterine fibroids. Samples of tumour tissue and blood were taken from each patient. After sequencing, the raw data was submitted to the Mutation Surveyor software version 5.0.1. Pathogenicity of mutations was evaluated with Polyphen software. To better understand the functional impact of missense mutations at the three-dimensional level, we simulated the structure of the protein by the ab-initio method using the I-Tasser web server. After cleaning, correcting and aligning the sequences with the BioEdit software version 8.0.5, the amino acid frequencies for the blood and tumour tissue samples were retrieved with the MEGA7 software. To see if there is a difference in the distribution of each amino acid between blood and tumour tissue, the average comparison test was performed with the R software version 3.3.1. Our results showed the presence of mutations of the *MED12* gene only in tumour tissues. All mutations are predicted to be deleterious. In comparison to the reference sequence, all the mutations show a conformational change in the 3D structure of the MED12 protein. In addition, the mutations p. Q43P, p. G44S, p. G44D, p. G44R, p. F45V, p. K60M give proteins of α-β structure different from the reference sequence which has an α structure. All mutations alter the predicted function of the MED12 protein, which further suggests their involvement in the pathobiology of uterine fibroids in Senegalese women. With regard to amino acid frequencies, the comparison of means between blood and tumour tissue samples shows different distributions for amino acids such as cysteine, aspartic acid, glycine, histidine, leucine, arginine and serine. The results obtained make it possible to better understand the molecular mechanisms involved in the etiology of uterine fibroids. They allow glimpsing applications for the screening of populations at risk, for a non-invasive diagnosis or even for preventive or curative treatment.

**Keywords:** Uterine Fibroids, *MED12* Mutations, Amino Acids, Biomarker

## 1. Introduction

Uterine fibroids, more commonly known as myomas, are the most common benign tumours of female reproductive organs. They are associated with significant morbidity and constitute a real public health problem. Fibroids develop at the expense of smooth muscle and are often separated from

the myometrium by a pseudocapsule associated with condensation of connective tissue [1]. The heterogeneity of the localization of uterine fibroids and their progression in the same patient illustrates the complex biological mechanism involved in their development.

In terms of prevalence, uterine fibroids affect 20 to 25% of women in reproductive activity and nearly 50% of women over 45 [2]. According to Okolo (2008) in reference [3], uterine fibroids affect millions of women worldwide and in 60% of cases occur in women aged 45 years. This prevalence varies by ethnicity, with African-born women having a 3- to 9-fold higher risk than Caucasian women. Nowadays, the known risk factors are age, ethnicity, early age of menarche and nulliparity [4, 5]. Several studies have also indicated the involvement of hormonal status in the development of uterine fibroids. And according to reports published in the National Institute of Environmental Health Science-Uterine Fibroid Study (NIEHS-UFS), the factors influencing steroid metabolism such as obesity, diet, among others are associated with a risk of occurrence of uterine fibroids [6]. Although the molecular mechanisms involved in their etiology remain incompletely elucidated, several studies have focused on the *MED12* gene [7-10]. In our previous study, the involvement of the *MED12* gene was reported in Senegalese women with uterine fibroids [11, 12]. In the first study it was reported a variable expressivity of the *MED12* gene in tumour tissues among Senegalese women in comparison to Caucasian women. In the second, our results highlighted a significant correlation between the mutations found and factors such as parity and diet. In this paper, we evaluate the mutant penetration of exon 2 of the *MED12* gene; investigations suggesting that amino acid substitution mutations in gene exon 2 could contribute to potential phenotype alterations as well as MED12 protein stability.

# 2. Methodology

### *2.1. Samples*

50 patients with uterine fibroids were recruited from the Grand Yoff General Hospital and the Ouakam Military Hospital (HMO). After obtaining informed consent, each patient was given a biopsy of the tumour tissue and a blood sample (to serve as a control) on an EDTA tube. To guarantee the anonymity of the patients and thus the respect of the professional secrecy, the samples were coded UF (Uterine fibroids).

