University of Siena

Department of Medical Biotechnologies

Doctorate in Genetics, Oncology and Clinical Medicine (GenOMeC)

XXIX cycle (2013-2016)

Coordinator: Professor Alessandra Renieri

Genetic and epigenetic analyses in thymomas of patients with Myasthenia Gravis

Scientific disciplinary sector: MED/03 – Medical Genetics

Tutor

Professor Lucia Migliore

PhD Candidate

Lopomo Angela

Academic Year 2016/2017

Alla mia famiglia,

quella di Sempre

e quella che sto per formare con Giuseppe

CENTRE de RECHERCHE en MYOLOGIE
UMRS 974 - UPMC Sorbonne Universités - INSERM - AIM
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Dr Rozen LE PANSE

PhD report for Angela Lopomo's PhD

University of Siena - Department of Medical Biotechnologies

Title: **Genetic and Epigenetic analyses in thymomas of patients with Myasthenia Gravis**

The aim of this phD work has been to investigate the genetic and epigenetic changes in thymoma associated myasthenia gravis (TAMG) with different objectives.

- To analyze the contribution of DNA methylation to the development of thymoma associated MG, she analyzed the methylation levels in promoters of genes involved in one carbon metabolism (MTHFR), in DNA methylation reaction (DNMT1, DNMT3A, DNMT3B), in autoimmunity (AIRE) and key cancer genes, such as genes of DNA repair (MGMT, hMLH1), and cell cycle (RASSF1A, CDKN2A). She has also analyzed the global methylation focusing on LINE1 methylation. These investigations were done on blood, tumor tissues, and adjacent thymic tissues from the same donor.
- To understand if methylation is an epigenetic mechanism involved in gene expression, she analyzed gene expression levels of MTHFR, DNMT3A, and AIRE genes in tumor and adjacent thymic tissues. She then investigated the correlation of expression levels with methylation levels but also of methylation status of chosen genes and both physical and clinic-pathological features of the patients.
- To understand if gene polymorphisms constituted a thymoma risk factor, she analyzed polymorphisms of genes required for DNA methylation processes in myasthenic patients with thymoma and myasthenic patients without thymoma. Next she searched for correlations among the studied polymorphisms and the methylation levels of MTHFR, DNMT1, DNMT3A, DNMT3B, MGMT, hMLH1, RASSF1A, CDKN2A, AIRE gene promoters.

Main comment concerning the manuscript

The document includes 5 main sections: abstract, introduction (56 pages), materials and methods (6 pages), results (12 pages), discussion (16 pages). The document also contains: a table of contents, a list of abbreviations, references, the original article with the main results of her PhD and the list of the publications in which she has been involved (9 articles: 3 as first author, 3 as second author).
The manuscript is well written but spelling errors should be corrected. My main advices for the manuscript are on the organization of chapters in the introduction (see my comment below).
The result section is well written and based on the recently published manuscript but the PhD student has managed to insert additional data.
The discussion is well written. A real effort has been made to take into account all the results and analyzed the impact of genetic and epigenetic changes in TAMG.

Detailed comments for revision

Abstract: It is well constructed but should be read carefully as it contains spelling errors.

Introduction: This introduction is well detailed and quite long (half of the manuscript) and includes 2 main parts:

1) Thymus and myasthenia gravis
2) Epigenetics changes.

Suggested revisions:

- Page 5 this sentence is not clear "*type B, subdivided on the basis of the proportional increase (in relation to the number of lymphocytes) in epithelial cells (thymocytes) and cellular atypia*".
- Page 10, spelling error on the 1.5 title
- A small paragraph explaining clearly the MG symptoms, is missing.
- In the paragraph "Myasthenia Gravis", it is a bit confusing as EOMG and TAMG have been mixed, subparagraphs should be done to help making the distinction as the etiological mechanisms involved in MG are different. Maybe 2 subparagraphs one on EOMG and TAMG could done.
- In the different paragraphs on *"Form that presents anti-AChR, anti-Musk and anti-LRP4 antibodies"*, the consequences of the autoantibodies effects should be mentioned. Are all these autoantibodies pathogenic?
- The paragraph on AIRE (a transcription regulator playing a key role in central tolerance) should be moved just after the 1.4 paragraph.
- Page 21: it should be specified that Barzago (2016) study was done on PBMCs while other data mentioned referred to the thymus.
- Page 23: it should be mentioned that CXCL13 and CCL21 are two chemokines involved in the abnormal recruitment of B cells in EOMG thymuses.
- Page 24: about hormonal influences the manuscript of Zhu et al. (Nat Commun. 2016) should be mentioned.
- Page 26: the sentence "The high frequency of thymoma-associated…" is very long and not that clear.
- Page 40, define TSG.
- Page 43: I suggest to remove the paragraph on miRNAs in MG. The previous one on miRNAs and TAMG is sufficient, especially as none experiments were done during the PhD on miRNAs.
- Page 45: Folate metabolism section could be moved just after the paragraph on 1.8 on Epigenetics.
- Page 59 and thereafter: about "*myasthenic patients with normal thymus* " it should be explained on which basis the thymus of these MG patients is considered as normal?

Materials and methods: A table with patient clinical data should be given

The work carried out by Angela Lopomo during her PhD has been done on an impressive number of patients. She has investigated thoroughly the hypothesis that epigenetic and genetic changes could be associated with thymoma MG patients. She has set up the path for future investigations, notably on the functional consequences of these changes.

An original article has been published based on her PhD work, and Angela Lopomo has also been involved in other publications. She has 9 publications altogether.

I totally agree with the PhD thesis defense of Angela Lopomo if she can manage to revise the manuscript.

Paris, 27/02/2017

Dr Rozen Le Panse

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UNIVERSITÀ DI PISA

Department of Translational Research and New Technologies in Medicine and Surgery
Director Gaetano Pierpaolo Privitera

Medical Genetics Laboratory,

Pisa, March 1st, 2017

To:
Doctor Rozen Le Panse
Centre de Recherche en Myologie
UMR 974 - UPMC Sorbonne Universités - INSERM - AIM

Dear Doctor Rozen Le Panse,
Thank you for detailed and accurate revision of my PhD thesis.

Please find below the responses on your requests.

- **Page 5 this sentence is not clear "type B, subdivided on the basis of the proportional increase (in relation to the number of lymphocytes) in epithelial cell (thymocytes) and cellular atypia".**
  Reply: The sentence has been rephrased (page 17, lines 1-2).
- **Page 10, spelling error on the 1.5 title.**
  Reply: Spelling error has been corrected, now it is the title of paragraph 1.6 (page 24, line 5).
- **A small paragraph explaining clearly the MG symptoms, is missing.**
  Reply: A small paragraph explaining clearly the MG symptoms has been added in the paragraph 1.6 Myasthenia Gravis (page 24, lines 12-22).
- **In the paragraph "Myasthenia Gravis", it is a bit confusing as EOMG and TAMG have been mixed subparagraphs should be done to help making the distinction as the etiological mechanisms involved in MG are different. Maybe 2 subparagraphs one on EOMG and TAMG could be done.**
  Reply: Thank you very much for this comment, two paragraphs, as indicated, have been included in the following order: 1.6 Myasthenia gravis; 1.6.1 Different forms of MG (with or without autoAbs); 1.6.2 EOMG forms; 1.6.2.1 Thymic abnormalities in EOMG form; 1.6.2.2 Genetics of EOMG form; 1.6.2.3 Etiological mechanisms in EOMG form; 1.6.3 Thymoma-associated myasthenia gravis; 1.6.3.1 Thymic abnormalities in TAMG; 1.6.3.2 Genetics of TAMG; 1.6.3.2 Etiological mechanisms in TAMG (pages 30-38).

- **In the different paragraphs on "Form that presents anti-AChR, anti-MuSK and anti-LRP4 antibodies", the consequences of the autoantibodies effects should be mentioned. Are all these autoantibodies pathogenic?**
  Reply: The pathogenic consequences of the presence of anti-AChR, anti-MuSK, and anti-LRP4 antibodies are detailed on page 26, lines 3-4, 9-11, page 27, lines 17-19, and page 27, lines 29-31) for anti-AChR, anti-MuSK, and anti-LRP4, respectively.
- **The paragraph on AIRE (a transcription regulator playing a key role in central tolerance) should be moved iust after the 1.4 paragraph.**
  Reply: The paragraph on AIRE has been moved, it now is 1.5 paragraph (pages 21-24).
- **Page 21: it should be specified that Barzago (2016) study was done on PBMCs while other data mentioned referred to the thymus.**
  Reply: The missing information has been added (page 34, lines 1-3).
- **Page 23: it should be mentioned that CXCL13 and CCL21 are two chemokines involved in the abnormal recruitment of B cells in EOMG thymuses.**
  Reply: The missing information on CXCL13 and CCL21 has been added (page 34, lines 29-30).
- **Page 24: about hormal influences the manuscript of Zhu et al. (Nat Commun. 2016) should be mentioned.**
  Reply: The manuscript of Zhu et al. (2016) has been added (page 30, lines 19-22).
- **Page 26: the sentence "The high frequency of thymoma-associated…" is very long and not that clear.**
  Reply: The sentence has been rephrased (page 37, lines 19-23).
- **Page 40: define TSG.**
  Reply: TSG acronym has been defined (page 59, lines 3-4).
- **Page 43: I suggest to remove the paragraph on miRNAs in MG.**
  Reply: The paragraph has been removed.
- **Page 45: Folate metabolism section could be moved just after the paragraph 1.8 on Epigenetics.**
  Reply: Folate metabolism paragraph has been moved after Epigenetics paragraph, it became 1.7 paragraph.
- **Page 59 and thereafter: about "myasthenic patients with normal thymus" it should be explained on which basis the thymus of these MG patients is considered as normal?**
  Reply: The thymus is defined normal according to the histological features (we read the report of our colleagues of the department of anatomo-pathology who performed the analysis on a piece of thymus previously taken after thymectomy) (page 65, line 10).

Materials and methods: A table with patients clinical data should be given.
Reply: Patients clinical data are given in Table 4 (page 65) of Materials and methods section.

Best regards,
Angela Lopomo

PhD Thesis Reviewer Report

Title: Genetic and Epigenetic analyses in Thymomas of patients with Myasthenia Gravis

Candidate: Lopomo, Angela

1. Evaluation about main contributions of the Thesis:

The thesis contains an extensive and original introduction with an excellent review on the literature.

2. Scientific quality of the Thesis:

Both the experiments as well as the analysis are convincing.

3. Organization of the Thesis:

The thesis is well organized.

4. Detailed technical comments:

See annotated Pdf document sent by email to Ms Lopomo.

5. Final recommendation:

I give a positive evaluation for the quality of the thesis

x PhD thesis **is admitted** to the final exam.

Significant additions / corrections requested:

I sent specific suggestions and corrections to Ms Popomo, with cc. to her supervisor, Prof Lucia Migliore, in an annotated pdf document.

Reviewer:

Dr Mario Losen

Signature:

Date:

29 - 3 - 2017

UNIVERSITÀ DI PISA

Department of Translational Research and New Technologies in Medicine and Surgery
Director Gaetano Pierpaolo Privitera

Medical Genetics Laboratory,

Pisa, March 29th, 2017

To:
Doctor Mario Losen
Department of Psychiatry and Neuropsychology Universiteitssingel
Maastricht University

Dear Doctor Mario Losen,

Thank you for detailed and accurate revision of my PhD thesis.

I took into account all your suggestions, and I showed to my supervisor, Professor Lucia Migliore all the changes.

Best regards,

Angela Lopomo

Table of contents

Abstract

Thymomas are uncommon neoplasms that arise from epithelial cells of the thymus and are often associated with myasthenia gravis (MG), an autoimmune disease characterized by autoantibodies directed to different targets at the neuromuscular junction. Little is however still known concerning epigenetic changes occurring in thymomas from MG individuals. The study aims: to shed light on the contribution of DNA methylation to the development of thymoma associated MG (TAMG) by the detection of gene-specific methylation levels in promoters of *MTHFR*, *DNMT1*, *DNMT3A*, *DNMT3B*, *AIRE*, *MGMT*, h*MLH1*, *RASSF1A*, *CDKN2A* genes and detection of global methylation by means of LINE1 methylation analysis in blood, tumor tissue, and adjacent thymus tissue; to understand if DNA promoter methylation influences gene expression and if it correlates with clinicopathological features of patients; to understand if some of polymorphisms in genes of folate metabolism, a key pathway involved in providing methyl groups necessary for DNA methylation, correlate with thymoma risk and if these polymorphisms influence the methylation levels of chosen genes.

Both *MTHFR* and *DNMT3A* promoters showed significant higher methylation levels in tumor tissue respect to blood, while *AIRE* showed an opposite trend. *MTHFR* showed also significantly higher methylation levels in tumor tissue respect to healthy adjacent thymic epithelial cells, while *AIRE* showed lower methylation levels in healthy tissue respect to blood. Moreover a negative correlation between *MTHFR* promoter methylation and gene expression in a small subgroup was found. All the other studied genes were largely hypomethylated both in blood and in tumor tissue, and the mean methylation levels were no higher than 2%. TAMG showed global DNA hypomethylation, in fact LINE1 resulted hypomethylated in tumor tissue respect to blood. Correlating clinicopathological features of patients with methylation levels, correlations between LINE1 and thymoma histology, Masaoka classification and MG Osserman one in tumor tissue were found. h*MLH1* methylation levels increased with age. Comparing genotype and allele frequencies between normal thymus patients and TAMG patients, significant decrease of *TYMS* 28bp 2R allele frequency in patients with thymoma with respect to normal thymus was observed. The *TYMS* 28bp 2R2R genotype was associated with decreased risk to develop a thymoma among MG patients. Moreover, it was noticed that polymorphisms of gene involved in one-

carbon metabolism could affect DNA methylation, in fact statistically significant correlations between the *MTRR* 66A>G polymorphism and *MTHFR* and h*MLH1* promoter methylation in tumor tissue and *DNMT1* in blood, between the *DNMT3B* -579TT genotype and higher promoter methylation levels of *DNMT3B* and h*MLH1* in tumor tissue and blood were found; the same happens for *DNMT3B* -149TT genotype related to *DNMT3B* methylation in thymoma tissue and blood.

The present study suggests that epigenetic changes, in term of gene specific methylation and global hypomethylation, could be involved in TAMG and that DNA methylation could influence gene expression and could be partially influenced by polymorphisms in folate metabolism genes.

Chapter 1: Introduction

1.1 Thymus

The thymus is a gland located in the chest, behind the breastbone and between the lungs; it is the central organ of the immune system and it is involved in the generation of many naive T cells, positively selected for their recognition of antigen associated with the major histocompatibility complex (MHC) expressed in the thymus, and negatively selected according to their response to self-antigens (Gwathmey and Burns et al., 2015). The result of these selections is that the T cells use the MHC expressed on the other cells of the body for recognition of foreign antigens but do not react with self-antigens (Fujii et al., 2013). Interactions between thymic stromal cells, that include epithelial cells, mesenchymal cells, and few myoid cells, expressing self-antigens and developing thymocytes lead to the elimination of autoreactive T cells (Suniara et al., 2000; Derbinski et al., 2001; Le Panse and Berrih-Aknin, 2005; Melzer et al., 2016). The self-tolerant T cells continue their differentiation before being exported to the periphery. Under physiological conditions, most thymic cells are thymocytes and stromal cells (Berrih-Aknin and Le Panse, 2014).

1.2 Thymic epithelial tumors: epidemiology and histology

1.2.1 Epidemiology

Thymic epithelial tumors (TETs) are rare mediastinal cancers arising from the epithelial cells of the thymus with an incidence of 1.7 per million persons/year in Europe and of 0.13 per 100000 person/year in the United States of America (Siesling et al., 2012). The incidence of TETs does not present a difference of gender but shows difference of age and geographical origins. The incidence increases with age, in fact there is an incidence of 0.4 per million/year in adolescent and young adults (<25 years of age), of 1.9 in the 25-64 years age patients, and of 4.2 in the over 65 years old patients (Siesling et al., 2012). Regarding geographical differences, TETs show lowest incidence, about 1, in Northern and Eastern Europe, United Kingdom and Ireland, and about the double in Central and Southern European Countries

(RARECAREnet, 2016). Among TETs, thymoma incidence in the United States of America is higher in Asian and Pacific Islanders and in black people than in Hispanic and white people and affect blacks at much younger age than whites (Engels, 2010). In Europe, about 7000 people have a diagnosis of epithelial tumor of the thymus (Girard, 2013).

Genetic and/or epigenetic features, modulated by environmental risk factors, may be a possible explanation of the increased risk in these groups of the population (Scorsetti et al., 2016). Another feature of this type of cancer is that patients with thymoma show an increased risk of developing second malignancies, such as lymphoma, gastrointestinal and lung cancers with a frequency between 8% and 28% (Filosso et al., 2013). This increased risk could be due to thymoma therapy, such as radiotherapy or surgery, which could cause immune deregulation, in fact thymectomy leads to immunosuppression (Scorsetti et al., 2016). Okumura and colleagues suggested that an autoimmune system alteration associated with thymoma might be the key to interprete and explain the increased risk of subsequent cancers (Okumura et al., 2008).

Survival from malignant thymic epithelial tumors diagnosed in Europe improved since the end of the nineties so much so that it is 84% at one year and 64% at five years (Siesling et al., 2012). In particular, five-year survival is higher for thymoma, followed by than squamous cell carcinoma, undifferentiated carcinoma and adenocarcinoma (Scorsetti et al., 2016). They have traditionally been treated using a multimodality approach consisting of surgery, radiotherapy, and platinum-based chemotherapy (Girard et al., 2013; Rajan et al., 2014).

1.2.2 Histology

Thymic epithelial tumors are very complex tumors, by this derives the difficult to classify them. In 1999, an international committee assembled by the World Health Organization (WHO) proposed the most recent classification system based on morphology and lymphocyte/epithelial cell ratio (Rosai, 1999), then updated in 2004 (Marx et al., 2004). The WHO system identifies six types of TET (Travis et al., 2004):

- *type A*, also called medullary or spindle-cell thymoma, without atypia or neoplastic lymphocytes,
- *type AB*, also called mixed thymoma, since are similar to type A but have foci of neoplastic lymphocytes,

- *type B*, subdivided on the basis of the proportional increase of thymocytes respect to lymphocytes and cellular atypia in:
  - *type B1*, also called lymphocyte-rich thymoma, lymphocytic thymoma, predominantly cortical thymoma, or organoid thymoma,
  - *type B2*, also called cortical thymoma,
  - *type B3*, also called epithelial, atypical, or squamoid thymoma or well-differentiated thymic carcinoma,
- *type C*, the thymic carcinomas.

The histological subtypes seem to have an independent prognostic significance, for example type A and type AB thymomas follow a benign clinical course, indeed types B1-3 are considered as low to moderate malignant neoplasms. Thymic carcinomas follow a malignant clinical course and display an aggressive behaviour, characterized by distant metastases (Zettl et al., 2000).

Thymoma are also classified in stages by Masaoka et al. (1981) staging system proposed in 1981 and then modified by Koga and colleagues in 1994 (Koga et al., 1994). This classification, usually indicated as "Masaoka system" describes tumor size and the spread degree of tumor:

- *stage I*: grossly and microscopically completely encapsulated tumor,
- *stage IIa*: microscopic transcapsular invasion,
- *stage IIb*: macroscopic invasion into thymic or surrounding fatty tissue, or grossly adherent to but not breaking through mediastinal pleura or pericardium,
- *stage III*: macroscopic invasion into neighbouring organ, such as pericardium, great vessel, or lung),
- *stage IVa*: pleural or pericardial metastases,
- *stage IVb*: lymphogenous or hematogenous metastasis.

1.3 Genetics of thymic epithelial tumors

1.3.1 Chromosome abnormalities

Copy number (CN) gains and losses of various chromosomes are TET hallmarks well described, in fact the frequency of these changes is associated with histological subtypes, and allows us to clustered TETs into various groups (Zettl et al., 2000; Inoue et al., 2003; Penzel et al. 2003; Lee et al., 2007a; Girard et al., 2009). Type A thymomas, which have an indolent behaviour and a good survival shows few CN aberrations and loss of heterozigosity (Inoue et al., 2003). All subtypes of thymomas, except B1, are characterized by the loss of 6q25.2–25.3 while the gain of 1q is common in type B2, B3 thymomas and thymic carcinomas; thymic carcinomas present the gain of 1q, 4, 5, 7, 8, 9q, 12, 15, 17q, 18, 20, and the loss of 3p, 6, 6p23, 9p, 13q, 14, 16q, 17p (Penzel et al., 2003; Inoue et al., 2003; Lee et al., 2007a; Girard et al., 2009). B3 thymomas and thymic carcinomas displayed a pattern of copy number aberrations similar to B2/B3 tumors except for the losses of 13q, 16q and 17p, that were not present in B2/B3 tumors (Penzel et al., 2003) and the loss of 13q was correlated with worse prognosis in B3 thymoma and thymic carcinomas (Zettl et al., 2000). The biological significance of chromosome changes could have a tumorigenic and autoimmune role, in fact the 6p23 region contains the *FOXC1* tumor suppressor gene, and chromosomal loss is correlated with lower protein expression. Tumors without *FOXC1* expression had a shorter time to progression and a trend for a shorter survival (Petrini et al., 2013a). In chromosome 6, the HLA (Human Leukocyte Antigens) locus harborours, so their loss might play a role in the pathogenesis autoimmunity characteristics of thymoma (Zettl et al., 2000). Copy number losses of *CDKN2A* and 13q were identified as potentially poor prognostic markers in TETs, in fact, CN loss of *CDKN2A* correlated with lack of expression of its related protein and identified tumors with poor prognosis (Petrini et al., 2012). Moreover, on chromosome 13q maps retinoblastoma 1 gene (*RB1*) a tumor suppressor gene, which deregulation may to have an important role in TET pathogenesis (Petrini et al., 2012).

Recently *EGFR* gene copy number was studied, in particular the non-coding CA simple sequence repeat 1 (CA-SSR-1) in intron 1 of *EGFR* gene. Two prevalent CA-SSR-1 genotypes were found, a homozygous 16 CA repeat and a heterozygous genotype, in which alleles have CA repeats from 16 to 20. Correlating the average combined allele length with tumor

subtypes, shorter sequences were significantly associated with the more aggressive thymoma subtype, such as B2/B3, B3 and B3/C histotypes. In thymomas, CA-SSR-1-allelic imbalance with short allele relative prevalence significantly correlated with increased *EGFR* gene copy number, advanced stage and metastatic behaviour (Conti et al., 2016).

1.3.2 Gene mutations

By new technologies, recent studies revealed mutations in thymic epithelial tumors. By sequence analysis of seventy-eight TET blood paired samples obtained from forty-seven thymic carcinoma and thirty-one thymoma patients revealed a total of eighty-six somatic non-synonymous sequence variations across thirty-nine genes in thirty-three (42%) TETs. The incidence of somatic non-synonymous mutations is higher in thymic carcinoma than thymomas. Among different genes, *TP53* is the most frequently mutated gene in TETs, especially in thymic carcinomas and was associated with a poorer overall survival. Moreover, genes involved in epigenetic modifications, such as genes for histone modification (*BAP1*, *SETD2*, *ASXL1*), chromatin remodelling (*SMARCA4*), and DNA methylation (*DNMT3A*, *TET2*, *WT1*) pathways, were recurrently mutated in thymic carcinomas, but not in thymomas, suggesting a potential disruption of epigenetic balance in thymic carcinomas (Wang et al., 2014b). By next-generation sequencing (NGS), in type A thymomas, a missense mutation (chromosome 7 c.74146970T>A) in *GTF2I* was identified and correlated with better survival. The *GTF2I* mutation was detected in type A (82%) and type AB (74%) thymomas and it was rare in the aggressive subtypes. Thymic carcinomas carried a higher number of mutations than thymomas, for example recurrent mutations in known cancer genes, such as *BAP1*, *CDKN2A*, *CYLD*, *PBRM1*, and *TP53* were identified in thymic carcinomas (Petrini et al., 2014). Mutations in *BCOR*, *FBN3*, *PCNXL3*, *PHF15*, *SFXN3*, *SRGAP1*, *VN1R5*, and *WDR70* were associated with thymic epithelial tumors. Among them *PHF15* is a gene that belongs to the PHF protein family, involved in chromatin-mediated gene regulation, and *BCOR* is a co-repressor of different transcription factors (Rajan et al., 2014). Thymic epithelial tumors show low frequency of mutation of key cancer genes, with different patterns between thymic carcinomas and thymomas. *TP53* was the most frequently mutated gene in thymic carcinomas, in which was associated with worse prognosis and *BCOR* was the gene mutated in B3 thymomas (Moreira et al., 2015).

By a recent analysis conducted on thymic epithelial tumors different mutations in thymoma and thymic carcinomas were found, in particular non-synonymous mutations in *ALK*, *ATM*, *CDKN2A*, *ERBB4*, *FGFR3*, *KIT*, *NRAS* and *TP53* were detected in carcinomas, while thymomas show mutations in *SMARCB1*, *STK11*, and *HRAS* in base of the histological subtype. By fluorescence in situ hybridization *CDKN2A*, *TP53* and *ATM* gene deletion in carcinomas were detected, whereas only one B3 thymoma exhibited a *CDKN2A* deletion, and none of the type A thymomas showed a gene loss (Enkner et al., 2016).

From these data, the picture emerges that thymic epithelial tumors have a unique mutational profile undetectable by gene panels used to analyze the profile common solid tumors, so the strategies could be useful to identify mutations in this rare tumor type (Rajan et al., 2014).

1.4 Thymoma associated with autoimmune diseases

The association between thymoma and different autoimmune diseases is known, especially with myasthenia gravis (MG), in fact the 10-20% of MG patients has a thymoma, and 30% of patients with thymoma develop MG (Bernard et al., 2016). Thymoma and myasthenia gravis are usually diagnosed at the same time. Moreover about 15% of MG patients develop a second autoimmune disease, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, or, in the major part of cases, autoimmune thyroiditis. In this paragraph, only the most frequent autoimmune diseases will be briefly described, a more exhaustive description of all autoimmune diseases associated to thymoma is reported in the review by Bernard and colleagues (2016).

Besides MG, thymoma is associated with other autoimmune diseases, including pure red cell aplasia (PRCA), SLE, polymyositis, limbic encephalitis, and Good's, Isaac's, and Morvan's syndromes. Thymectomy do not always have a positive effect on the outcome of autoimmune diseases, in fact, in some cases, it may worsen the evolution of the disease (D'Andrea et al., 1993; Boonen et al., 2000).

Isaac's syndrome, also known as acquired neuromyotonia, is a rare disorder due to the presence of autoantibodies against presynaptic voltage-gated potassium channels (VGKCs), in particular against to leucin-rich glioma-inactived protein 1 (Lgi 1) and contactin-associated protein 2 (Casp 2), leading to hyperexcitability of peripheral nerves (Hart et al., 2002;

Vernino and Lennon, 2002; Irani et al., 2010; Ahmed and Simmons, 2015). Autoantibodies against VGKCs are present not only in the peripheral nervous system, but also in the central nervous system, where they play an important role in neuronal excitability. Thymoma is present in about 20% of Isaac's syndrome patients, and the major part of them have antibodies direct to Casp2 (Vincent and Irani, 2010). Isaac's syndrome is the second most frequent autoimmune disease after MG in thymoma patients, for which thymectomy has little positive effects on the clinical severity (Evoli et al., 2007; Ahmed and Simmons, 2015).

Morvan's syndrome is characterized by acquired neuromyotonia, sleep disorder with long periods of insomnia, and encephalitis (Fleisher et al., 2013). In a study, thymoma was found in 37% of the patients with Morvan's syndrome, among which about 50% had Casp 2 antibodies, while no patient had antibodies against Lgi1 (Irani et al., 2012). Patients treated with rituximab had a complete resolution of disturbs in anti-Casp 2 patients (Ong et al., 2013).

About 1.5-2% patients affected by s*ystemic lupus erythematosus* have thymoma, while about 2-10% of thymoma patients develop SLE. Thymoma and SLE are diagnosed simultaneously in 35% of cases; they have a diagnosis of SLE before or after the diagnosis of thymoma. The clinical symptoms of SLE include articular involvement, skin involvement, and serositis (Zhang et al., 2009). The effect of thymectomy on SLE activity is not clear, since in some patients there was no effect, and in others SLE activity increased (Boonen et al., 2000).

1.5 AutoImmune REgulator - AIRE

AIRE, the AutoImmune REgulator, a transcriptional regulator factor expressed in medullary thymic epithelia, promotes the promiscuous expression of more than one thousand tissue restricted antigens (TRA), such as thyroglobulin and insulin, in order to delete auto-aggressive T cells (Liston et al., 2003; Mathis and Benoist, 2009). Mature T cells are generated in the thymus, and their antigen receptors are generated through random somatic DNA rearrangement, which provides a molecular basis to recognize and eliminate an essentially unlimited number of pathogens (Anderson and Takahama, 2012; Hogquist et al., 2005; Klein et al., 2014). After positive selection by cortical thymic epithelial cells (cTECs) based on the proper affinity to MHC molecules, T cells migrate to the medulla and undergo negative selection. In the medullary thymic epithelial cells (mTECs) AIRE enforces the self-

tolerance by promoting the expression of tissue restricted-antigens (TRA), so autoreactive T cells that recognize these TRAs undergo negative selection preventing the release of autoreactive T cells in the periphery (Derbinski et al., 2001; Metzger et al., 2011; Klein et al., 2014). In absence of AIRE, autoimmune reactions could develop, in fact, Autoimmune PolyEndocrinopathy Candidiasis Ectodermal Dystrophy (APECED), also known as autoimmune polyglandular syndrome type 1 (APS1), an autosomic recessive autoimmune disease, is caused by mutations in the *AIRE* gene that cause its loss and is characterized by the loss of immunological self-tolerance towards several endocrine organs (Akirav et al., 2011). Although patients with APS1 harbour *AIRE* mutations on both alleles, it is emerging that individuals with single *AIRE* point mutations on only one allele have increased susceptibility to autoimmune diseases (Oftedal et al., 2015).

Aire expression is regulated by conserved non-coding sequences upstream the gene, activated by nuclear factor-kβ (LaFlam et al., 2015; Haljasorg et al., 2015) and its transcription seems to undergo spatiotemporal regulation, since it is ubiquitously transcribed during the earliest stages of embryogenesis, during which it is restricted to mTECs, extrathymic AIRE-expressing cells, thymic B cells, and becomes upregulated in mature mTEC (Anderson and Su, 2016). It was described that AIRE regulates TRAs expression in two ways, stochastic and ordered; stochastic because a small percentage of the total number of mTECs expresses a particular TRA, since AIRE promotes the expression of diverse sets of TRA within individual mTEC (Derbinski et al., 2008; Villaseñor et al., 2008) and ordered because a particular set of TRA genes are frequently co-expressed in an individual mTEC (Pinto et al., 2013).

AIRE has not a DNA-binding motif but recognizes genes that possess silenced chromatin states, in fact, it recognizes the unmethylated histone H3 lysine 4 (H3K4), a repressive epigenetic marker. AIRE interacts with histone core proteins, either directly or through its interactions with ATF7IP-MBD1 (activating transcription factor 7-interacting protein-methyl-CpG-binding domain protein 1) complex normally associated with gene repression (Waterfield et al., 2014). Moreover, AIRE promotes TRA expression through the release of stalled RNA polymerase to elongate RNA transcripts (Giraud et al., 2012) and interacts with proteins that exert control over the function of AIRE through post-translational modification, for example, it interacts with the protein deacethylase sirtuin 1 that deacetylates lysine residues on AIRE and with CREB-binding protein and p300, that add acetyl groups to AIRE (Incani et al., 2014; Chuprin et al., 2015).

The loss of *AIRE* expression can allow the autoreactive T cells to escape negative selection; patients with thymoma have very often autoantibodies, in fact in thymomas *AIRE* expression is very low (Suzuki et al., 2008). This could be due to fact that *AIRE* is expressed in the medullary epithelial cells while most thymomas are to the cortical epithelial cells of the thymus, suggesting that they origin from cortical epithelial cells. Moreover, there is a different frequency of MG associated with various histological types of thymoma that cannot be explained by the difference in the expression of *AIRE*. Type B thymomas have a very high frequency of associated myasthenia gravis, but type AB, although having an equally high number of immature T cells as the B type thymomas, have a low frequency of associated myasthenia gravis, suggesting that other factors could be implicated in the pathogenesis (Okumura et al., 2001).

However the existence of AIRE-independent TRA suggested that a non-AIRE factor control TRA expression in mTEC (Derbinski et al., 2005). Recently a new key transcription factor Fezf2 (also known as Zfp312 and Fezl) was identified. Fezf2 was originally discovered in the developing forebrain of *Xenopus* and zebrafish and has been shown to be a neuronal transcription factor required for the differentiation and specification of corticospinal neurons and subcerebral projection neurons in the brain (Shim et al., 2012; Eckler and Chen, 2014). Recently it has been demonstrated that Fezf2, expressed in mTECs of the thymus, regulates TRA expression independently of Aire, in fact mice that specifically lack Fezf2 in mTECs develop severe autoimmune phenotypes, with autoantibody production and inflammatory cell infiltration. Fezf2 and Aire do not play redundant roles in the TRA expression regulating different TRAs (Takaba et al., 2015). This was demonstrated by genome-wide analysis of mRNAs expressed in the mTECs isolated from Fezf2-/- mice. The authors selected genes that were expressed more than four times higher in the wild-type than the Fezf2-/- mTECs and observed that they belong to TRAs, and are regarded as Fezf2-dependent TRAs. Moreover, five of them, such as Krt10, Resp18, Fabp9, Maoa, and Timd2, had been reported to be induced in the absence of Aire, suggesting that Fezf2 regulates the expression of a distinct subset of TRAs in the thymus (Derbinski et al., 2005). The expression of Fezf2 is regulated by lymphotoxin beta receptor (LTβR) signalling pathway, a different pathway than that regulates Aire (Takaba et al., 2015). Fezf2 act binding directly to the promoter region of Fezf2-depenedent TRA genes, some of which, such as Mbp, Gad1, Col2a1, and Muc1, are known to be autoantigens or tumor antigens (Roulois et al., 2013; Komatsu et al., 2014). The

transcriptional program in the thymus is governed by Fezf2 and Aire, both of which contribute in an essential manner to the suppression of autoimmune diseases (Takaba et al., 2015).

1.6 Myasthenia Gravis

Myasthenia gravis is an autoimmune disorder of neuromuscular junction (NMJ) characterized by fatigability and muscular weakness primarily mediated by autoantibodies to the nicotinic acetylcholine receptor (AChR), the postsynaptic muscle neurotransmitter receptor. Other targets, such as muscle-specific kinase (MuSK) and low-density lipoprotein receptor-related protein 4 (LRP4), have been described. Some MG patients have striational antibodies, such as antibodies against titin or rynanodin receptors (Figure 1). Myasthenia gravis is characterized by specific symptoms, such as pronounced weakness and fatigue of voluntary muscles, ptosis, diplopia, facial paresis, bulbar disorders, neck weakness, dysphonia, muscular damage without alterations in central nervous system alterations (Sieb, 2014). In the major of patients, at the onset of MG are evident eye muscle weakness, this causes diplopia and ptosis; if these weakness will remain limited to the ocular muscles, it will be defined ocular myasthenia. While in generalized myasthenia gravis, weakness is more pronounced in the proximal limb than in the distal (Sieb, 2014). All these symptoms can cover a period from weeks to months and are characterized by short duration variation during the daytime with an aggravation during the evening, menstruation or the occurrence of fatigue (Sieb, 2014). MG has a worldwide prevalence of 40-180 per million people, and an annual incidence of 4-12 per million people and occurs in patients of all ages and both sexes (Gilhus and Verschuuren, 2015). The number of MG patients is increased, more than double, in the last twenty years in Western countries as well as Eastern one, probably due to a better ability of neurologists to distinguish MG symptoms from classical fatigability due to aging (Gilhus and Verschuuren, 2015). Increased number of cases could be also due to increased longevity of population, since autoimmunity disease risk increases with age. Myasthenia gravis is a multifactorial disease, in fact it is generally believed that a combination of genetic, epigenetic and environmental factors are involved in disease generation.

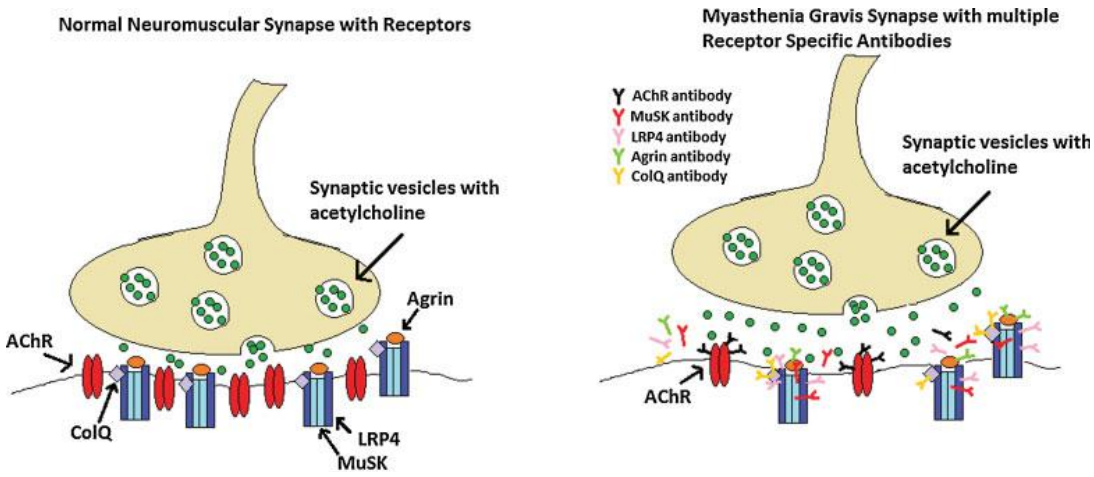

Figure 1. Neuromuscular junction simplification in normal neuromuscular synapse and in a myasthenia gravis synapse (AChR: acetylcholine receptor; ColQ: collagen-tail subunit of AChE; LRP4: lipoprotein receptor-related protein 4; MuSK: muscle-specific kinase) (from Townsel et al., 2016).