### *2.2. DNA Extraction, MED12 Gene Amplification and Sequencing*

The total DNA of each sample was extracted using the Qiagen protocol (Qiagen Dneasy Blood and Tissues kit). After extraction, a portion of interest of the *MED12* gene was amplified, namely exon 2 and its flanking regions. The amplification was carried out in a reaction volume of 50 µl with the primers 5'GCCCTTTCACCTTGTTCCTT3 'and 5'TGTCCCTATAAGTCTTCCCAACC3'. The PCR took place in an Eppendorf-type thermocycler under the conditions previously described by Mäkinen *et al*., [7]. An electrophoretic migration on 1.5% agarose gel was also performed to check the quality of the amplicons. Sequencing reactions were performed in a MJ Research PTC-225 Peltier thermocycler with ABIPRISM BigDye TM Terminator Cycle kits. Each sample was sequenced using forward primer. The fluorescent fragments were purified with the BigDye Xterminator purification protocol. The samples were suspended in distilled water and electrophoresed in ABI 3730xl sequencer (Applied Biosystems).

### *2.3. Detection of Mutations*

To determine the presence of any mutation and its position relative to the *MED12* gene, the raw sequencing data were submitted to the Mutation Surveyor software version 5.0.1 (www.softgenetics.com), which compares the submitted chromatograms with the reference sequence of *MED12* gene (NT_011669_70337906).

### *2.4. Prediction of the Pathogenicity of Mutations*

To see whether the non-synonymous substitutions (mutations that induce an amino acid change) of exon 2 of the *MED12* gene are benign or deleterious, the nucleotide sequences have been translated into protein sequences using the MEGA 7 software [13] and these have been submitted to the Polyphen2 software (http://genetics.bwh.harvard.edu/pph2/). It is a probabilistic classifier that calculates the functional significance of a change of allele by Naive Bayes, a set of supervised learning algorithms to identify deleterious mutations. The input options for this method are the acquisition number of the database or the protein sequence as well as the details of the variants. Any mutation with a score $\geq 0.9$ (independent number of specific positions) are classified as "potentially damaging mutations".

### *2.5. Analysis of the Protein Structure*

To better understand the functional impact of missense mutations at the three-dimensional level, we simulated the structure of the protein by the ab-initio method using the I-Tasser web server [14]. Starting from the target amino acid sequence, I-Tasser first generates full-length anatomical structural models from multiple alignments and iterative structural assembly simulations. To derive the biological function of the target proteins, the I-Tasser models are mapped to the proteins of the BioLIP library [15], which is a semi-manual protein function database. Functional information including ligand binding and gene ontology are derived from BioLIP models that are classified according to composite scores combining structural, global, and local similarity [16]. The 3D dimensional models for the mutant-type protein models were constructed by manually inserting specific amino acid modifications into the MED12 protein reference sequence.

### *2.6. Amino Acid Frequency*

After cleaning, correcting and aligning the sequences with BioEdit software version 8.0.5 [17], the amino acid frequencies for blood and tumour tissue samples were retrieved using MEGA7 software [13]. To see if there is a difference in the frequency distribution of each amino acid between blood and tumour tissue, the database was submitted to R version 3.3.1 software [18]. Shapiro Wilk's normality test was performed to see if the data follows a normal distribution. In the case of a normal distribution, the Student's t-test is performed for the comparison of averages; otherwise the Wilcoxon test is used. The confidence interval is fixed at 5%.

## 3. Results

### *3.1. Nature and Position of Mutations*

Chromatogram analysis with the Mutation Surveyor software indicated the presence of mutations only for tumour tissue. The blood samples show no genetic variability of the *MED12* gene compared to the reference sequence (Figure 1). All single-position mutations affecting exon 2 induce an amino acid change. Long-chain deletions, which can induce a shift of the reading frame during the translation of exon 2, have also been found (Table 1). At the level of the region upstream of exon 2, a mutation 1285_1286delA was found for the first time in our patients (Table 1).