1.6.1 Different MG forms

MG is classified on the basis of the location of the affected muscles, ocular versus generalized, the age of onset of symptoms, early versus late, and the autoantibody profile. The specific origin of the autoimmune dysfunction in MG patients is unknown, but thymic abnormalities, defects in immune regulation, sex hormones, chronic inflammation, characterized by high expression of inflammatory cytokines which may increase AChR expression, and impaired regulatory T cell (Treg) function may play important roles in patients with anti-AChR antibodies (Le Panse et al., 2008; Cufi et al., 2013; Townsel et al., 2016).

Myasthenia Gravis is classified in different forms:

- form that presents anti-AChR antibodies (AChR-MG) that include:
  - ocular forms;
  - generalized forms;
- form that presents anti-MuSK antibodies (MuSK-MG);
- form that presents anti-LRP4 antibodies (LRP4-MG);
- form without detectable AChR, MuSK, or LRP4 antibodies.

Form that presents anti-AChR antibodies

MG forms with anti-AChR antibodies are the most common form of MG; in fact about 50% of ocular MG and 85% of generalized MG patients have antibodies against AChR. The anti-AChR antibodies belong to the IgG1 and IgG3 subclasses that bind the complement (Berrih-Aknin and Le Panse, 2014). Acetylcholine receptors, located on the surface of muscle cells, at the synapse between nerve cells and muscle cells, are composed of five protein chains, 2 α, β, δ, and ε, arranged into a channel that passes through the cell membrane. The acetylcholine (ACh) binds the α chains causing the opening of channel by a conformational change that allows positively charged ions to cross the membrane, generating endplate potentials and leading to muscle contraction (Berrih-Aknin and Le Panse, 2014). In MG patients the autoantibodies directed to AChR causes the reduction of AChR density at the NMJ resulting in decreased endplate potentials. AChR-associated MG has a bimodal age pattern of incidence related to gender, with a peak in young adults aged about 30 years highly frequent in women, and then an increase in incidence with age older than 50 years slightly more frequent in men (Berrih-Aknin, 2014; Drachman, 2016).

Ocular form

Among forms that present anti-AChR antibodies, ocular form affects about 15% of patients and it is diagnosed when symptoms are restricted to ocular signs for at least two years. The signs of this form are double vision and ptosis generally bilateral and asymmetric, in fact ocular MG affects the extra ocular muscles but not the pupils. After the first two years, the disease turns into generalized form in 25% of cases, while remains confined to ocular movement in 75% of cases (Townsel et al., 2016).

Generalized form

According to the age of onset, generalized forms can be divided into three subgroups:

- early-onset form;
- late-onset form;
- very late-onset form.

The *early-onset form* (EOMG) is characterized by the onset of first symptom before age 50 years, by high level of anti-AChR antibodies and by thymic follicular hyperplasia. This form is more frequent in female than male with a ratio female:male of 3:1, suggesting a role of sex

hormones. EOMG patients respond to thymectomy and can develop other autoimmune diseases, such as thyroiditis (Berrih-Aknin, 2014).

The *late-onset form* (LOMG) is characterized by the onset of first symptom after age 50 years and by the presence of a thymoma. This form does not show a strong difference of gender but is slightly more frequent in males than females. Patients have generalized symptoms with bulbar signs and frequent severe respiratory crises and they usually show autoantibodies against striated muscle proteins, such as rynanodin or titin (Romi et al., 2000).

The *very late-onset* form is characterized by age of onset after 60 years, by the absence of thymoma, and it affects mainly males (Aragones et al., 2003). After 1990, the incidence of patients older than 50 years without thymoma is tripled in Italy, probably due to increased longevity of population (Casetta et al., 2010).

Form that presents anti-MuSK antibodies (MuSK-MG)

About 40% of MG patients without anti-AChR antibodies and with generalized symptoms have anti-MuSK antibodies. Muscle-specific tyrosine kinase is a protein expressed on the postsynaptic membrane, which play a key role in AChR cluster induced by agrin and NMJ formation. The function of MuSK is activated when agrin, a proteoglycan, binds to MuSK via the co-receptor LRP4. In MuSK-MG patients, autoantibodies bind the Ig-like regions in the MuSK ectodomain, blocking assembly and activation of the agrin-LRP4-MuSK complex. This form is characterized by severe clinical symptoms that involve facial, bulbar and respiratory muscles and by muscle atrophy (Evoli et al., 2003), it is frequents among young females (Lavrnic et al., 2005), and patients do not show thymic pathology (Evoli et al., 2003; Leite et al., 2005). Unlike other MG forms, this group shows a strong association with HLA, in particular with HLA-DQ5 (Marino et al., 2014).

Form that presents anti-LRP4 antibodies (LRP4-MG)

About 20% of MG patients have anti-LRP4 antibodies. LRP4, a member of the LDLR family, binds the neural isoforms of agrin and is required to stimulate MuSK phosphorylation. LRP4 has a role in the endplates formation and LRP4 antibodies bind to the membrane protein *in vivo*, block the agrin-LRP4 interaction and thereby also inhibit AChR clustering in the membrane. Mice carrying mutations in *lrp4* show grave defects in neuromuscular synapse formation, leading to neonatal lethality (Weatherbee et al., 2006).

MG-like symptoms, such as impaired neuromuscular transmission and reduced muscle action potentials appear when IgG purified from LRP4-immunized rabbits are transferred into naive mice (Shen et al., 2013). The thymus is atrophic and normal for age, but few cases of hyperplasia have been reported (Berrih-Aknin, 2014).

Form without detectable AChR, MuSK, or LRP4 antibodies

Patients without detectable AChR, MuSK, or LRP4 antibodies represents a heterogeneous group and may have ocular or generalized form of MG (Cossins et al., 2013). Seronegative patients probably have pathogenic antibodies against yet undefined antigens in the postsynaptic membrane. Recently new targets have been discovered, such as agrin, cortactin, titin and ryanodine, sometimes together with known antibodies (Zhang et al., 2014; Gallardo et al., 2014). Agrin antibodies have been detected in a few MG patients with AChR, MuSK, or LRP4 antibodies (Gasperi et al., 2014; Zhang et al., 2014), while cortactin auto-antibodies have been reported both in MG patients with and in patients without known neuromuscular autoantibodies (Gallardo et al., 2014). Antibodies direct to titin have been reported in about 40% of all AChR-MG. The presence of antibodies direct to titin, a filamentous protein muscle protein involved in the maintenance of cell structure flexibility, is age-related, since it has a prevalence of 6% in EOMG and of 50-80% in non-thymomatous LOMG (Stergiou et al., 2016). Antibodies to ryanodine receptor are found in LOMG patients, which have thymoma, high percentage of bulbar respiratory and neck weakness (Romi et al., 2007).

Congenital Myasthenic Syndrome

Congenital Myasthenic Syndromes (CMSs) comprises a group of rare, heterogeneous and inherited disorders caused by mutations in genes that encode for essential proteins involved in the formation of neuromuscular junction. Genetically confirmed CMS show a prevalence of 9.2 cases per million children less than 18 years of age in the United Kingdom (Parr et al., 2014). Usually the prevalence ranges from 2.8 to 15.5 per million with the highest values in England and Northern Ireland (Abicht et al., 2012). In southern Brazil, a low prevalence of 0.18/100.000 of CMS is estimated (Mihaylova et al., 2010). Clinical signs of the disease are usually evident at or soon after birth, even if in some cases the onset of clinical signs may be late with involvement in adolescence or adulthood, and include muscle weakness, hypotonia, bulbar and facial paresis, hypoventilation or episodic apnoea, and ptosis. CMS

are not defined by well-defined diagnostic criteria and are suspected in cases of early-onset fatigable muscle weakness, a positive family history of a specific disorder, clinical myasthenic findings with a negative antibody testing profile, a presence of a specific clinical syndromic phenotype (Engel, 2014; Rodríguez Cruz et al., 2014; Engel et al., 2015).

Mutations affect about 20 genes, involved in pre- and post synaptic neuromuscular junction and synaptic basal lamina, impairing acetylcholine signal in the muscle. However, by new technologies, new causative genes which are ubiquitously expressed have been identified, suggesting that CMS is not only due to defects in the neuromuscular junction (Belaya et al., 2012; Cossins et al., 2013; Das et al., 2014). Genetic inheritance patterns include both autosomal dominant, with mutations in genes encoding the AChR subunits, such as *CHRNA1*, *CHRNB1*, *CHRND*, and *CHRNE*, and recessive forms, with mutations in *RAPSN*, *CHAT*, *AGRN*, *COLQ*, and *DOK7* (Rodriguez Cruz et al., 2014; Souza et al., 2016). Following are listed (Table 1) all principal congenital myasthenic syndrome subtypes but for a well description is possible to consult the reviews by Rodriguez Cruz et al. (2014) and Souza et al. (2016).

CMS type	CMS type cause	Gene	Gene location
Pre-synaptic syndromes	Choline acetyltransferase deficiency	*CHAT*	10q11.23
	SNAP25B deficiency	*SNAP25*	20P12.2
	Synaptotagmin-2 deficiency	*SYT2*	1q32.1
Synaptic basal lamina	Endplate acetylcholinesterase deficiency	*COLQ*	3p25.1
	Laminin-β2 deficiency	*LAMB2*	3p21.31
	Agrin deficiency	*AGRN*	1p36.33
Post-synaptic syndromes	Acetylcholine receptor deficiency Slow-channel syndrome Fast-channel syndrome	*CHRNA*	2q31.1
		CHRND	2q37.1
		CHRNB, CHRNE	17p13.2
	Escobar syndrome	*CHRNG*	2q37.1
	LRP4 deficiency	*LRP4*	11p11.2
	MuSK deficiency	*MuSK*	9q31.3
	Dok-7 deficiency	*DOK-7*	4p16.3
	Rapsyn deficiency	*RAPSN*	11p11.2
	COL13A1-related CMS	*COL13A1*	10q22.1
Glycosylation pathway	GFPT1 deficiency	*GFPT1*	2p13.3
	DPAGT1 deficiency	*DPAGT1*	11q23.3
	ALG2 and ALG14 deficiency	*ALG2*	9q22.33
		ALG14	1p21.3

	GMPPB deficiency	*GMPPB*	3p21.31
Other myasthenic syndromes	PRELP Deletion Syndrome	*PRELP*	2p21
	Plectin deficiency	*PLEC*	8q24.3
	Defects in sodium channels	*SCN4A*	17q23.3

Table 1. Congenital Myasthenic Syndrome subtype and associated genes, and genes location.

1.6.2 EOMG form

The early onset form (EOMG) is characterized by the onset of first symptoms before age 50 years, thymic follicular hyperplasia, marked by ectopic germinal centers (GCs), and high titers of anti-AChR antibodies. About 80% of patients with follicular hyperplasia are women (Berrih-Aknin, 2014). The high frequency of EOMG among women suggests a role of sex hormones, in fact different studies suggest an important role of estrogens in MG (Nancy and Berrih-Aknin, 2005). Estrogens receptors are expressed on thymocytes and thymic stromal cells (Kawashima et al., 1992). In MG patients, it was observed an increased expression of estrogens receptor-α in thymocytes, causing an higher sensibility to the effects of estrogens (Nancy and Berrih-Aknin, 2005). Dragin and colleagues (2016) proposed a model, according to which, during puberty, estrogens downregulate thymic AIRE expression, reducing the efficiency of the tolerance process regulated by AIRE. The decreased AIRE expression results in increased export of autoreactive cells. Consequently, the equilibrium between autoreactive T cells and Tregs is weak and it can become unbalanced, resulting in the development of autoimmune diseases in the presence of a triggering external factor (Dragin et al., 2016). In males, androgens have an opposite role respect estrogens against autoimmunity. Androgens recruit androgen receptor to *Aire* promoter regions, with consequent upregulation of Aire transcription, playing a protective role against autoimmunity (Zhu et al., 2016). Moreover, HLA-region genes contain estrogens response elements and polymorphisms in these response elements might influence transcription in a gender specific manner (Kaur et al., 2008), in fact recently in a genetic association study of thirty-five candidate genes a gender bias association for SNPs in the HLA locus was observed (Gregersen et al., 2012).

1.6.2.1 Thymic abnormalities in EOMG form

The thymus of EOMG patients shows thymic hyperplasia, which is characterized by the B lymphocyte infiltrates invading thymic medulla or present in perivascular spaces that are fused with the thymic medulla. In the first case the B lymphocyte infiltrates are present in form of ectopic B-cell follicles with inclusion of germinal centers (GCs) forming follicular hyperplasia; in the second case B lymphocytes infiltrates are distributed through the medullary parenchyma, forming diffuse hyperplasia and thymitis (Cavalvante et al., 2013; Figure 2). When GCs are created, they lead to hypermutation of genes encode for B cell receptor and to intrathymic production of high affinity myastogenic anti-AChR antibodies, explaining the reason for which thymectomy has a positive effect on EOMG patients (Marx et al., 2013). Therefore, EOMG thymus is characterized by the presence of germinal centers, intrathymic production of autoantibody, increased number of B cells and plasma cells. Expansion of perivascular spaces, the rupture of continuous epithelial cell layer and lamina basal caused by the formation of germinal centers, and alterations in regulatory T cells function are all follicular hyperplasia hallmarks (Marx et al., 2013).

To explain intrathymic pathogenesis, Marx and colleagues (2013) proposed a two-step model of the according to which, at first time, unfolded AChR subunits expressed by MHC class I and II positive hyperplastic medullary epithelial cells trigger helper T cells, and next antibodies elicited by T cells attack nearby myoid cells and active complement with release of AChR complex that actives antigen presenting cells (Marx et al., 2013). So myoid cells could have an important role in intrathymic autosensitisation to AChR (Marx et al., 2013).

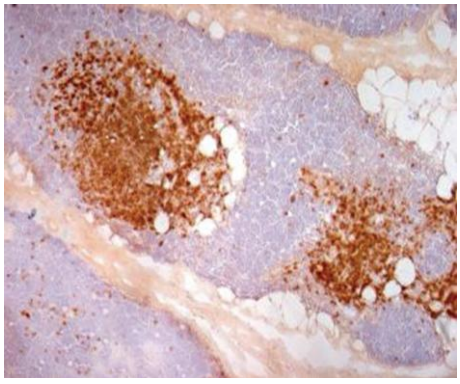

Figure 2. Thymus with follicular hyperplasia. CD20+ B cells aggregate to form germinal center (in brown) in the thymic medulla (from Cavalcante et al., 2011c).

1.6.2.2 Genetics of EOMG form

Among the genetic factors involved in EOMG, the role of Human Leukocyte Antigen (HLA) system is well established. The association between HLA haplotypes and MG shows a heterogenic pattern in base of population ethnicity and pathological subgroups (Ehsan et al., 2015). One of the most consistent associations is the correlation of HLA A1-B8-DR3 haplotype with anti-AChR positive patients and EOMG in Caucasian ethnicity (Vandiedonck et al., 2005). This haplotype is not closed to MG but correlates also with other autoimmune diseases, such as celiac disease and SLE (Candore et al., 2002; Candore et al., 2003). By genome-wide association study (GWAS), it was found that, HLA-B*08 is the major associated allele with EOMG hyperplastic thymus in North Europeans (Gregersen et al., 2012). Moreover studies on different populations found an association between MG and MHC class II, such as HLA-DRB1 and -DQB1; for example, EOMG patients had a tendency toward associations with HLA DR3, DRB1*09, DQB1*02, DQB1*05:03, and DQB1*06:04 (Giraud et al., 2001; Franciotta et al., 2001; Saruhan-Direskeneli et al., 2006; Deitiker et al., 2011; Xie et al., 2011).

The HLA are particularly important since they contain genes related to humans immune system function. For example, the tumor necrosis factor-alpha (TNF-α), a proinflammatory cytokine, is located within the HLA locus closely linked to AH8.1 haplotype. Moreover, a functional polymorphism located in the promoter region, *TNF-α* -308G>A, has been linked to higher expression and serum levels of TNF-*α* in EOMG (Elahi et al., 2009; Barrett, 2012). HLA and non-HLA genetic factors are involved in specific MG subgroups among different populations, such as HLA B8, *TNIP1*, and *PTPN22* in North European EOMG patients (Gregersen et al., 2012); HLA DRA, *TNF-α*, *BAFF*, and *VAV1* in Caucasian EOMG patients (Avidan et al., 2014); HLA DRB1/HLA DQA1, *TNFRSF11A*, and *CTLA4* in North American and Italian anti-AChR positive MG patients (Renton et al., 2015).

Polymorphisms in genes encoding for auto-antigens and immune-modulating proteins might contribute to the susceptibility and severity of MG, by altering the three-dimensional structure and expression of proteins, and influencing the interaction with their modulating proteins (Li et al., 2016). One of them is *PTPN22*, associated with MG and other autoimmune diseases (Lefvert et al., 2008). PTPN22, protein tyrosine phosphatise non-receptor 22, is an intracellular protein tyrosine phosphatise bound to c-src tyrosine kinase (Csk), involved in T-

cell activation (Lee et al., 2007b). A SNP, *PTPN22* 1858C>T causes the change of amino acid at position 620 from an arginine (R) to a tryptophan (W) disrupting the interaction between PTPN22 and Src family kinases (Csk) in T cell activation. *In vitro* experiments have shown that the mutant T-allele causes the formation of a weak bind between PTPN22 and Csk (Bottini et al., 2004; Begovich et al., 2004). The 620W variant was significantly overrepresented in MG patients (Greve et al., 2009), that show a higher titer of anti-AChR antibodies respect to wild-type (Lefvert et al., 2008). A different SNP in the promoter region, *PTPN22* -1123G>C, that disrupts an AP-4 binding site, was associated with low autoantibody titers and a mild MG in the Italian cohort (Provenzano et al., 2012).

Polymorphisms in *CTLA4*, cytotoxic T lymphocyte associated antigen 4, gene have been associated to different autoimmune diseases, such as rheumathoid arthritis, Grave's disease, celiac disease, type 1 diabetes, and also to myasthenia gravis, suggesting that these SNPs could contribute to pathogenesis causing aberrant splicing of *CTLA4* and cellular abnormalities of MG T-cells (Strobel et al., 2008; Wang et al., 2008; Chuang et al., 2014).

Sequencing of the genomic region of *CHRNA1* identified a unique SNP, rs16862847, located in the promoter region that might disrupt a transcription binding motif. This SNP was found to be more twice more frequent in MG cases versus control in two different cohorts from France and UK (Giraud et al., 2007).

By GWAS emerged that, in TNF-α-induced protein 3 (TNFAIP3)-interacting protein 3, also called TNIP1, the variant rs2233290, encoding a non-conservative Pro151Ala change, is associated with EOMG in northern Europe (Gregersen et al., 2012; Seldin et al., 2015).

1.6.2.3 Etiological mechanisms of EOMG form

Pathogens infections

The existence of a chronic inflammatory state in the MG thymus could alter innate immune responses and lead to the failure of self-tolerance (Cufi et al., 2013; Cavalcante et al., 2011a; Cavalcante et al., 2013; Cordiglieri et al., 2014). The inflammatory state could be due to persistent viral replications; viruses proposed to be associated to MG are Epstein Barr virus (EBV), cytomegalovirus, human foamy virus, and Nile virus even if MG symptoms appear after a long time from a possible infection, so it is a problem to fix a link between MG and a specific infection (Barzago et al., 2016; Cavalcante et al., 2016). Recently, an upregulation of

infectious transcripts related to disease, such as *ETF1*, *NFKB2*, *PLK3*, and *PPP1R15A*, and a downregulation of *CLC* and *IL4* in the peripheral blood mononuclear cell (PBMC) of AChR-EOMG patients was observed. Moreover the upregulation of inflammatory categories, *ABCA1*, *FUS*, and *RELB* was found, suggesting that these molecules could have a role in MG immunological dysfunctions (Barzago et al., 2016). Pathogen infections are suspected to play a role in autoimmune diseases through the induction of dysregulated Toll-like receptor (TLR)-mediated innate immune responses, which can lead to dysregulated innate immune responses and long-term inflammation, rendering the thymus vulnerable to auto-sensitization (Barzago et al., 2016; Cavalcante et al., 2016). This hypothesis was supported by the observation that TLR4 and TLR4-positive poliovirus-infected macrophages are overexpressed in MG thymus (Bernasconi et al., 2005; Cavalcante et al., 2010a).