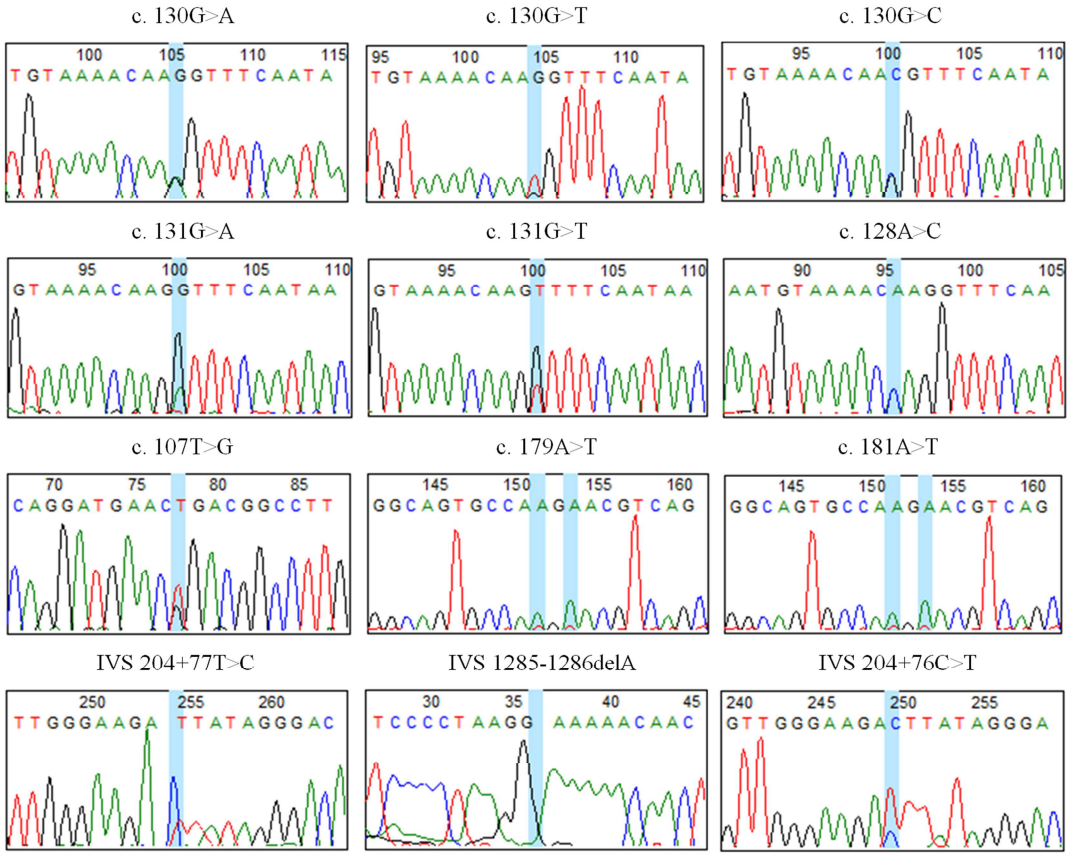


***Figure 1.*** *Heterozygous single nucleotide polymorphism of MED12 gene in uterine fibroids.*

***Table 1.*** *MED12 mutations status in Senegalese women with uterine fibroids.*

| ID | Nucleotide change | Mutation status | Amino acid change |
|---|---|---|---|
| UF3 | 1348G>A | Missense | p. Gly44Ser |
| UF4 | 1348G>T | Missense | p. Gly44Cys |
| UF6 | 1285_1286delA | Loss of splice acceptor | |
| UF7 | 1349G>A | Missense | p. Gly44Asp |
| UF8 | 1349G>T | Missense | p. Gly44Val |
| UF9 | 1285_1286delA | Loss of splice acceptor | |
| UF10 | 1285_1286delA | Loss of splice acceptor | |
| UF12 | 1319_1358del_39nt | Frame-shift mutation | p. delAsp34_Asn47 |
| UF13 | 1349G>A<br>1285_1286delA | Missense<br>Loss of splice acceptor | p. Gly44Asp |
| | 1349G>A | Missense | p. Gly44Asp |
| UF14 | 1397A>T | Missense | p. Lys60Met |
| | 1399A>T | Missense | p. Asn61Tyr |
| UF15 | 1348G>A | Missense | p. Gly44Ser |

| ID | Nucleotide change | Mutation status | Amino acid change |
|---|---|---|---|
| | 1285_1286delA | Loss of splice acceptor | |
| UF16 | 1348G>A | Missense | p. Gly44Ser |
| UF17 | 1348G>T | Missense | p. Gly44Cys |
| | 1285_1286delA | Loss of splice acceptor | |
| UF18 | 1356_1379del_23nt | Frame-shift mutation | p. delAsn46_Asp54 |
| UF21 | 1348G>A | Missense | p. Gly44Ser |
| UF23 | 1349G>A | Missense | p. Gly44Asp |
| UF24 | 1348G>C | Missense | p. Gly44Arg |
| UF30 | 1357_1363del_6nt | Frame-shift mutation | p. delAsn47_Pro49 |
| UF31 | 1348_1350delGGT | Frame-shift mutation | p. delGly44 |
| UF35 | 1349G>A | Missense | p. Gly44Asp |
| UF37 | 1349G>A | Missense | p. Gly44Asp |
| | 1285_1286delA | Loss of splice acceptor | |
| UF38 | 1325T>G | Missense | p. Leu36Arg |
| UF39 | 1346A>C | Missense | p. Gln43Pro |
| UF40 | 1349_1372del_23nt | Frame-shift mutation | p. delGly44_Ser52 |
| UF41 | 1344_1352del_8nt | Frame-shift mutation | p. delLys42_Phe45 |
| UF43 | 1348G>A | Missense | p. Gly44Ser |
| | 1285_1286delA | Loss of splice acceptor | |
| UF44 | 1349G>T | Missense | p. Gly44Val |
| | 1285_1286delA | Loss of splice acceptor | |
| UF45 | 1344_1367del_23nt | Frame-shift mutation | p. delLys42_Ala50 |
| UF46 | 1349G>T | Missense | p. Gly44Val |
| UF47 | 1348G>C | Missense | p. Gly44Arg |
| UF50 | 1349G>T | Missense | p. Gly44Val |
| UF54 | 1326_1366del_40nt | Frame-shift mutation | p. delLeu36_Ala50 |
| UF55 | 1348G>A | Missense | p. Gly44Ser |
| UF57 | 1349G>A | Missense | p. Gly44Asp |
| UF61 | 1349G>A | Missense | p. Gly44Asp |
| UF63 | 1348G>A | Missense | p. Gly44Ser |
| | 1285_1286delA | Loss of splice acceptor | |
| | 1329G>A | Synonymous | p. Thr37Thr |
| UF64 | 1349G>C | Missense | p. Gly44Ala |
| | 1351T>G | Missense | p. Phe45Val |

### *3.2. Pathogenicity of Mutations*

In uterine fibroids, codon 44 of *MED12* exon 2 gene is the most mutated. This nucleotide triplet, which codes for glycine is mutated into serine, arginine, cysteine, aspartic acid, alanine and valine. Mutations inducing the change of leucine_36 to arginine, glutamine_43 to proline, lysine_60 to methionine and asparagine_61 to tyrosine have also been found. 63.63% of these mutations are in the 2nd position of the codons and 36.37% in the 1st position. All these mutations with score ≥ 0.9 are predicted to be pathogenic (Table 2).

***Table 2.*** *Pathogenicity of mutations in exon 2 of the MED12 gene in uterine fibroids.*

| CDS position | Codon | Amino acid change | Score | Prediction of pathogenicity |
|---|---|---|---|---|
| c.107T>G | CTG>CGG | p. L36R | 0.999 | Damaging |
| c.128A>C | CAA>CCA | p. Q43P | 0.999 | Damaging |
| c.130G>A | GGT>AGT | p. G44S | 1.000 | Damaging |
| c.130G>C | GGT>CGT | p. G44R | 1.000 | Damaging |
| c.130G>T | GGT>TGT | p. G44C | 1.000 | Damaging |
| c.131G>A | GGT>GAT | p. G44D | 1.000 | Damaging |
| c.131G>C | GGT>GCT | p. G44A | 1.000 | Damaging |
| c.131G>T | GGT>GTT | p. G44V | 1.000 | Damaging |
| c.133T>G | TTC>GTC | p. F45V | 0.989 | Damaging |
| c.179A>T | AAG>ATG | p. K60M | 0.983 | Damaging |
| c.181A>T | AAC>ATC | p. N61Y | 0.997 | Damaging |

### *3.3. Analysis of the Protein Structure*

With respect to the prediction of the structure of exon 2 of the *MED12* gene according to each type of non-synonymous substitutions found, the results are listed in Figure 2. In comparison with the reference sequence, all the mutations show a conformational change in the 3D structure of the MED12 protein; in other words, there is a deformation of the protein and to a large extent a modification of the biological activity of the MED12 protein in uterine fibroids.