Epstein Barr virus (EBV), a human γ herpes virus that infects most of the world's population, is one of the main candidates suspected to play a role in initiation of autoimmune diseases, due to its ability to promote abnormal activation and survival of B cells, and to disrupt critical B-cell tolerance checkpoints (Swanson-Mungerson and Longnecker, 2007; Niller et al., 2011). In MG thymuses, the presence of antiviral signatures, such as the overexpression of IFN (interferon)-I-induced genes, in particular IFN-β and TLR4, as well as dsRNA signalling molecules, such as IRF5 and IRF7 (IFN Regulatory Factor) was demonstrated injecting into mice a synthetic analog of dsRNA, poly(I:C), which was able to increase in the number of B lymphocytes and to induce overexpression of α-AChR in the thymus, mediated by TLR3, protein kinase R, and the release of IFN-β (Bernasconi et al., 2005; Le Panse et al., 2006; Cizeron-Clairac et al., 2008). Moreover TLR7/9 signalling may be involved in antiviral type I IFN production and long-term inflammation in EBV-infected MG thymuses, hypothesis that arises by the observation of higher *TLR7* and *TLR9* mRNA levels in EBV-positive MG respect to EBV-negative normal thymuses, and high protein levels in B and plasma cells of MG thymic germinal centers and lymphoid infiltrates, where the two receptors co-localized with EBV antigens (Cavalcante et al., 2016). Regarding INF, it was noticed that IFN-I, particularly IFN-β, could play a central role in the thymic follicular hyperplasia in MG patients inducing the overexpression of α-AChR and chemokine CXCL13 in thymic epithelial cells and of the chemokine CCL21 in endothelial lymphatic cells, two chemokines involved in the abnormal recruitment of B cells in EOMG thymuses. IFN-β also

increases the expression of B cell activating factor (BAFF), which favours autoreactive B cells (Cufi et al., 2014a).

All these data could be useful for a therapeutic goal, in fact, drugs able to regulate TLR7/9 signalling, already tested in animal models where have showed high efficiency, could be good candidates for the development of autoimmune and inflammatory diseases therapy (O'Brien et al., 2010; Hennessy et al., 2010; Gambuzza et al., 2011; Kanno et al., 2015).

Drugs

IFN-α or IFN-β therapies can prime the development of autoimmune diseases, for example IFN-β overexpression in MG thymus can mediate the effects of dsRNA activation and causes the overexpression of α-AChR subunit, suggesting that IFN-β can play a central role in MG development (Cufi et al., 2013). Moreover, myasthenia gravis is reported in 1% to 7% of patients treated with D-penicillamine, and even if the symptoms are mild, the presence of anti-AChR antibodies is clear. After D-penicillamine treatment in scleroderma patient, the development of an anti-AChR and anti-MuSK MG was reported (Poulas et al., 2012).

1.6.4 Thymoma associated myasthenia gravis

As described above, the autoimmune disease more frequently associated with thymoma is the myasthenia gravis, in fact, about 30% of thymoma patients have also myasthenia gravis, and 10-20% of MG patients have a thymoma (Sakaguchi et al., 2008; Marx et al., 2010; Berrih-Aknin and Le Panse, 2014). As mentioned in the "Myasthenia gravis" section, patients with thymoma associated MG (TAMG) typically show generalized myasthenic symptoms and almost all TAMG patients have antibodies to the AChR; rare exceptions showed anti-MuSK antibodies or were siero-negative, while anti-LRP4 autoantibodies have not been reported (Maggi et al., 2008; Rigamonti et al., 2011; Zisimopoulou et al., 2014). TAMG mainly occurs after 50 years of age, does not show neither gender bias nor strong HLA association.

1.6.4.1 Thymic abnormalities in TAMG

Thymus of TAMG patients show different characteristics respect to EOMG thymus. First of all, thymomas lack myoid cells, usually present in a thymopoietically active normal

thymus and express AChRs and striational antigens, and have no or reduced medullary area implying a clear deficiency of the anatomical structure where negative selection takes place (Roxanis et al., 2001; Marx et al., 2010; Figure 3). The neoplastic epithelial cells of thymomas variably express epitopes of striational antigens and AChR subunits and whole functional receptors are absent, causing the alteration of positive selection of maturing thymocytes or activate mature T cells involved in the recognition of these epitopes (Romi et al., 2002; Maclennan et al., 2008). Moreover the neoplastic epithelial cells express reduced levels of HLA-class II, alterations in positive T cell selection, impairing negative selection and interfering with the generation of regulatory T cells (Strobel et al., 2004; Strobel et al., 2007; Strobel et al., 2008; Scarpino et al., 2007). In thymoma, thymocytes-rich areas is like a disorganized thymic cortex (Strobel et al., 2014). TAMG in comparison to non-myasthenic thymoma shows different mRNA expression levels of chemokines, cytokines, and double-stranded RNA sensing molecules (Cufi et al., 2014b). Recently, an oncogenic role of human polyomavirus 7 (HPyV7) in thymomas was assessed; HPyV7 DNA has been detected in the neoplastic epithelial cells of 62% of thymomas, both in patients with MG and in ones without MG, suggesting its potential role in initiation in thymoma pathogenesis (Rennspiess et al., 2015). Another pathogenic feature of thymomas is defective expression of the autoimmune regulator AIRE, as described in paragraph 1.4 (Strobel et al., 2007).

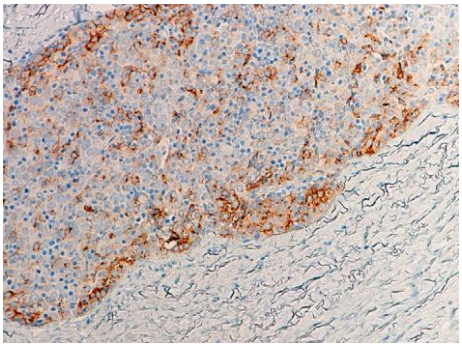

Figure 3. Thymoma (type B3) associated with myasthenia gravis (from Alexiev et al., 2007).

1.6.4.2 Genetics of TAMG

Also thymoma shows a correlation with the HLA locus, in particular at 6p21, which loss is present in all histological subtypes (Zettl et al., 2000; Strobel et al., 2008). The loss of this chromosome region in TAMG implicates MHC hemizygosity of neoplastic epithelial cells

and the consequent impaired epithelial presentation of autoantigenic motives (Strobel et al., 2008). While HLA DRB1*03-DQA1*05:01-DQB1*02:01 haplotype showed a negative association with thymomatous MG (Eshan et al., 2015). Moreover, polymorphisms in genes as *CTLA4* and *PTPN22,* involved T cell receptor signalling, might impair negative selection in thymomas (Vandiedonck et al., 2006; Chuang et al., 2005; Chuang et al., 2009; Gianchecchi et al., 2013). By a recent meta-analysis emerged that *PTPN22* R620W polymorphism was significantly associated with an increased risk of MG in thymoma patients (Xiong et al., 2015). T cell immunoglobulin and mucin domain-3 (Tim-3), a surface molecule on T cells, is important in immune regulatory functions and is strongly correlated with various tumors, suggesting that could be associated to TAMG, in fact, in the Han population of North China, a polymorphism in the promoter region of gene, *Tim-3* -574G>T, was found to be a protective factor in TAMG (Xu et al., 2015). Different results were obtained for another member of Tim family, since a study found that -1637A>G polymorphism in the promoter region of the Tim-1 gene is associated to thymoma with MG; the same study found an higher Tim-1 expression in thymoma patients with MG respect them without MG (Zheng et al., 2014).

1.6.4.3 Etiological mechanisms of TAMG

The high frequency of thymoma-associated autoimmune phenomena (>50%) compared to their rarity («5%) respect to other cancer types let us to suppose that it cannot be a coincidence that autoimmune diseases are so prevalent in patients with thymic epithelial tumors, considered that thymus is the site of central self-tolerance induction (Marx et al., 2010; Holbro et al., 2012). Recently Marx and colleagues (2015) proposed three hypotheses, trying to explain the mechanisms that lead to TAMG (Marx et al., 2015). The first hypothesis involves the faulty negative selection of non-tolerant naïve effector T cells with potential AChR or titin reactivity that are exported from the thymoma. In the extrathymic periphery the progeny of these escaped T cells starts to react against related targets, attacking neuromuscular targets, especially with AChR and titin specificity. This could be due to the absence of myoid cells that cause the selective failure of cross-presenting dendritic cells necessary to delete T cells specific for muscle autoantigens (Marx et al., 2010). The loss of myoid cells is an hallmark of almost all in thymomas (>90%), while only 30% of them are TAMG, suggesting that additional mechanisms are involved in the pathogenesis (Strobel et

al., 2002). The second hypothesis takes in account AIRE expression, which deficiency in thymoma causes specific bias in T-cell selection determining an aberrant expression of neuromuscular auto-antigens. So selected T cells are pre-primed to induce autoantibody responses after export to the periphery (Marx et al., 2015). Recent studies found low levels of AIRE and AChR α subunit RNA in two different cohorts, Chinese and Caucasian thymoma patients, correlated with a high prevalence of TAMG (Liu et al., 2014; Wolff et al., 2014). The third mechanism could be operative in cases of rare thymopoietically inactive thymomas but associated with MG, where mature T cells from the normal AChR-antibody reactive repertoire could be activated after recirculation to the thymoma (Marx et al., 2010; Marx et al., 2014).

The substantial export of mature CD4+ T cells from the thymoma seems to be indispensible to elicit TAMG and the high numbers of autoreactive T cells could replace the patient's normal T cell repertoire in the periphery, reason for which, once initiated, is self-sustaining also after thymectomy (Buckley et al., 2001; Marx et al., 2010; Marx et al., 2015). Moreover high levels of anti-IFN-I neutralizing antibodies were detected also in TAMG in response to viral infections (Maeger et al., 2003). Thymuses of TAMG patients exhibit high levels of IFN-I, particularly IFN-α2, IFN- α8, IFN-ω, and IFN-β, and dysregulated expression of dsRNA signalling molecules (Cufi et al., 2014a).

1.8 Epigenetics

The modern polymath Conrad H. Waddington was the first to coin the term 'epigenetics' to describe heritable changes that influence gene expression without any change in the DNA sequence (Boland et al., 2014). DNA wraps around histone octamer to form nucleosomes, the fundamental unit of chromatin. Each histone octamer involved in the organization of nucleosomes contains two copies of four histones (H2A, H2B, H3, and H4). When the DNA is well packed in these nucleosomes to form condensed chromatin, genes will be switched off, whereas when the DNA is uncoiled to form a decondensed chromatin, it is more accessible to transcription factors (TFs) and genes will be switched on. The level of compaction of these nucleosomes is influenced by chemical tags or epigenetic modifications, which associate with the histones or directly with the DNA. The epigenetic modifications involve DNA methylation, histone modifications and RNA associated silencing (Egger et al.,

2004; Figure 4) and are mainly studied in eukaryotes, especially humans, for their role in embryogenesis, cellular differentiation, genomic imprinting (Egger et al. 2004; Portela and Esteller 2010). Epigenetic factors guarantee the activation and maintenance of specific differentiation programs in adult somatic cells (Berdasco and Esteller, 2010).

Epigenetic modifications are placed by epigenetic 'writers' and removed by 'erasers' in a dynamic but highly regulated manner and has DNA and histones as targets (Boland et al., 2014). Many chromatin regulators are also 'reader' due to their ability to recognize the epigenetic landscape by specific domain, leading to recruitment of functional complexes regulating DNA transcription (Boland et al., 2014; Hamidi et al., 2015). This role is executed by three families of proteins, the methyl-CpG-binding domain (MBD) family, zinc finger (ZnF) proteins and SET and RING finger-associated (SRA) domain proteins, that are able to bind methylated DNA (Hamidi et al., 2015). The MBD family includes MeCP2 and MBD1-4. Among them, MBD3 is unable to bind DNA, while MeCP2, MBD1 and MBD2 bind to methylated DNA repressing gene transcription, and MBD4 binds to regions of base pair mismatch and enhances DNA mismatch repair (Du et al., 2015; Hamidi et al., 2015).

Epigenetic marks can be environmentally induced as a response to external stimuli, occur during the life-span in healthy tissues and can have a crucial role in the development and progression of human complex diseases, such as cancer, autoimmune disorders, neurodegenerative diseases, obesity, psychiatric disorders, and many others (Brunet and Berger, 2014). More interestingly variability of epigenetic marks exists among individuals and among the different cell types of each individual. This is not surprisingly since epigenetic changes are largely viewed as the missing link between the environment and the genome, likely representing a cellular response to environmental exposures.

Epigenetic abnormalities are reversible and as a result novel therapies that work by reversing epigenetic effects are being increasingly explored. The biggest clinical impact of epigenetic modifying agents in neoplastic disorders and the efficacy of DNA methyltransferases and histone deacetylates inhibitors in blood cancers clearly attest to the principle that therapeutic modification of the cancer cell epigenome can produce clinical benefit. Although the efficacy of epigenetic therapy in solid tumors remains as yet unproven, we can believe that the use of existing agents will improve the therapeutic index of this approach (Hatzimichael and Crook, 2013).

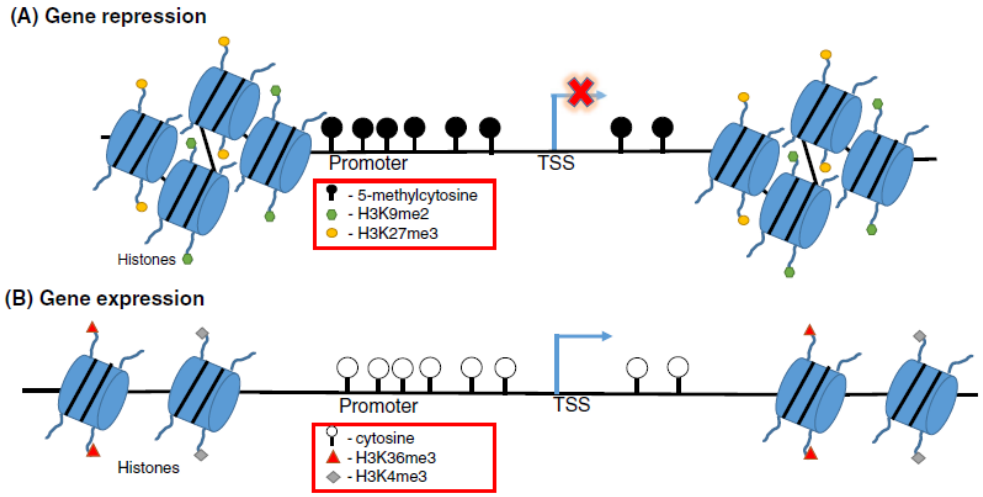

Figure 4. Epigenetic regulation of A) gene repression and B) gene expression. TSS, transcription start site. Amino acids subjected to post-translational modification are denoted according to the histone protein, single letter amino acid and specific modification (from Paluch et al., 2016).

1.8.1 DNA methylation

DNA methylation is currently the most widely studied form of epigenetic modifications and consists of a covalent addition of a methyl group (CH_3) to the 5′ position of cytosine residues in CG dinucleotides by DNA methyltransferases (DNMTs), the key enzymes for DNA methylation that catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to cytosine, thus forming 5-methylcytosine (5mC). DNA methylation is a physiological mechanism required for embryonic development, cell differentiation, X-chromosome inactivation, genomic imprinting, repression of repetitive elements, and maintenance of the cellular identity and its function is intrinsically linked to the mechanisms for establishing, maintenance and removing the methyl group (Martin-Subero, 2011).

DNA methylation is frequently described as a 'silencing' epigenetic mark, but it is becoming more clear that the function of DNA methylation seems to vary with context, especially related to location and time of methylation. Methylation occurs in transposons, gene bodies, and intergenic regions, while regions of high CpG density, the so called CpG islands (CGIs) show different pattern of methylation (Paska and Hudler, 2015). CpG islands are DNA sequences more than 200 base pairs long, with CG content greater than 50% (Sandoval and Esteller, 2012; Kim, 2009). In mammals, most of the DNA CpG sites are methylated (85-100%), but CpG-islands associated with promoter regulatory regions of

almost all housekeeping genes as well as with half of tissue-specific genes are usually kept free of methylation (Esteller, 2011). Methylation of CpG islands in the promoter region of a gene might induce chromatin conformational modifications and inhibits the access of the transcriptional machinery, thus altering gene expression levels: promoter hypermethylation has been associated with a decreased gene transcription and promoter demethylation with gene expression (Esteller, 2011). Methylated promoter CGIs are usually restricted to genes at which there is long-term stabilization of repressed states; for example imprinted genes, genes located on the inactive X chromosome and genes that are exclusively expressed in germ cells and that would presumably be inappropriate for expression in somatic cells (Jones, 2012).

Methylation is mainly located at transposons, bodies of coding regions (intragenic), and intergenic regions. Methylated CGIs at transcription start sites (TSSs) cannot initiate transcription after the DNA has been assembled into nucleosomes (Kass et al., 1997; Hashimshony et al., 2003) and are marked with trimethylation of lysine 27 on histone 3 (H3K27me3), associated to an inactive status (Taberlay et al., 2011), while genes with unmethylated CGIs at their transcription start site are active and their promoters are usually characterized by nucleosome-depleted regions (NDRs), often flanked by nucleosomes containing the histone variant H2A.Z, are marked with trimethylation of histone H3 at lysine 4 (H3K4me3) (Kelly et al., 2010). CpG-poor genes at the TSS show a different pattern of promoter methylation, in particular genes with non-CGI TSS that are expressed in primordial germ cells are unmethylated at the TSS, whereas genes that are exclusively expressed in embryonic stem cells or tissue-specific genes often show methylation in sperm but not in oocytes or in expressing somatic cells (Farthing et al., 2008). All is complicated by the observation that most genes have at least two TSSs, so the downstream start sites are within the 'bodies' of the transcriptional units of the upstream promoters. These alternative promoters can be CGIs or non-CGIs, or there can be combinations of an upstream non-CGI and a downstream CGI, or vice versa (Jones, 2012). Methylation of a downstream promoter would only block transcription and it would allow the elongation of a transcript that emanates from an upstream promoter (Jones, 2012).

Although many CGIs are located at gene promoters, CGIs also exist within the bodies of genes and within gene deserts (Jones et al., 1999); these regions may represent 'orphan promoters' and might be used at early stages of development (Illingworth et al., 2010). Gene body methylation is a feature of transcribed genes. CGIs situated in intragenic regions

usually remain unmethylated but it was observed that about 34% of all intragenic CGIs are methylated in the human brain, even if the role of this tissue-specific methylation is not yet clear (Maunakea et al., 2010) and that intragenic CGIs can also be preferential sites for *de novo* methylation in cancer (Nguyen et al., 2001).