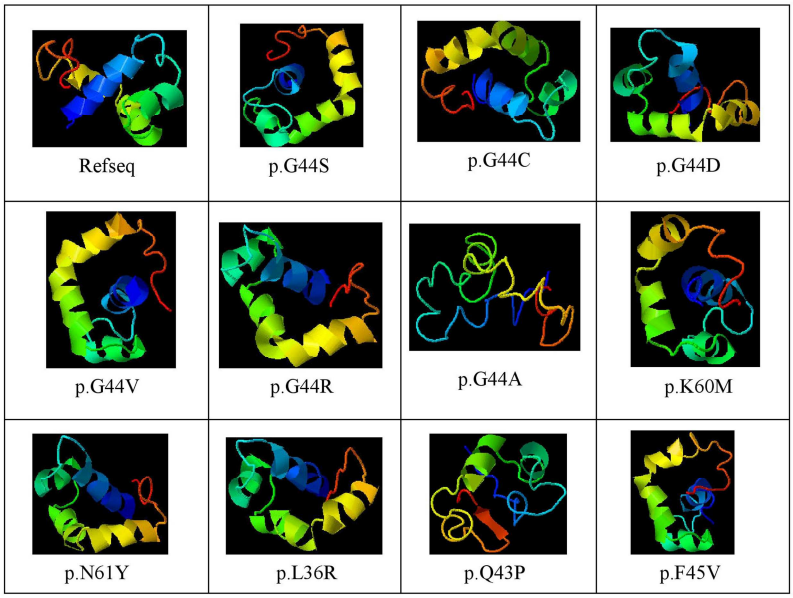


***Figure 2.*** *3D conformation of mutated amino acids in uterine fibroids.*

Moreover, for each mutation, the secondary structure of the protein, the function predicted in UniProt, the molecular function and the biological process are presented in Table 3. In comparison with the reference sequence (Refseq) which has a secondary structure α, mutations p. Q43P, p. G44S, p. G44D, p. G44R, p. F45V, p. K60M give proteins of α-β structure. All mutations alter the predicted function of the MED12 protein (Table 3), which further suggests their involvement in the pathobiology of uterine fibroids in Senegalese women.

***Table 3.*** *Prediction of the Structure, Function and Biological Processes of Exon 2 Mutations in the MED12 Gene.*

| Mutant | Secondary structure of protein | Predicted function in UniProt | Molecular function | Biological process |
|---|---|---|---|---|
| p. L36R | Protein α | Trp operon repressor (ID_P0A881) | DNA Binding | Transcription regulation |
| p. Q43P | Protein α-β | Nitrate reductase (ID_P71186) | Electron transfer activity | Cellular respiration |
| p. G44S | Protein α-β | Phosphosystem II (ID_P0A444) | DNA Binding | Transcription regulation |
| p. G44C | Protein α | DNA gyrase subunit A (Q99XG5) | DNA Binding | Transcription regulation |
| p. G44D | Protein α-β | Trp operon repressor (ID_P0A881) | DNA Binding | Transcription regulation |
| p. G44V | Protein α | Trp operon repressor (ID_P0A881) | DNA Binding | Transcription regulation |
| p. G44R | Protein α-β | DNA gyrase subunit B (ID_P66937) | DNA Binding | Transcription regulation |
| p. G44A | Protein α | DNA repair protein (ID_Q94BZ9) | Serine type carboxypeptidase | Antibiotic catabolic process |
| p. F45V | Protein α-β | Brinker brk (ID_Q9XTN4) | DNA Binding | Transcription regulation |
| p. K60M | Protein α-β | DNA subunit alpha (ID_Q5SH26) | DNA Binding | Transcription regulation |
| p. N61Y | Protein α | Trp operon repressor (ID_P0A881) | DNA Binding | Transcription regulation |

With regard to amino acid frequencies, the comparison of means between blood and tumour tissue samples shows different distributions for amino acids such as cysteine, aspartic acid, glycine, histidine, leucine arginine and serine (Table 4).