The DNA methylation reaction is catalyzed by DNMTs; currently, there are five known mammalian DNMTs and DNMT-like proteins: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L (Robertson, 2002). DNMT3A and DNMT3B are *de novo* methyltransferases and establish DNA methylation patterns in the germ line or early embryo while DNMT1 is involved in the maintenance of DNA methylation patterns during development and cell division, recognizes hemimethylated DNA and carefully copies the pattern of DNA methylation from parent to daughter strand during DNA replication, in fact it is localized at DNA replication loci during the S phase (Goll and Bestor, 2005). DNMT2 and DNMT3L do not have the same catalytic activity of the others, in particular human DNMT2 does not methylate DNA but instead methylates a small RNA (Goll et al., 2006), and DNMT3L is an enzymatically inactive regulatory factor and acts as a regulatory factor in germ cells (Jurkowska et al., 2011). The expression of Dnmt1 mRNA varies in cell-cycle dependent manner, reaching a maximum in S phase and its disruption in mice results in extensive demethylation of the genome and embryonic lethality, indicating the fundamental role in early development (Li et al., 1992; Kimura and Shiota, 2003). Dnmt3a and Dnmt3b are required for genome wide *de novo* methylation and are essential for mammalian development. Dnmt3a -/- mice normally developed and were in healthy at birth but died at about 4 weeks of age. Dnmt3b -/- mice died *in utero* (Okano et al., 1999). Emerging evidence suggests that these functions are not exclusive but a functional overlap may exist, such that DNMT3A and DNMT3B may correct errors made by DNMT1 after DNA replication (Jones and Liang, 2009). Mutations in human *DNMT3B* could lead to centromeric instability, developmental abnormalities and immune deficiencies, such as the Immunodeficiency, Centromere instability and Facial anomalies (ICF) syndrome (Moarefi et al., 2011). Polymorphisms of the *DNMT3B* gene may influence DNMT3B enzyme activity on DNA methylation, thereby modulating the susceptibility to cancer. DNMT3B usually appears with lower expression in normal adult tissues, whereas significant up-regulation of this gene could be observed in many kinds of human tumors.

Aberrant DNA methylation patterns, as well as abnormal expression of components of the DNA methylation machinery, are associated with numerous human diseases, including cancer, immunological diseases and neurological disorders.

Recently, the role of another modified base, 5-hydroxymethylcytosine (5hmC), now considered the sixth base of the genome, was identified (Munzel et al., 2011). 5hmC shows the highest levels in the central nervous system, where play important roles in brain function, such as neuronal differentiation and development (Kriaucionis and Heintz, 2009; Hahn et al., 2013). 5hmC is generated from the oxidation of 5mC, a reaction that is catalyzed by the ten-eleven translocation (TET) enzymes and is thought to be an intermediary toward 5mC demethylation (Tan and Shi, 2012). 5hmC can be further catalyzed to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Both 5fC and 5caC are decarboxylated by thymine DNA glycosylase (TDG) and replaced by an unmodified cytosine through a base excision repair (BER) pathway, completing the DNA methylation/demethylation cycle. The levels of 5hmC varies in different organs. The content of 5hmC is highest in brain, following the rectum, liver and colon and lower in heart, breast and placenta with a content that ranges from 0.6% to 1.5% (Li et al., 2011). 5hmC is one of the intermediates in a process that results in the demethylation of 5mC at gene promoter sites, a process that can be both active and passive. Unlike 5mC, associated with gene repression, 5hmC is supposed to facilitate transcription by opening chromatin (Mendonca et al., 2014). Ten-eleven translocation 1 enzyme downregulation results in a decrease in the level of 5hmC, cancer metastasis and tumor growth in certain types of cancers such as prostate, colorectal and breast cancers (Yang et al., 2013).

1.8.2 Histone tail modifications

In eukaryotic cells, DNA is wrapped with histones and formed a highly organized and condensed structure, so called nucleosomal core particle. Each nucleosome contains four core histones, H2A, H2B, H3, H4, with 145-147bp (base pairs) of DNA wound around it, and one linker histone (H1). Histones can carry many post-translational modifications, which influence chromatin compaction and accessibility in many different ways. Those modifications regulate gene expression, DNA repair, replication and recombination processes via directly influencing the overall chromatin structure or by regulating the

binding of effectors molecules (Bannister and Kouzarides, 2011). Despite that acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, and other post-translational modifications can occur on histone tails, acetylation and methylation are the two best-characterized epigenetic marks, the first resulting in an open chromatin structure that allows transcription, and the latter associated with either chromatin condensation or relaxation, due to the fact that several sites for methylation are present on each tail (Berger, 2007). Acetylation removes the positive charge on the histones, which leads to a decrease in the interaction of the N-termini of histones with the negatively charged phosphate groups of DNA. Subsequently condensed chromatin (heterochromatin) is transformed into a more relaxed structure (euchromatin), which leads to increased levels of gene transcription (Bannister and Kouzarides, 2011). Histone acetylases and histone deacetylases (HDACs) add and remove acetyl groups from histones and are critical regulators of gene expression (Bannister and Kouzarides, 2011). Indeed, histone methylation by histone methyltransferases can lead to transcriptional activation or repression depending on the number of methyl groups added and the position of modified lysine residue, for example methylation of lysine 4 on histone 3 (H3K4) is linked to transcriptional activation, while methylation of lysine 27 at histone 3 (H3K27) is linked to transcriptional repression. Moreover, methylation of lysine residues on histone 3 (H3K4, H3K36, and H3K79) and histone acetylation promote transcription, whereas histone hypo-acetylation and increased di- and trimethylation at H3K9 and H3K27 are regarded as repressive marks (Berger, 2007; Goel and Boland, 2012). Protein modifications are less stable biomarkers than DNA methylation and therefore less useful as non-invasive cancer biomarkers (Goel and Boland, 2012).

1.8.3 Noncoding RNA molecules

Several classes of noncoding RNAs are nowadays regarded as epigenetic regulators of gene expression levels. Among them, microRNAs (miRNAs) are small noncoding RNAs of about 22 nucleotides in length that bind to the 3′ untranslated region (UTR) of target mRNAs mediating post-transcriptional regulation by their degradation or translational inhibition (Szafranski et al., 2015). Other noncoding RNAs involved in targeted gene silencing include short interfering RNAs (siRNAs: 20–25 nucleotides), PIWI interacting RNAs (piRNAs: 24–31 nucleotides), and long noncoding RNAs (lncRNAs: at least 200 nucleotides) (Moazed, 2009;

Saxena and Carninci, 2011). piRNAs act by inhibition of RNA polymerase II (Holoch and Moazed, 2015), and lncRNAs have been implicated in transcriptional and post-transcriptional regulation interacting with other epigenetic mechanisms (Szafranski et al., 2015). Genetic and epigenetic alterations in genes that code for noncoding RNAs can modify the expression profile and alter the mechanisms that they regulate (Kawahara, 2014; Szafranski et al., 2015).

In last years, circulating miRNAs have been the centre of attention in discovery of cancer biomarker (He et al., 2015), and increasing evidence points to a role of lncRNAs in malignant transformation (Huarte, 2015).

1.9 Folate metabolism

Folates are a group of water-soluble B vitamins entirely derived from dietary sources, mainly from consumption of green vegetables, especially leafy vegetables, fruits, cereals, peas, poultry, and meat. They maintain genomic stability donating one-carbon units required for important intracellular processes, as the synthesis of DNA and RNA precursors necessary for DNA replication and repair, the synthesis of amino-acids, and the conversion of homocysteine (Hcy) to methionine, which is then used to form S-adenosylmethionine (SAM), the major DNA methylating agent (Duthie, 2011).

After intestinal adsorption, dietary folates are reduced and methylated into liver to form 5-methyltetrahydrofolate (5-MTHF), which is released into the blood and taken up by the cells. Specific membrane transport systems are required for the cellular uptake of folate, including the reduced folate carrier (RFC1), folate receptor (hFR) and proton-coupled folate transporter (PCFT). Methylentetrahydrofolate reductase (MTHFR), an enzyme involved in regulating DNA methylation reactions, reduces 5,10-methylentetrahydrofolate (5,10-MTHF) to 5-methylTHF, the one-carbon donor for the remethylation of Hcy to methionine, mediated by the methionine synthase (MTR) enzyme. Cobalamin or vitamin B12 are cofactors in this reaction and methionine synthase reductase (MTRR) is required for the maintenance of MTR in its active state. Methionine is then converted into SAM, the substrate for methyltrasferase enzymes, that transfer the methyl group from SAM to diverse biological acceptors (Coppedè, 2014). SAM levels are regulated by glycine N-methyltransferase, enzyme that catalyzes the

transfer of a methyl group from SAM to glycine to form sarcosine and S-adenosyl-homocysteine. This enzyme is expressed in liver, prostate, exocrine pancreas and tissues with active secretion, as the proximal kidney tubules, intestinal mucosa, cortical neurons and Purkinje cells. Since the SAM is required for many biological processes, the regulation of GNMT plays a critical role in cellular homeostasis. When metionine levels are high, SAM levels are also high, so it is not necessary to synthesize methyl groups. High SAM levels inhibit the activity of MTHFR, leading to a decrease of 5-methyl-THF. In these conditions, GNMT is active and SAM is converted to sarcosine and SAH. On the other hand, when methionine is low, SAM will be low, and inhibition of MTHFR is relieved, producing elevated amounts of 5-MTHF and greater inhibition of GNMT (Luka et al., 2009). Mice Gnmt-/- showed high SAM levels within the hippocampus, reduced neurogenic capacity, memory impairment and spatial learning (Carrasco et al., 2014).

Folate derivatives can be also used for the synthesis of nucleic acid precursors. Thymidilate synthase (TYMS) converts deoxyuridine monophosphate (dUMP) and 5,10-MTHF to deoxythymidine monophosphate (dTMP) and dihydrofolate (DHF) in the *de novo* synthesis of pyrimidines. TYMS impairment might result in uracil misincorporation into DNA, critical for point mutation formation and carcinogenesis. For *de novo* production of purines is used 10-formylTHF (Coppedè, 2014; Figure 5).

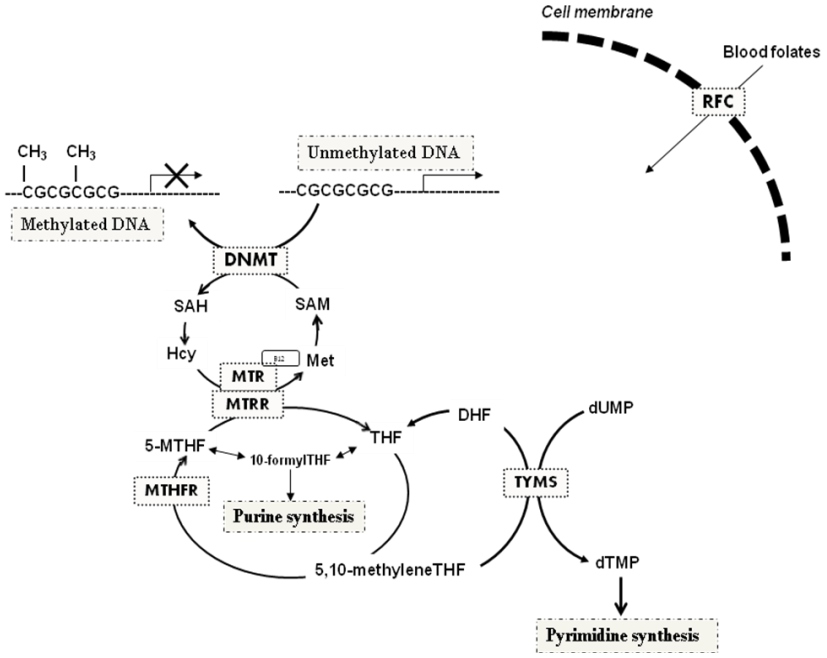

Figure 5. Simplified representation of folate metabolism (RFC, reduced folate carrier; hFR, human folate receptor; MTR, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; TYMS, thymidylate synthase; THF, tetrahydrofolate; DHF, dihydrofolate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate) (from Coppedè et al., 2015).

Humans are unable to biosynthesize folate *de novo*, so they derive all the necessary folate from their diet (Persson et al., 2013). Folate can be obtained from natural food sources, including citrus fruits, green leafy vegetables, cruciferous vegetables, legumes and cereals (He et al., 2016). An excess of folic acid as well as folate deficiency may impair DNA stability, gene expression, and cell proliferation. Cancer data are contrasting, in fact, many some suggest that folate have a protective effect against cancer development (Larsson et al., 2007; Kennedy et al., 2011; Rycyna et al., 2013), others suggest that increased risk of cancer is related to folic acid fortification (Hirsch et al., 2009; Mason et al., 2007). An effect of folate on carcinogenesis depending on the dosage and the time of exposure was supposed. Regarding the time of exposure, animal experiments demonstrated that folate supplementation could promote cancer progression if pre-neoplastic lesions are previously established (Ulrich et al., 2007; Lee et al., 2011).

Regarding the dosage, folate deficiency can have genetic and epigenetic consequences, supporting tumor development. Low cellular 5,10-methyleneTHF retards conversion of dUMP to dTMP, leading to thymidine depletion and elevated uracil concentrations. As uracil and thymidine differ only by a single methyl group, uracil is misincorporated into DNA in place of thymine during DNA synthesis but is quickly removed by DNA repair enzymes, leaving a single-strand break in the DNA molecule. If folate deficiency is persistent, this "catastrophic cycle" of DNA breakage and repair continues, leading to DNA double-strand breaks, chromosomal aberrations and malignant transformation (Cabelof et al., 2004; Duthie et al., 2010). Uracil misincorporation, genome-wide DNA strand breakage and chromosomal instability in response to experimental folate deficiency have been observed in various cell culture models, including human lymphocytes, human colonocytes and Chinese hamster ovary cells (Duthie et al., 2010). Folate deficiency could also modulate the epigenome. DNA methylation is an important determinant of genomic stability, mutagenesis and gene expression. Folate deficiency, by attenuating remethylation of S-adenosylhomocysteine to SAM in the methionine cycle, may disrupt this function. Under conditions of low dietary folate, SAM is reduced whereas SAH is elevated, resulting in hypomethylation of new synthesized DNA and, potentially, increased proto-oncogenes expression (Kim, 2007). Surprisingly, folate deficiency is also associated with hypermethylation in specific gene regions, notably in tumor suppressor genes. Folate deficiency may induce both gene-specific DNA hypermethylation and global hypomethylation, the two common features in

tumorigenesis, since DNA methyltransferase is sequestered away from the DNA replication fork to regions of folate deficiency induced DNA damage (Arasaradnam et al., 2008). Generally, severe and prolonged folate deficiency in rodents causes global DNA hypomethylation in the liver and in certain regions of the colon (James et al., 2003). Folate deficiency significantly increased hepatic OGG-1 and MGMT repair activity; the up-regulation of these two proteins indicates the occurrence of DNA damage lending further support to the finding that increased DNA damage is a consequence of folate deficiency (Duthie et al., 2010). Deficiency of folate may also result in elevated homocysteine levels. Folate deficiency is significantly more associated with oncogenesis when combined with hyper-omocysteinemia (increased risk of 17 times of carcinogenic lesions); moreover, inflammatory bowel disease patients with folate deficiency and hyperhomocysteinemia might be associated with increased cancer risk (Phelip et al., 2008). Also polymorphisms in one carbon metabolism genes could influence global methylation, in fact a recent study conducted on histological normal breast tissues observed that variation in *MTHFR*, *MTR*, and *MTRR* were significantly associated with LINE-1 methylation. Variant allele carriers of *MTHFR* 1289A>C had 4% lower LINE-1 methylation, while variant allele carriers of *MTR* 2756A>G and *MTRR* 66A>G had 3% higher LINE-1 methylation, compared to those carrying the more common genotypes of these SNPs, suggesting that polymorphisms in folate metabolism genes could affect DNA methylation of LINE-1 elements in histological normal breast tissues (Llanos et al., 2015).

Folate derivatives are cofactors in nucleotide synthesis and high levels could promote the proliferation of rapidly diving cells. As a consequence, folate dietary supplements or food fortification might accelerate carcinogenesis in individuals who already harbour neoplastic lesions (Mason, 2011). The role of folate supplementation in our cells is controversial, in fact, while folate supplementation is recommended for pregnant women to prevent birth defects, the benefit of supplementation in adults remains unclear, since elevated homocysteine levels are correlated with diseases of aging, such as cardiovascular disease and cognitive decline, and clinical trials have not shown a benefit in supplemental vitamin B intake for these conditions (Kim, 2003; Ami et al., 2016). Even if regarding the folate supplementation during pregnancy, it is emerging that high levels of it could cause epigenetic alterations in offspring, in fact a recent study demonstrated that maternal methyl-group donor intake, by diet and supplement use, before and during each trimester of pregnancy can influence offspring DNA

methylation in genes related to metabolism, in particular, in offspring higher *LEP* and *RXRA* cord blood methylation levels were found. The two genes are involved in metabolism, in particular *LEP* is has several functions including regulation of food intake (inhibition), body weight, energy homeostasis, and *RXRA* is involved in insulin sensitivity, adipogenesis, and fat metabolism (Pauwels et al., 2016). Moreover, in mice high folate intake during pregnancy leads to MTHFR deficiency, disturbed choline/methyl metabolism, embryonic growth delay and memory impairment in offspring (Bahous et al., 2017).

Folate effect was studied in different type of cancer, such as colorectal, breast, pancreatic, oesophageal cancer; in the normal colorectum, folate deficiency can increase the development of colorectal cancer, whereas folic acid supplementation suppresses it, even if the results are conflicting, in fact in a recent meta-analysis the authors asserted that folic acid supplementation did not affect the colorectal cancer risk (Qin et al., 2015). Moreover, it was also observed that once aberrant crypt foci are established, folate deficiency could inhibit the progression and induces regression of pre-existent cancer lesions (Kim, 2007; Kim, 2008). A study on oesophageal cancer found that greater dietary intake of folate and higher serum folate levels are protective against oesophageal cancer. And the dose-response analysis of dietary intake of folate shows that every 100 μg/day increase of folate can reduced 12% risk of oesophageal cancer (Zhao et al., 2017). In mice, the 2.5 times supplemental level of folic acid was associated with higher weight and volume of all mammary tumors (Deghan et al., 2014).

1.9.1 Functional polymorphisms of folate metabolism genes

Polymorphisms in folate metabolism genes, have been associated with cancer risk in different studies, often in combination with folate intake (Guo et al., 2010; Lee et al., 2012; Zhou et al., 2012; Fang et al., 2014; Kim et al., 2015; Singh et al., 2015; Hajiesmaeil et al., 2016; Kaya et al., 2016; Zara-Lopes et al., 2016; Huang et al., 2016; Yi et al., 2016).

1.9.1.1 Methylenetetrahydrofolate reductase

Methylenetetrahydrofolate reductase (MTHFR) catalyses the irreversible conversion of 5,10-methyleneTHF, the methyl donor involved in the conversion of dUMP to dTMP, into 5-

methyl THF, which remethylates homocysteine to methionine (Goyette et al., 1994; Figure 5). The gene *MTHFR* is located at 1p36.3, is composed of 11 exons and the promoter region have not a TATA box but contains CpG islands, multiple potential SP1 -binding sites, and binding sites for other transcription factors (Ghaugan et al., 2000). Thus, MTHFR controls whether folate is partitioned towards DNA precursor synthesis or DNA methylation. Polymorphisms in the *MTHFR* gene influence the risk of human cancers. The most common variants of the *MTHFR* gene, 677C>T and 1298A>C, are associated with reduced enzyme resulting in slower folate metabolism. The most common variant of *MTHFR* gene 677C>T (rs1801133), in exon 4, leads to the amino acid substitution of alanine by valine at codon 222 ($Ala^{222}Val$) which causes a thermolable enzyme and a reduced enzyme activity (about 70%) resulting in 30% of CC wild-type activity (Eaton et al., 2005).

To date, many association studies investigated the contribution of *MTHFR* polymorphisms in cancer risk with different results, often depending on ethnicity, in fact 677T allele prevalence varies in different geographical regions and ethnic groups, ranging from 2% to 65% for (Gueant-Rodriguez et al., 2006; Rajeevan et al., 2012). The mutant genotype is associated with changes in the distribution of blood folates, in fact, decreased plasma folate and vitamin B12 levels, increased homocysteine levels, low 5-methylTHF, and high formyl THF levels were found in TT individuals (Dekou et al., 2001; Ueland et al., 2001; Strandhagen et al., 2004). Mechanistically, reduced MTHFR activity would be expected to increase cancer risk due to low blood 5-methyl THF, DNA hypomethylation and proto-oncogene activation but data on different type of cancers are not concordant (Narayanan et al., 2004). For example, in colorectal cancer the TT genotype is associated with reduced cancer risk, as well as in cervical cancer (Sharp and Little, 2004; Hajiesmaeil et al., 2016). Low MTHFR activity, increasing the availability of 5,10-methylene THF necessary for thymidine and purine synthesis, may reduce cancer risk providing nucleoside precursors for normal DNA synthesis and repair and preventing uracil misincorporation and chromosomal breakage (Kono and Chen, 2005). In other cancer types this SNP is associated to increased risk, for example thyroid and lung breast cancer, even if these association depend on ethnicity (Liang et al., 2014; Kumar et al., 2015; Yang et al., 2016; Zara-Lopes et al., 2016). In Turkish population *MTHFR* 677TT genotype was associated to increased recurrence risk in patients with lymph node-positive breast cancer (Suner et al., 2016). Moreover, individual homozygotes TT had a significant risk of DNA hypermethylation of *MGMT* in cancer tissue

and hypomethylation of *IGF-2* gene (Chen et al., 2012; Cheng et al., 2012), but no association between global DNA methylation and *MTHFR* 677C>T genotypes (Gomes et al., 2012). Alterations of the human sperm epigenome associated with high-dose folic acid supplementation, increased by *MTHFR* 677C>T polymorphism, were observed (Aarabi et al., 2015). The protective contribution of the *MTHFR* 677TT genotype could be influence by folate intake levels. MTHFR enzyme, in fact, works as a dimer protein and the presence of the 677T polymorphism may render the dimer unstable in case of reduced folate levels, therefore individuals bearing the *MTHFR* 677TT genotype require higher folate levels for the stabilization and the activity of the protein (Haghighi et al., 2009).

The other SNP, *MTHFR* 1298A>C, in exon 7, results in the substitution of glutamate by alanine ($Glu^{429}Ala$). The 1298A>C transversion is located in the presumed regulatory domain and leads to a decrease of 40% in enzyme activity in the individuals carrying homozygosity of 1298CC and heterozygosity of 1298AC (Chen et al., 2002). The *MTHFR* 1298C allele frequency is approximately 20-70% in Asia, 24-46% in Europe, 0-15% in America (Rajeevan et al., 2012). A strong significant association between *MTHFR* 1298CC genotype and the risk of cervical cancer was observed, in particular, *MTHFR* 1298CC genotype seems more susceptible to HPV type 16 infection. Combination of *MTHFR* 677C>T and 1298A>C polymorphisms revealed that combined *MTHFR* 677CC and 1298CC genotypes are strongly associated with a risk of cervical cancer (Hajiesmaeil et al., 2016). A recent meta-analysis suggested that there is no significant association between *MTHFR* 1298A>C polymorphism and breast cancer susceptibility for overall population (Zhang et al., 2016).

de Vogel and co-workers hypothesized that MTHFR may act as a switch being able to shift the balance between DNA methylation and DNA synthesis, both of which have distinct consequences for carcinogenesis. Moreover they suggest that *MTHFR* polymorphisms may influence cancer risk but that a change in promoter hypermethylation, as measured by CpG island methylator phenotype or h*MLH1* hypermethylation, may not be the primary contributor to carcinogenesis in these individuals (de Vogel et al., 2009).

1.9.1.2 Methionine synthase

Methionine synthase catalyzes the methylation of homocysteine to methionine with simultaneous conversion of 5-methylTHF to tetrahydrofolate, reaction that requires

methylcobalamin (MeCbl), a derivative of cobalamin, or vitamin B12, for activity. The gene *MTR* is located on chromosome 1 (1q43) and consists of 33 exons, coding for a protein of 1256 amino acids (Watkins et al., 2002). A common polymorphism, A to G at position 2756, leading to the replacement of aspartic acid at position 919 with glycine ($Asp^{919}Gly$) has been identified and may affect plasma homocysteine levels (van der Put et al., 1997; Chen et al., 2001). Some studies but not others have found that homocysteine concentrations tend to decrease linearly across genotype, with the AA genotype associated with the highest homocysteine (Slattery et al., 1999). *MTR* 2756AA genotype was found as a risk factor for coronary heart disease (Hyndman et al., 2000), while the *MTR* 2756GG genotype had about 3 fold increased risk for retinoblastoma (Akbari et al., 2015).

Moreover, carriers of the variant *MTR* 2756G allele showed a lower rate of CpG island hypermethylation in tumor suppressor genes, including *CDKN2A* and h*MLH1* (Paz et al., 2002), even if a study on breast cancer did not find any association between methylation status and *MTR* polymorphism (Tao et al., 2009). This polymorphism increases the enzymatic activity of the MTR protein, leading to increased SAM synthesis and DNA methylation (Weiner et al., 2014). The discrepancies could be explained by the evidences that the interaction between different polymorphisms may modify their individual effects, and that the same genotypes combination may have different effects in individuals, depending on environmental factors (Alessio et al., 2004).

1.9.1.3 Methionine synthase reductase

Methionine synthase reductase is involved in the regeneration of methionine synthase enzyme, since after its work the cob(I)alamin cofactor becomes oxidized to cob(II)alamin, rendering the MTR enzyme inactive (Leclerc et al., 1998). The *MTRR* gene is located on chromosome 5p15.31 and encodes a protein of 698 amino acids (Leclerc et al., 1998). By purified recombinant human proteins, Yamada and colleagues found that that MTRR maintained MTR activity at a 1:1 stoichiometric ratio. In the presence of MTRR and NADPH, holoenzyme formation from MTR apoenzyme (apoMTR) and methylcobalamin was significantly enhanced due to stabilization of apoMTR in the presence of MTRR. MTRR was also able to reduce aquacobalamin to cob(II)alamin in the presence of NADPH. So MTRR works as a chaperone for MTR and as an aquacobalamin reductase (Yamada et al., 2006).

The most common polymorphism is *MTRR* 66A>G, resulting in an isoleucine to methionine change at position 22 ($Ile^{22}Met$) that led to formation of a variant with 4-fold lower activity respect to the wild type (Wilson et al., 1999). *MTRR* 66GG genotype was inversely associated with plasma homocysteine levels (Alessio et al., 2004), and decreased SAM availability by reducing the levels of active MTR, inducing DNA hypomethylation (Wettergren et al., 2010). By a recent meta-analysis, it is emerged that this SNP was significantly associated with an increased overall cancer risk, in particular, the strongest associations were found for head and neck cancer, Caucasians, Africans (Wang et al., 2017.)

MTR 2756A>G was associated with reduced risk for colorectal cancer with CpG methylator phenotype among men and the G allele was associated with reduced risk of colorectal cancers with h*MLH1* hypermethylation among women (de Vogel et al., 2009). Moreover a recent meta-analysis on effect of *MTRR* 66A>G polymorphism on colorectal cancer risk, revealed a protective effect of A allele in Asian population (Pabalan et al., 2015). Among cancers, *MTRR* 66A>G is associated with a reduced risk of developing leukemia (Fang et al., 2014), could influence the risk of adult meningioma in Chinese Han population (Zhang et al., 2013) but it is not associated with breast cancer susceptibility (Hu et al., 2014). Moreover it is associated with increased risk of having a child with Down syndrome (Coppedè et al., 2014).

1.9.1.4 Thymidilate synthase

Thymidylate synthetase is a key enzyme in the folate metabolism, as it catalyzes the reaction that converts the deoxyuridine monophosphate in deoxythymidine monophosphate. The conversion is essential for the production of thymine, a nucleotide required for DNA repair and synthesis. The *TYMS* gene is located at 18p11.32, contains 7 exons and shows a length of about 30 kb (Kaneda et al., 1990). In humans the *TYMS* gene is widely polymorphic, and variable number of tandem repetitions (VNTR) are found in the promoter enhancer region five primer-untranslated region (5'-UTR). Although there have been reports of up to 9 copies of the VNTR in some populations, the majority of *TYMS* alleles contain only 2 or 3 repeats, and have been indicated as 2R or 3R, respectively (Horie et al., 1995; Luo et al., 2002). The frequencies of 3R3R, 2R3R, and 2R2R genotypes are about 20%, 30-55%, and 20-40%, respectively, depending on the studied population (Mandola et al.,

2003). Individuals that are homozygous for the 3R showed high intra-tumoral TYMS mRNA (Pullarkat et al., 2001) and protein levels compared with 2R homozygotes (Kawakami et al., 1999), in fact both i*n vitro* and in tumor tissue, less cellular thymidilate synthase protein production in 2R2R genotype respect to 3R3R one was observed (Kawakami et al., 1999). Polymorphisms in *TYMS* would affect plasma folate levels since TYMS competes with MTHFR for the availability of 5,10- methylenetetrahydrofolate (5,10-MTHF). Increased enzyme activity due to the presence of 3R3R genotype could remove substrate to MTHFR resulting in increased homocysteine levels; this was demonstrated by a study in which *TYMS* 3R3R genotype was associated with reduced plasma folate and with elevated plasma homocysteine levels in individuals with low dietary folate intake, suggesting that TYMS and MTHFR compete for folate indispensable for the remethylation of homocysteine (Trinh et al., 2002). This variable number tandem repeat was shown to predict risk of colorectal adenomas (Ulrich et al., 2002), and of acute lymphoblastic leukemia (ALL) (Krajinovic et al., 2002; Skibola et al., 2002). In ALL the *TYMS* 2R2R genotype was associated with a lower total leukocyte count and blasts' percentage respect to 2R3R and 3R3R genotypes, suggesting that the VNTR reduces risk for ALL (Dunna et al., 2014). Moreover, *TYMS* 2R2R genotype was significantly associated with both hematologic and gastrointestinal toxicity in response to drug treatment, suggesting a possible role as a predictive marker of toxicity in patients treated with low-dose capecitabine (Romiti et al., 2016).

A single nucleotide polymorphism at the 12th nucleotide of the VNTR has been identified *in vivo* as a G>C change in the second repeat of the 3R allele and a C>G change in the last repeat of the 2R allele. This SNP is located within the USF (Upstream transcription factor) consensus element and alters the ability of USF proteins to bind inhibiting the transcriptional activation of *TYMS* (Mandola et al., 2003). The 3R G>C has been shown to be present in approximately half of all Caucasian 3R alleles, and is indicated as 3RC to discriminate it from the wild type 3RG. The 3RC allele frequency varied by 2.2-fold across the four races and was lowest (15%) in African Americans and highest (33%) in whites (Mandola et al., 2003). Colorectal cancer patients with the homozygous wild type 3RG3RG genotype showed higher thymidilate synthase expression compared to 2R2R, 2R3RC or 3RC3RC individuals (Mandola et al., 2003; Kawakami and Watanabe, 2003; Lincz et al., 2007). TYMS 2R3RG, 3RC3RG or 3RG3RG variants were associated with worse progression-free survival in

colorectal cancer patients and worse overall survival in the advanced gastroesophageal cancer (Joerger et al., 2015).

Expression of *TYMS* is also influenced by a -6bp deletion at position 1494 in the 3'-UTR. The -6bp allele has been reported to result in low *TYMS* mRNA stability and low *TYMS* expression compared with the wild type allele (Mandola et al., 2004). These genotypic effects may contribute to individual susceptibility to cancer (Romiti et al., 2016; Tecza et al., 2016), coronary artery disease (Vijaya et al., 2011), and birth defects (Blanton et al., 2011).

In thymoma thymidilate synthase mRNA levels were significantly higher in the tumor tissue than in the normal adjacent thymus ones (Sasaki et al., 2003). Among tumor tissue, *TYMS* mRNA expression levels were significantly higher in thymomas compared to thymic carcinoma, and the reduction in thymic carcinoma correlated linearly with protein expression levels (Monica et al., 2013).

The expression of *TYMS* is important for the response to chemoterapic 5-fluoro uracil (5-FU), an chemotherapeutic agents used for the treatment of different cancers. 5-FU itself is inactive and requires the intracellular conversion to 5-fluoro-2-deoxyuridine 5-monophosphate (FdUMP), which forms a ternary complex with thymidilate synthase and 5,10-MTHF, and inhibitions thymidilate synthase and blocks the DNA synthesis. A close relationship between the overexpression of *TYMS* and the resistance of 5-FU was demonstrated in different types of cancer and the presence of VNTR or SNPs could influence its expression causing a bad response to chemotherapy. Recently it was observed that microRNA could mediate the response of cancer cells to 5-FU by regulating TYMS expression. In colorectal cancer was showed that miR-197 directly binds to and negatively regulates TYMS expression altering the sensitivity to 5-fluorouracil (Sun et al., 2015). in colorectal cancer also miR-203 increases 5-FU chemosensitivity via the downregulation of TYMS, in fact the inhibition of miR-203 expression enhances 5-FU chemoresistance, while its overexpression increases 5-FU chemosensitivity (Li et al., 2015).

1.10 Epigenetics and cancer

In contrast to all previously theories, according to which cancer was seen as a diseases driven by the accumulation of genetic mutations, considered the major causes of neoplasia (Hanahan and Weinberg, 2011), nowadays the disruption of epigenetic regulatory mechanisms are seen as the most prevalent alterations in cancer (Baylin and Jones, 2011; Sandoval and Esteller, 2012). In fact, it is clear that both mutations and epigenetic changes play a fundamental role in the onset and progression of cancer, in the tendency toward metastasis, and in the response to treatment (Baylin and Jones, 2011). More interestingly, it is believed that virtually all tumors harbour mutations in proteins that control the epigenome, and for every genetic mutation in a given patient's tumor the expression of hundreds of genes is epigenetically deregulated to drive tumorigenesis (Herman and Baylin, 2003; Azad et al., 2013). Therefore, epigenetic and genetic alterations are two mechanisms participating in carcinogenesis and between them there is a strong crosstalk. Genetic alteration of the epigenome contributes to cancer as well as epigenetic process can cause point mutations and disable DNA repair functions (You and Jones, 2012; Burgio and Migliore, 2015). Deleterious epigenetic profiles could be a consequence of mutations in the enzymes that place epigenetic marks; germline mutations of epigenetic modifiers may contribute to the development of cancer, in fact, defective epigenetic machinery has been observed in cancer initiation and progression (Berdasco and Esteller, 2013).

Global changes in DNA methylation correlate with altered gene expression and genomic instability in cancer. Human cancers are characterized by global loss of DNA methylation coupled to promoter hyper-methylation and silencing of selected genes (Miousse and Koturbash, 2015). A high density of methylated cytosine in the CpG islands of the tumor suppressor gene promoter can lead to a complete block of transcription, and many types of cancer use this mechanism to inactivate tumor suppressor genes (Migheli and Migliore, 2012). Cancers display also global DNA hypomethylation that contribute to carcinogenesis by inducing activation of potential oncogenes and leading to chromosomal instability (Ogino et al., 2008; Igarashi et al., 2010; Martinez et al., 2012). Particularly, long interspersed nuclear elements (LINEs) are the most abundant mammalian transposable elements that cover about 20% of the human genome, and DNA methylation plays a pivotal role in the suppression of their expression and retrotransposition (Miousse and Koturbash, 2015). In cancer

development and progression, the loss of global DNA methylation is closely associated with LINE1 hypomethylation (Miousse and Koturbash, 2015).

Impairments of DNMTs have been often observed in various type of cancer. It has been shown that loss of *de novo* DNA methylation activity at advanced tumor stages leads to the promoter DNA demethylation dependent expression of specific oncogenes. These data support the notion that *de novo* DNMTs have also an important role in the maintenance of DNA methylation and suggest that, in addition to acting as oncogenes, they also behave as tumor suppressors. It was proposed that in healthy tissues, tumor suppressor genes are unmethylated and oncogenes are methylated. In early tumor stages, DNMTs seem to lead to methylation-associated repression of tumor suppressor genes and promote tumor initiation. In advanced tumor stages, the downregulation of *de novo* DNMTs seem associated with promoter DNA hypomethylation of specific oncogenes and, consequently, promotes tumorigenesis (Fernandez et al., 2012).

Over the years the interest in DNA methylation increased since it is a stable mark that can be easily assessed in DNA samples obtained by means of minimally invasive procedures from cancer patients, including serum, urine, sputum and faecal material, and is increasingly investigated as a promising biomarker for the development of diagnostic and prognostic assays (Coppedè, 2014; Costa-Pinheiro et al., 2015).

Similar to 5mC, global loss of 5hmC was observed in large numbers of cancer, such as prostate, breast, lung, and colon, in which ten-eleven translocation enzymes dysregulation was detected (Haffner et al., 2011; Jin et al., 2011; Kudo et al., 2012; Chen et al., 2013; Du et al., 2015; Barazeghi et al., 2016). In solid cancers, ten-eleven translocation 1 enzyme decreased expression accompanied by a reduction of global 5hmC could cause the accumulation of aberrant DNA methylation and consequent alterations in gene expression, promoting cell proliferation and facilitating cell invasion and cancer metastasis. Ten-eleven translocation 1 enzyme binds to hypomethylated, high CpG-dense regions to prevent DNMT's activity and resist methylation. Ten-eleven translocation 1 enzyme also binds to dispersed CpGs, limiting the accessibility of DNMTs or MBDs (Tian et al., 2016). Alterations in *ten-eleven translocation* genes were observed also in haematological malignancies, where *ten-eleven translocation 2* gene is frequently mutated, causing the loss of function of the protein (Delhommeau et al., 2009; Jankowska et al., 2009; Langemeijer et al., 2009; Ko et al., 2010). It was demonstrated that *ten-eleven translocation 2* mutations in patients with acute myeloid leukaemia were

associated with a DNA hypermethylation phenotype (Figueroa et al., 2010). In general, the loss of the TET proteins leads to the accumulation of stochastic aberrant DNA methylation at CpG islands that would provide cells with a growth advantage. This would lead to clonal expansion of the cells and could promote cancer development (Williams et al., 2011).