***Table 4.*** *Distribution of the amino acid frequencies of exon 2 between blood and tumour tissue.*

| Amino acid | Wilcoxon test | P-value |
|---|---|---|
| Ala | 662,5 | 1 |
| Cys | 550 | 0,032 |
| Asp | 525 | 0,032 |
| Glu | 625 | 0,424 |
| Phe | 520 | 0,080 |
| Gly | 912 | 0,002 |
| His | 562 | 0,04 |
| Ile | 587 | 0,129 |
| Lys | 637 | 0,618 |
| Leu | 875 | 0,006 |
| Met | 650 | 0,509 |
| Asn | 712 | 0,314 |
| Pro | 550 | 0,096 |
| Gln | 712 | 0,314 |
| Arg | 550 | 0,04 |
| Ser | 512 | 0,02 |
| Thr | 625 | 0,424 |
| Val | 650 | 0,831 |
| Trp | 575 | 0,060 |
| Tyr | 650 | 0,509 |

## 4. Discussion

In Eukaryotes, RNA transcription requires 2 steps. First, specific factors bind to DNA and recruit cofactors to modify the histone that results from DNA relaxation. Secondarily, a multiprotein complex called Mediator Complex associates with DNA and recruits transcription factors and RNA

polymerase II that initiates transcription [19]. *MED12*, a gene of 45 exons, located on the X chromosome (Xq13), expressed in all Eukaryotes, forms with Cyclin C, MED13 and CDK8, a subunit of this multiprotein complex. In this study, the *MED12* gene has been investigated in Senegalese women with uterine fibroids. Analysis of the chromatograms revealed a presence of mutations of the *MED12* gene only in tumour tissues with a frequency of 74% (37/50). This confirms the hypothesis that the *MED12* gene is involved in the occurrence of uterine fibroids. Mäkinen *et al.* [8] first described the link between *MED12* mutations and fibroids. In 2011, and according to him, mutations in the *MED12* gene represent the largest genetic defect in uterine fibroids. In addition, mutations of the *MED12* gene in tumours other than uterine fibroids are rare. Only 0.3 to 0.5% of colorectal cancers have mutations in the *MED12* gene, stating that they are only passenger's mutations [20, 21]. 5% of prostate cancers have different *MED12* mutations [22, 23]. No *MED12* mutations have been detected in malignant breast and ovarian tumours or any other carcinoma. The involvement of *MED12* in uterine fibroids could be explained by the fact that it is a gene that has a significant role in various cell signaling mechanisms by interacting with multiple receptors, particularly estrogen receptors [24]; and it is now accepted that uterine fibroids are dependent on steroid hormones including estrogen. Indeed, estrogens are considered the main agent inducing the growth of uterine fibroids. These findings are that uterine fibroids are rare during pubertal years, they increase or show minimal changes during reproductive age, and generally decline during postmenopausal period. In addition, studies have shown an accumulation of estrogen receptors in fibrotic tissues such as uterine fibroids [25-27].

The role of *MED12* in the occurrence of uterine fibroids is also demonstrated by analysis of the pathogenicity of the mutations found. Indeed, all the mutations affecting exon 2 (88.89%) appear as deleterious mutations, in particular those affecting codon 44 (score = 1.000). In other words, all the mutations affecting exon 2 cause an amino acid change and therefore an aberrant function of the MED12 protein. A study by Bourbon et al. [28], involving 39 different species, showed that codon 44 is the most conserved codon of the *MED12* gene, which states that this codon plays an important role in the biological process of the protein. Thus the missense mutations observed on this codon 44 in particular can render the translated protein non-functional, indicating the specific importance of this amino acid for the *MED12* function with respect to leiomyoma and suggesting that these mutations could represent alleles gain-of-function. Moreover, according to the work in reference [29], the binding domain of Cyclin C resides at the level of the N-terminal region encoded by exons 1 and 2 of the *MED12* gene and codon 44 would play a role in this membership. This further confirms the transcriptional activation of MED12 aberrant function in uterine fibroids and that codon 44 is essential for this process.