Other frequently observed epigenetic changes in cancer are post-translational modifications of the histone tails of nucleosomes, including global deacetylation and regional demethylation at lysine 4 of histone H3 (H3K4) at DNA hypermethylated genes, leading to a condensed chromatin configuration (Hashimoto et al., 2010), as well as altered expression of several non-coding RNAs occurring within disease progression, invasion and metastasis (Hayes et al., 2014). In development and progression of cancer, microRNAs may function as tumor suppressors, which down-regulation is associated with increased malignancy or metastatic potential, and also as oncogenes, promoting proliferation and cell cycle progression, senescence, apoptosis, invasion, and metastasis (Vogt et al., 2011; Fabbri et al., 2013; Guo et al., 2015; Wang et al., 2014; Saki et al., 2015). For example miRNA-17, and miRNA-155 might have oncogenic properties, while miRNA-34, miRNA-16 and let-7 might to function as tumor suppressor (Calin and Croce, 2006; Johnson et al., 2007).

Therefore, a complex interplay between DNA methylation, chromatin remodelling, and other mechanisms that regulate gene expression levels, occurs in cancer and is responsible for the inactivation of tumor suppressor genes, DNA repair genes, and many others to allow escape from immune response and apoptosis and resistance to chemotherapy (Azad et al., 2013).

1.10.1 DNA methylation in thymic epithelial tumors

The epigenetic mechanisms underlying thymic epithelial tumors have not yet been fully explored, and only a few studies to date have investigated the DNA methylation in TETs (Table 2). *CDKN2A* promoter methylation was reported in up to 11% of thymomas and 25% of thymic carcinoma (Hirabayashi et al., 1997), and by immunohistochemical analysis it was demonstrated that copy number loss of *CDKN2A*, accompanied by lack of expression of its related protein, identified tumors with poor prognosis (Petrini et al., 2012). Nine tumor suppressor genes, such as *APC1A*, *CDH1*, *CDKN2A*, *DAPK*, *FHIT*, h*MLH1*, *MGMT*, *RARβ2*, and *RASSF1A* were found downregulated in a promoter hypermethylation-dependent

fashion, and methylation frequencies were significantly correlated with tumor severity as assessed by modified Masaoka stage score and WHO histological type. Therefore, promoter hypermethylation and consequent downregulation of specific tumor suppressor genes (TSGs) may play an important role in the development of aggressive and invasive TETs (Chen et al., 2009). Moreover, it was observed that the methylation frequency of *DAPK*, *CDKN2A*, *MGMT*, and *HPP1* promoters are significantly lower in thymomas (29%) than in thymic carcinomas (86%), and the frequency of tumors with methylation of multiple genes in thymic carcinoma was higher than in thymoma (60% vs 20%). In particular, *MGMT* methylation was detected only in 23% of thymoma and in 83% of thymic carcinoma (Hirose et al., 2009). The same data was observed in another study, in which *MGMT* methylation (17/23, 74% versus 13/44, 29%) and loss of its protein expression (20/23, 87% versus 10/44, 23%) has been reported to be significantly more frequent in thymic carcinoma than in thymoma and in advanced of thymic epithelial tumors (stages III/IV) compared with early stage tumors (stages I/II) (Mokhtar et al., 2014).

Also *AIRE* methylation status was investigated, since CpG methylation in the promoter of the gene has been supposed to control its tissue-specific expression pattern. In human AIRE-positive medullary and AIRE-negative cortical epithelium, the *AIRE* promoter is hypomethylated, whereas in thymocytes, the promoter had high level of CpG methylation. Likewise, in mouse mTECs the *AIRE* promoter was uniformly hypomethylated. The *AIRE* promoter was hypomethylated in AIRE-negative thymomas and in several peripheral tissues. In contrast, a positive correlation between AIRE expression and histone H3 lysine 4 trimethylation, an active chromatin mark, was found in the *AIRE* promoter in human and mouse TECs (Kont et al., 2011).

A better understanding of alterations in DNA methylation can have important role in the discovery of biomarkers of pathology and in therapeutic implications.

Gene	Function	Epigenetic alteration	Reference
APC	Cell cycle regulation	Promoter hypermethylation	Chen et al., 2009
CDH1	Cell adhesion	Promoter hypermethylation	Chen et al., 2009
CDKN2A	Cell cycle regulation	Promoter methylation reported in up to 11% of thymomas and 25% of thymic carcinoma	Hirabayashi et al., 2009; Hirose et al., 2009
DAPK	Cell apoptosis	Promoter hypermethylation and	Chen et al.,

		methylation frequency lower in thymoma than thymic carcinoma	2009; Hirose et al., 2009
FHIT	Cell cycle regulation	Promoter hypermethylation	Chen et al., 2009
MGMT	DNA repair	Promoter hypermethylation associated with the loss of protein	Chen et al., 2009; Hirose et al., 2009; Mokhtar et al., 2014
h*MLH1*	DNA repair	Promoter hypermethylation	Chen et al., 2009
RARβ2	Cell cycle regulation	Promoter hypermethylation	Chen et al., 2009
RASSF1A	Signal transduction	Promoter hypermethylation	Chen et al., 2009
AIRE	Autoimmune regulation	Promoter hypomethylation	Kont et a., 2011

Table 2. List of genes deregulated in thymic epithelial tumors by DNA methylation.

1.10.2 MicroRNA in thymic epithelial tumors

MicroRNAs are important regulators of genetic programs in thymic epithelial cells (Table 3), as miRNA-deficient TECs are severely defective, for example miR-205 is highly expressed in medullary thymic epithelial cells (mTECs) during both embryonic development and in the postnatal thymus, suggesting its functional importance for TEC biology. In thymic epithelial tumors miRNAs are differentially expressed between tumor tissues and normal thymic ones, between thymomas and thymic carcinomas, and among different histotypes groups (Khan et al., 2015). Upregulation of miR-21-5p, miR-34b-5p, and miR-141-3p, as well as down-regulation of miR-486-5p and miR-145-5p have been observed in TETs in comparison with normal tissue (Ganci et al., 2014). Among them, miR-21 has been shown to have oncogenic properties due to its ability to target tumor suppressor genes, such as PTEN, and to promote cell proliferation, survival and migration (Meng et al., 2007). The down-regulation of tumor suppressor miR-145-5p is thought to lead to epidermal growth factor receptor (EGFR) signalling pathway overexpression (Ganci et al., 2014), in fact, EGFR protein was also reported to be up-regulated in thymic tumors (Aisner et al., 2010; Weissferdt et al., 2012). Among the miRNAs down-regulated in thymic carcinoma, miR-142-5p, miR-363-3p and miR-16-2-3p are predicted to target three mRNA of anti-apoptotic signature, such as BIRC3, CCL20, and MYC (Ganci et al., 2014). Looking for blood plasma circulating non-invasive biomarkers for TET management, miR-21-5p and miR-148a-3p resulted significantly up-

regulated in blood plasma collected from TET patients at the time of surgery compared to healthy donors samples, and were significantly reduced in follow-up samples, suggesting that they could represent novel non-invasive biomarkers to evaluate the efficacy of therapy and the prognosis of TET (Bellissimo et al., 2016).

Some miRNAs are tumor type specific, in fact a large microRNA cluster on chr19q13.42 was significantly overexpressed in all A and AB tumors and their expression was absent in the other thymomas and normal tissues. Overexpression of this microRNA cluster activates the PI3K/AKT/mTOR pathway (Radovich et al., 2016).

In thymoma associated MG, thymic stromal lymphopoietin (TSLP), post-trancriptionally regulated by microRNA-19b, showed a significantly decreased (Wang et al., 2015). TSLP, expressed mainly by epithelial cells and an interleukin 7-like cytokine, was a key regulator for the generation of Treg and Th17 cells (Spadoni et al., 2012). The expression of microRNA-19b was negatively correlated with TSLP mRNA and protein expression in MG thymomas, indicating that the increasing of microRNA-19b could influence T cell development in thymomatous MG (Wang et al., 2015). miR-125a-5p was significantly dysregulated in thymoma compared with normal thymus controls. Foxp3 3'-untranslated region (UTR) was identified as a target of miR-125a-5p, which expression was negatively correlated with Foxp3 expression in normal tissue adjacent to the thymoma from TAMG patients (Li et al., 2016).

MicroRNA	Dysfunction	Reference
miR-21-5p miR-34b-5p miR-141-3p	Upregulation in thymic epithelial tumors respect to normal thymic tissue	Ganci et al., 2014
miR-486-5p miR-145-5p	Downregulation in thymic epithelial tumors respect to normal thymic tissue	Ganci et al., 2014
miR-142-5p miR-363-3p miR-16-2-3p	Downregulation in thymic carcinoma	Ganci et al., 2014
miR-21-5p miR-148a-3p	Upregulated in blood plasma of tumor patients compared to healthy donors samples	Bellissimo et al., 2016
microRNA-19b	Upregulation in thymoma associated MG, targeting TSLP that shows a significantly decreased	Wang et al., 2015
miR-125a-5p	Dysregulated in thymoma associated MG respect to normal thymus, targeting Foxp3	Li et al., 2016

Table 3. MicroRNA in thymic epithelial tumors.

Chapter 2: Aim of the study

This project aims to study the genetic and epigenetic changes in thymoma associated myasthenia gravis (TAMG) and for this aim a large number of TAMG patients were recruited from November 2013 to October 2016.

The study comprises the following items:

1. the detection of gene-specific methylation levels in promoters of genes involved in one carbon metabolism (*MTHFR*), DNA methylation reaction (*DNMT1*, *DNMT3A*, *DNMT3B*), autoimmune regulation (*AIRE*), and key cancer genes, such as genes of DNA repair (*MGMT*, h*MLH1*), and cell cycle (*RASSF1A*, *CDKN2A*) and detection of global methylation by means of LINE1 methylation analysis in blood, tumor tissue, and adjacent thymic one in order to shed light on the contribution of DNA methylation to the development of thymoma associated MG;
2. gene expression analysis of *MTHFR*, *DNMT3A*, and *AIRE* genes in tumor tissue and adjacent thymic one, and correlation of expression levels with methylation levels in order to understand if methylation is an epigenetic mechanism involved in their regulation;
3. correlation analysis among the methylation status of chosen genes and both physical and clinicopathological features of the patients;
4. analysis of polymorphisms of genes required for DNA methylation processes, such as *MTHFR* 677C>T, *MTRR* 66A>G, *MTR* 2756A>G, *TYMS* 28 bp repeats, *DNMT3B* -149C>T, *DNMT3B* -579G>T in myasthenic patients with thymoma and myasthenic patients with normal thymus to understand if some of them constitute a thymoma risk factor;
5. correlation among the studied polymorphisms and the methylation levels of *MTHFR, DNMT1, DNMT3A, DNMT3B, MGMT, hMLH1, RASSF1A, CDKN2A, AIRE* gene promoters.

Chapter 3: Materials and methods

3.1 Study population

A total of 372 AChR$^+$ MG patients were recruited at the Myasthenia Clinic, Department of Neuroscience and Cardiac and Thoracic Department, of the Pisa University Hospital. The diagnosis of patients was based on characteristic signs and symptoms of MG coupled with anti-AChR antibody positive test. The patients were divided on the basis of thymic pathology, so 132 patients (35.5%) had normal (involuted) thymus, and 240 patients (64.5%) had a thymoma. The thymus is defined normal according to the histological features. Demographic and clinicopathological characteristics of the population under study are reported in Table 4. About two millilitres of blood was collected from each patient in EDTA tubes and stored at -20° C until assayed. Each patient gave an informed written consent for genotype analysis before blood drawing. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Pisa University Hospital (Protocol number 21302/2015).

	Normal thymus	**Thymoma**
N. patients (%)	132 (35.5)	240 (64.5)
Age±SD	62.8+15.1	55.7±13.4
Gender (%)	F: 57 (43); M: 75 (57)	F: 125 (52); M: 115 (48)
MG onset (%)	<50 years: 55 (41.7) >50 years: 77 (58.3)	<50 years: () >50 years: ()
MG Osserman Classification (%)	I: 39 (29.4) IIA: 37 (28.0) IIB: 54 (41.0) III: 1 (0.8) IV: 1 (0.8)	I: 13 (5.4) IIA: 46 (19.2) IIB: 144 (60) III: 22 (9.2) IV: 11 (4.5) V: 4 (1.7)
Thymoma histology (%)		A: 28 (11.7) AB: 44 (18.3) B1: 32 (13.3) B2: 60 (25) B3: 24 (10) B2-B3: 15 (6.3) NS: 37 (15.4)

Table 4. Demographic and clinicopathological characteristics of the studied population (MG: myasthenia gravis, F: females, M: males, NS: no specified tumor histology).

3.2 Extraction of genomic DNA

Genomic blood and tissue DNA was extracted using QIAmp DNA Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instruction. The extracted DNA was quantified using a Nano Drop ND 2000c spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, DE, USA).

3.3 Genotyping analysis

The genotyping of *MTHFR* 677C>T (rs1801133), *MTRR* 66A>G (rs1801394), *MTR* 2756A>G (rs1805087), *DNMT3B* -149C>T (rs2424913), and *DNMT3B* -579G>T (rs1569686) were performed according to PCR-RFLP methods described in Coppedè et al., 2006 and Coppedè et al., 2012. The genotyping for *TYMS* 28 bp repeats (rs34743033) was performed by means of PCR without RFLP. The PCR produced a 243 bp product from the 3R allele and 215bp product from the 2R allele. The PCR conditions with annealing temperature e primers sequences were reported in Table 5. The digestion conditions were described in Table 6.

Polymorphism	Primer sequence	T_a*	Product size
***MTHFR* 677C>T**	F: 5′TGAAGGAGAAGGTGTCTGCGGGA-3′ R: 5′AGGACGGTGCGGTGAGAGTG-3′	62°C	198bp
***MTR* 2756A>G**	F: 5′-TGTTCCCAGCTGTTAGATGAAAATC-3′ R: 5′-GATCCAAAGCCTTTTACACTCCTC-3′	60°C	211bp
***MTRR* 66A>G**	F: 5′-GCAAAGGCCATCGCAGAAGACAT-3′ R: 5′-TGGTGGTATTAGTGTCCTTTTG-3′	56°C	182bp
***TYMS* 28bp repeats**	F: 5′-GTGGCTCCTGCGTTTCCCCC-3′ R: 5′-GGCTCCGAGCCGGCCACAGGCATGGCGCGG-3′	63°C	243/215bp
***DNMT3B* -149C>T**	F: 5′-TGCTGTGACAGGCAGAGCAG-3′ R: 5′-GGTAGCCGGGAACTCCACGG-3′	62°C	380bp
***DNMT3B* -579G>T**	F: 5′-GAGGTCTCATTATGCCTAGG-3′ R: 5′-GGGAGCTCACCTTCTAGAAA-3′	56°C	225bp

Table 5. PCR conditions (*T_a: annealing temperature).

Polymorphism	Enzyme	Digestion condition	Fragment products
***MTHFR* 677C>T**	Hinf I	37°C 2h	C: 198bp T: 175bp, 23bp
***MTR* 2756A>G**	Hae III	37°C 2h	A: 211bp G: 131bp, 80bp
***MTRR* 66A>G**	Nde I	37°C 16h	A: 160bp, 22bp G: 182bp
***DNMT3B* -149C>T**	Xmaj (AvrII)	37°C 16h	C: 380bp T: 207bp, 173bp
***DNMT3B* -579G>T**	PvuII	37°C 16h	G: 225bp T: 132bp, 93bp

Table 6. RFLP conditions.

Digestion products were visualized after electrophoresis on a 3% agarose gel containing ethidium bromide.

3.4 DNA methylation analysis: Methylation Sensitive-High Resolution Melting (MS-HRM)

200 ng of DNA from each sample were treated with sodium bisulfite using the EpiTectH Bisulfite Kit (Qiagen) according to the manufacturer's protocol. Sodium bisulfite treatment converts all unmethylated cytosines into uracil, while methylated cytosines are left unchanged. MS-HRM analysis was performed in a subgroup of 69 AChR+ myasthenic patients with thymoma for which was available DNA obtained from both surgically resected tumor tissues and the adjacent normal tissue, available from forty-four of them. The demographics characteristics of the subgroup were showed in Table 7.

TAMG patients	Age±SD	Gender (%)	MG onset (%)	MG Osserman Classification (%)	Thymoma hystology (%)	Masaoka stage (%)
69	55.5±13.2	F: 37 (54) M: 32 (46)	<50 years: 26 (38) ≥50 years: 43 (62)	I: 7 (10.0) IIA: 11 (16.0) IIB: 34 (49.5) III: 13 (19.0) IV: 3 (4.0) V: 1 (1.5)	A: 13 (18.8) AB: 13 (18.8) B1: 5 (7.2) B2: 23 (33.4) B3: 8 (11.6) B2-B3: 5 (7.2) NS: 2 (3.0)	I: 7 (10) IIa: 15 (22) IIb: 29 (42) III: 7 (10) IVa: 9 (13) NS: 2 (3)

Table 7. Demographic and clinicopathological characteristics of the studied population (MG: myasthenia gravis, F: females, M: males, NS: no specified tumor histology).

Promoter methylation was assessed by means of methylation sensitive-high resolution melting (MS-HRM) analysis in a CFX96 Real-Time PCR detection system (Bio-Rad). For the MS-HRM analysis we developed protocols according to literature (Coppedè et al, 2014; Migheli et al., 2013). All analyses were run according to the following conditions: 1 cycle of 95° C for 12 min, 60 cycles of 95° C for 30s, T_a for 30s and 72° C for 15s; followed by an HRM step of 95° C for 10s and 50° C for 1 min, 65° C for 15s, and continuous acquisition to 95° C at one acquisition per 0.2° C. PCR was performed in a final volume of 25 μl, containing 12.5 μl of master mix (Qiagen), 10 pmol of each primer and 10 ng of bisulfite modified DNA template. Each reaction was performed in duplicate. We analyzed 10% of the samples independently on separate occasions to verify the inter-assay variability and we observed a good reproducibility. Table 8 shows the conditions used for each gene, such as primers, annealing temperature, CpG sites, and amplicon length; for *MTHFR* and *DNMT3A* we used primers derived from literature (Vaisseire et al. 2009; and Jost et al., 2014), such as *MGMT*, h*MLH1*, *CDKN2A*, and *RASSF1A* (Huang et al., 2010), while *AIRE*, *DNMT31* and *DNMT3B* primers were designed by us using Meth-primer software (available at http://www.urogene.org/methprimer).

Fully methylated and unmethylated DNA (EpiTectH methylated and unmethylated human control DNA, bisulfite converted, Qiagen) were mixed to obtain the following ratios of methylation: 0%, 12.5%, 25%, 50%, 75%, and 100%. Standard curves with known methylation ratios were included in each assay and were used to deduce the methylation ratio of each blood, adjacent healthy tissue and tumor one. Validation of the MS-HRM assays was performed by means of pyrosequencing, as detailed elsewhere (Migheli et al., 2013; Tannorella et al., 2014). In order to obtain single methylation percentage values from MS-HRM assays, we applied an interpolation method developed and described by us, which allowed to obtain precise HRM methylation values comparable to those obtained by pyrosequencing (Migheli et al., 2013).