Our results show that all the mutations found alter the predicted function of the MED12 protein (conformational change in the 3D structure), which further suggests their involvement in the pathobiology of uterine fibroids in Senegalese women. In Eukaryotes, the Mediator Complex consists of at least 30 proteins [28], structurally divided into four modules, which are the head, the middle, the tail and the kinase modules. The head and middle modules interact directly with RNA polymerase II while the tail module associates with several cofactors to facilitate transcription. The Kinase module interacts with the Mediator Complex to suppress transcription [30]. Indeed, the Mediator Complex exists in 2 forms. The L. mediator form contains 4 modules of the kinase subunit (MED12, MED13, Cyclin C, CDK8 or CDK19) and acts as a receptor. The S. mediator form (without the CDK8 module) stimulates basal transcription. The MED12 domain plays a vital role in connecting Cyclin C-CDK8 to the core of the complex, which activates CDK8 kinase. In this regard, Mediator serves as a regulatory signal chain for activating and repressing proteins by affecting changes in gene expression programs that control various physiological processes including cell growth and homeostasis, development, and differentiation. Given its key role in the interactions between transcription factors of RNA polymerase II, the mediator complex may be indispensable in the transcription of all genes encoding proteins. Changes in the biological function of MED12 in uterine fibroids are also highlighted in protein function prediction analysis. Indeed, the mutations of exon 2 seem to induce gains and/or losses of function of the MED12 protein. The mutations p. L36R, p. G44D, p. G44V, p. N61Y make exon 2 of *MED12* Trp operon repressor. It is an aporepressor protein, that is, when it is supplemented with L-Tryptophan, binds the operator region and prevents the initiation of transcription. The p. G44C and p. G44R mutations are predicted to play a DNA gyrase role. DNA gyrase is a family of class II topoisomerase. They break and simultaneously connect 2 strands of DNA in an ATP dependent manner. Other protein functions such as Photosystem II (p. G44S), DNA repair protein (p. G44A) and Nitrase reductase are also predicted. These modifications constitute a proof of the biological modifications of the MED12 protein in women with uterine fibroids and therefore their implication in the occurrence and/or progression of these tumour cells.

This pathogenicity of the exon 2 mutations of the *MED12* gene is also highlighted in the comparison of the amino acid frequencies between the blood samples used as controls and the tumour tissues. A statistically significant difference is observed on cysteine, aspartic acid, glycine, histidine, leucine, arginine and serine. Codon 44 glycine is the codon most impaired in uterine fibroids and is mutated to serine, cysteine, aspartic acid, valine and arginine. The mutation of leucine_36 to arginine is also common in uterine fibroids. Proteins make up the largest part of the protoplasm of cells by ensuring their structure. Constituent amino acids are used to make enzymes and hormones that catalyze and regulate all metabolic functions in both the cell and the whole organism. Indeed, cysteine is a non-essential sulfur amino acid, synthesized from methionine, the only sulfur amino acid

essential in healthy adults. It is also likely that cysteine becomes conditionally essential in adult aggression, since it is the limiting amino acid for the synthesis of glutathione (glutamyl-cysteinyl-glycine), tripeptide that is the keystone the defense system against oxidative stress [31]. Arginine is a precursor and activator of ureogenesis in the liver, but it has a host of other functions [32]. It appears to have a trophic effect, since it increases the skin synthesis of collagen and it is now accepted that uterine fibroids are characterized by an accumulation of collagen fibers.

# 5. Conclusion

Results obtained show a significant genetic alteration of the *MED12* gene with a high frequency of mutations noted in particular codon 44 of exon 2. All these mutations being predicted as deleterious testify to their implication in the pathobiology of uterine fibroids. In addition, the noted alterations lead to instability of the MED12 protein and thus a change in its biological function in uterine fibroids.

The results obtained thus open up avenues for understanding the molecular mechanisms involved in the occurrence and/or progression of uterine fibroids. They also provide a glimpse of treatment strategies because *MED12* proves to be an indispensable biomarker in the progression of uterine fibroids.

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# Conflict of Interest

The authors declared no conflict of interest.

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