Gene	Primer sequence	T_a	Amplicon length	CpG sites
MTHFR	F: 5′-TTTTAATTTTTGTTTGGAGGGTAGT-3′ R: 5′-AAAAAAACCACTTATCACCAAATTC-3′	54°	155 bp	7
DNMT1	F: 5′-GGTATCGTGTTTATTTTTTAGTAA-3′ R: 5′-ACGAAACCAACCATACCCAA-3′	52°	114 bp	9
DNMT3A	F: 5′-GGTTTGGGTTTATTGTAGGAAGGTTATTAAGGT-3′ R: 5′-AATCCAAAACCCCCCTATCACGAAA-3′	58°	199 bp	7
DNMT3B	F: 5′-TGGTGTTGTGTGATTATAGTGG-3′ R: 5′-TCACCCTAAAAAATCAAAAACC-3′	55°	174 bp	6
MGMT	F: 5′-GCGTTTCGGATATGTTGGGATAAGT-3′ R: 5′-AACGACCCAAACACTCACCAAA-3′	58°	110 bp	12
h*MLH1*	F: 5′-AGTTTTTAAAAACTGAATTAATAGGAAGAG-3′ R: 5′-ACTACCCGCTACCTAAAAAAATATAC-3′	56°	81 bp	5
CDKN2A	F: 5′-CGGAGGAAGAAAGAGGAGGGGT-3′ R: 5′-CGCTACCTACTCTCCCCCTCT-3′	62°	93 bp	7
RASSF1A	F: 5′-TCGGGTTTTATAGTTTTTGTATTTAGGTTTT-3′ R: 5′-CCTCCCCCAAAATCCAAACTAA-3′	60°	87 bp	7
AIRE	F: 5′TTTTGGTGGGTGAGTTAGGTTAG-3′ R: 5′ CCAATCAAAACCAAAACCTC-3′	60°	111 bp	5

Table 8. Primer sequences and annealing temperatures (T_a) used during MS-HRM analysis, as well as amplicon length and number of CpG sites for each gene.

3.5 Gene expression analysis: qPCR

Total RNA was extracted from human thymic fragments using AllPrep DNA/RNA/miRNA Universal (Qiagen). Between 500 ng and 1 μg of total RNA was used for reverse transcription using Reverse Transcriptase AMV (Roche kit). One microliter of the reverse transcriptase reaction was subsequently subjected to PCR amplification on the LightCycler 480 system using PCR primers listed in Table 9 (Eurogentec, Seraing, Belgium). Each RNA sample was run in triplicate. These experiment were performed at Center of Research in Myology (Sorbonne Universités, Pierre and Marie Curie University - Paris 6).

Gene	Forward primer	Reverse primer
28S	5′-GGTAGGGACAGTGGGAATCT-3′	5′-CGGGTAAACGGCGGGAGTAA-3′
AIRE	5′-ATGACACTGCCAGTCACGAG-3′	5′-AGGAGGTGTCCTTCTCAGCA-3′
DNMT3A	5′-TCCAACCCTGTGATGATTGA-3′	5′-GCCCTGCTTTATGGAGTTTG-3′
MTHFR	5′-TGAAGCACATCCGAAGTGAG-3′	5′-GAAAAGCTGCGTGATGATGA-3′

Table 9. List of primers used for gene expression analysis.

3.6 Statistical analysis

To verify whether allele frequencies were in Hardy-Weinberg equilibrium and to assess differences in allele distributions between groups, the Chi square (χ^2) analysis was used. Logistic regression analysis was used to examine the associations between the studied polymorphism and thymoma risk by estimating odds ratios (ORs) and 95% confidence intervals (CIs) adjusted for age and gender. All individual values were analyzed with the MedCalc 12.5 statistical package for Windows.

Differences in mean methylation levels among the three groups were evaluated with analysis of variance (ANOVA) test, differences group by group were evaluated by Student's test for paired sample. The effect of clinical and pathological features, such as onset of MG (early or late), MG Osserman classification, thymoma histology, and Masaoka classification on mean methylation levels was assessed by means of ANOVA, including age at sampling and gender as covariates. Linear regression analysis was performed to search for a correlation between age and methylation levels of chosen genes in the studied tissues, as well as to search for a correlation of methylation levels among the different tissues, and to correlate methylation levels with gene expression.

The statistical power of the study was evaluated with the clinical tool and calculators for medical professionals, ClinCalc (available at http://clincalc.com/Stats/Power.aspx). The sample size was chosen to have an a priori power of >80% to detect mean methylation differences of 5% or higher.

Analyses were performed with the STATGRAPHICS 5.1 Plus software package for Windows and Graphpad software. *P-value* for considering a result to be statistically significant was set at 0.05.

Chapter 4: Results

4.1 Genotype and allele frequencies in TAMG patients

Polymorphisms of one-carbon metabolism genes, such as *MTHFR* 677C>T, *MTRR* 66A>G, *MTR* 2756A>G, *TYMS* 28 bp repeats, *DNMT3B* -149C>T, *DNMT3B* -579G>T, were analyzed in blood of AChR+ MG patients with thymoma respect to AChR+ MG patients with normal thymus to understand if some of them constitute a thymoma associated MG risk factor. In these cells, this metabolism maintains genomic stability providing one-carbon units required for several intracellular processes, that include the synthesis of DNA and RNA precursors necessary for DNA replication and repair, the synthesis of amino-acids, and the conversion of homocysteine to methionine, which is then used to form the main DNA methylating agent S-adenosylmethionine.

Table 10 shows the distribution of genotype and allele frequencies in 132 myasthenic patients with normal thymus and 240 myasthenic patients with thymoma. Comparing genotype and allele frequencies between two groups, we observed a significant decrease in *TYMS* 28bp 2R allele frequency in patients with thymoma with respect to normal thymus (0.41 vs 0.49, $P = 0.04$). Moreover, the *TYMS* 28bp 2R2R (OR = 0.51; 95% CI = 0.27-0.97, $P = 0.04$) was associated with decreased risk to develop a thymoma among AChR+ MG patients. Adjusting the OR for age and gender the statistical significance is borderline.

Genotypes/ Alleles	Normal thymus MG patients N. 132 (%)	TAMG patients N. 240 (%)	Crude OR (95% IC)	*P-value*	Adjusted OR[a] (95% IC)	*P-value*
***MTHFR* 677C>T (rs1801133)**						
CC	36 (27%)	62 (26%)	1.00[b]	--	1.00[b]	--
CT	68 (52%)	117 (49%)	0.99 (0.60-1.66)	0.99	0.71 (0.45-1.12)	0.15
TT	28 (21%)	61 (25%)	1.26 (0.69-2.32)	0.45	1.14 (0.61-2.14)	0.68
Allele C	0.53	0.50	1.00[b]	--	1.00[b]	--
Allele T	0.47	0.50	1.12 (0.83-1.51)	0.46	1.06 (0.78-1.45)	0.70
***MTRR* 66A>G (rs1801394)**						
AA	40 (30%)	81 (34%)	1.00[b]	--	1.00[b]	--
AG	72 (55%)	120 (50%)	0.82 (0.51-1.33)	0.43	0.78 (0.48-1.28)	0.33
GG	20 (15%)	39 (16%)	0.96 (0.50-1.86)	0.91	0.93 (0.47-1.84)	0.84
Allele A	0.58	0.59	1.00[b]	--	1.00[b]	--
Allele G	0.42	0.41	0.95 (0.70-1.29)	0.76	0.92 (0.67-1.26)	0.59

***MTR* 2756A>G (rs1805087)**						
AA	86 (65%)	155 (65%)	1.00[b]	--	1.00[b]	--
AG	40 (30%)	82 (34%)	1.14 (0.72-1.80)	0.58	1.13 (0.70-1.82)	0.60
GG	6 (5%)	3 (1%)	0.28 (0.07-1.14)	0.07	0.34 (0.08-1.43)	0.14
Allele A	0.80	0.82	1.00[b]	--	1.00[b]	--
Allele G	0.20	0.18	0.92 (0.63-1.34)	0.65	0.94 (0.64-1.40)	0.78
***TYMS* 28bp (rs34743033)[c]**						
3R3R	31 (24%)	80 (34%)	1.00[b]	--	1.00[b]	--
3R2R	70 (54%)	120 (50%)	0.66 (0.40-1.10)	0.12	0.69 (0.41-1.16)	0.16
2R2R	**28 (22%)**	**37 (16%)**	**0.51 (0.27-0.97)**	**0.04**	**0.52 (0.27-0.99)**	**0.05**
Allele 3R	0.51	0.59	1.00[b]	--	1.00[b]	--
Allele 2R	**0.49**	**0.41**	**0.73 (0.54-0.98)**	**0.04**	**0.73 (0.53-1.00)**	**0.05**
***DNMT3B* -149 C>T (rs2424913)**						
CC	65 (49%)	102 (42%)	1.00[b]	--	1.00[b]	--
CT	48 (36%)	112 (47%)	1.48 (0.94-2.35)	0.09	1.52 (0.94-2.47)	0.08
TT	19 (15%)	26 (11%)	0.87 (0.45-1.70)	0.69	0.92 (0.45-1.85)	0.81
Allele C	0.67	0.66	1.00[b]	--	1.00[b]	--
Allele T	0.33	0.34	1.07 (0.78-1.48)	0.66	1.09 (0.78-1.53)	0.58
***DNMT3B* -579 G>T (rs1569686)**						
GG	66 (50%)	109 (45%)	1.00[b]	--	1.00[b]	--
GT	51 (39%)	101 (42%)	1.20 (0.76-1.89)	0.43	1.13 (0.71-1.81)	0.61
TT	15 (11%)	30 (13%)	1.21 (0.61-2.42)	0.59	1.30 (0.63-2.70)	0.47
Allele C	0.69	0.66	1.00[b]	--	1.00[b]	--
Allele T	0.31	0.34	1.14 (0.83-1.58)	0.43	1.14 (0.81-1.59)	0.44

Table 10. Distribution of genotypes and allele frequencies of the studied polymorphisms in myasthenic patients with normal thymus and those with thymoma. [a] Adjusted for age and gender. [b] Reference value for OR. [c] For three patients with normal thymus the genotype was 2R4R and for one patient 3R5R; for two patients with thymoma the genotype was 3R5R and for one patient 3R4R.

4.2 Gene-specific methylation analysis

4.2.1 Methylation levels among tissues

In order to investigate the involvement of epigenetic changes, in term of DNA methylation, in thymoma associated MG pathogenesis, the methylation levels of genes involved in one carbon metabolism (*MTHFR*), DNA methylation reaction (*DNMT1*, *DNMT3A*, *DNMT3B*), autoimmune tolerance (*AIRE*), and in key cancer genes, such as genes of DNA repair (*MGMT*, h*MLH1*), and cell cycle (*CDKN2A*, *RASSF1A*) were detected.

DNA obtained from both blood and surgically resected tumor tissue of sixty-nine AChR+ myasthenic patients with thymoma was available; for forty-four of them the tissue adjacent to tumor one was available. The mean methylation levels of the studied genes in blood and

tumor tissue DNA of all TAMG patients are reported in Table 11. *MTHFR* shows higher methylation levels in tumor tissue respect to blood ($P = 0.007$), and *DNMT3A* shows about 10% promoter methylation in tumor tissue but it is completely demethylated in blood ($P < 0.0001$). *DNMT3B* and *DNMT1* studied regions were largely hypomethylated both in blood and tumor tissue DNA, with higher methylation levels of *DNMT1* in blood respect to tumor tissue ($P = 0.04$) even if the mean methylation levels were no more than 2%.

AIRE, the autoimmune regulator, showed lower methylation levels in tumor tissue respect to blood and this difference was statistically significant ($P = 0.0008$).

In our population, genes involved in DNA repair and cell cycle, such as *MGMT*, h*MLH1*, *CDKN2A*, and *RASSF1A* were largely hypometylated both in blood and in tumor tissue, particularly the mean methylation levels were no higher than 2%. h*MLH1* showed lower methylation levels in tumor respect to blood ($P = 0.0007$), such as *MGMT* ($P = 0.02$) but they were very low, no higher than 1%.

Gene	Blood (mean±SEM)	Tumor tissue (mean±SEM)	*P-value*
MTHFR	24.28±0.99	29.66±1.55	**0.007**
DNMT3A	0.35±0.08	9.78±0.91	**<0.0001**
DNMT3B	1.14±0.24	0.95±0.26	0.41
DNMT1	1.67±0.12	1.43±0.13	**0.04**
MGMT	0.32±0.04	0.20±0.03	**0.02**
h*MLH1*	0.57±0.09	0.22±0.06	**0.0007**
CDKN2A	0.60±0.07	0.57±0.11	0.79
RASSF1A	1.06±0.21	1.65±0.31	0.09
AIRE	30.96±1.95	24.30±1.85	**0.0008**

Table 11. Methylation levels (%) of the studied genes in blood and tumor tissue of total thymoma-associated MG (TAMG) patients ($n = 69$). Blood and tumor tissues were taken from the same donors. *P-value* refers to a t-test for paired samples.

For forty-four TAMG patients also adjacent thymic tissue was available. The mean methylation levels in blood, tumor tissue and adjacent one are listed in Table 12. *MTHFR*, *DNMT3A*, and *AIRE* showed statistically significant different methylation levels among the three analyzed tissues.

Gene	Blood (mean±SEM)	Adjacent thymic tissue (mean±SEM)	Tumor tissue (mean±SEM)	*P-value*
MTHFR	22.69±0.99	19.62±1.54	27.49±1.34	**0.001**
DNMT3A	0.24±0.09	8.85±0.82	10.09±1.17	**<0.0001**
DNMT3B	1.07±0.24	2.14±0.79	0.85±0.25	0.148
DNMT1	1.66±0.18	1.69±0.20	1.38±0.16	0.423
MGMT	0.30±0.04	0.32±0.06	0.16±0.03	0.045
h*MLH1*	0.64±0.12	0.41±0.19	0.20±0.07	0.079
CDKN2A	0.57±0.08	0.42±0.12	0.47±0.13	0.643
RASSF1A	0.85±0.19	1.33±0.35	1.42±0.35	0.367
AIRE	30.85±1.91	16.31±1.70	22.60±1.95	**<0.0001**

Table 12. Methylation levels (%) of the studied genes in blood, adjacent thymic tissue and tumor one for TAMG patients for which adjacent tissue was available (n = 44). Blood, adjacent tissue and tumor one were taken from the same donors. *P-value* refers to a ANOVA test.

Figure 6 shows graphically the mean methylation levels of the studied genes in blood, tumor tissue, and adjacent thymic one (listed in Table 12). To compare the single differences between each tissue a t-test for paired sample was applied.

MTHFR shows higher methylation levels in tumor tissue respect to blood (P = 0.004, Figure 6A) and to adjacent thymic tissue (P < 0.001, Figure 6A). *DNMT3A* shows higher methylation levels in tumor tissue and adjacent thymic tissue respect to blood that results completely demethylated (P < 0.001, Figure 6B), but no significant difference was observed between thymomas and adjacent thymic tissue. *AIRE* shows higher methylation levels in blood respect tumor tissue (P = 0.003, Figure 6I) and adjacent thymic one (P < 0.0001, Figure 6I). h*MLH1* methylation levels are higher in blood respect tumor tissue but they are very low, no higher than 1% (P = 0.001, Figure 6F). The other genes, such as *DNMT3B*, *DNMT1*, *MGMT*, *CDKN2A*, and *RASSF1A*, showed very low methylation levels, and no statistically significant difference was observed among analysed tissues (Figure 6C-E, G-H).

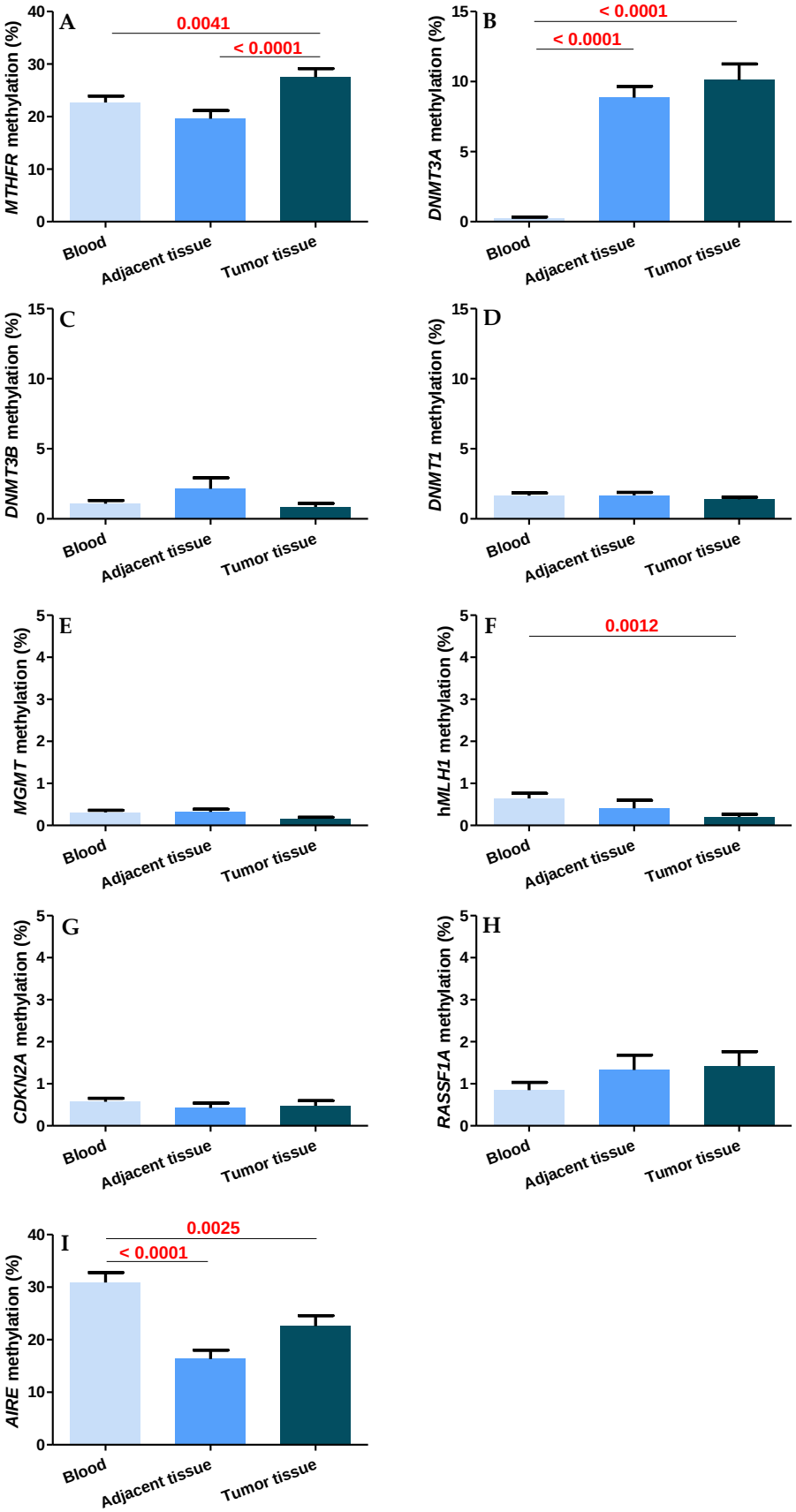

Figure 6. Methylation levels (%) of the studied genes (**A**: *MTHFR*, **B**: *DNMT3A*, **C**: *DNMT3B*, **D**: *DNMT1*, **E**: *MGMT*, **F**: h*MLH1*, **G**: *CDKN2A*, **H**: *RASSF1A*, **I**: *AIRE*) in blood, tumor tissue and adjacent thymic tissue of thymoma-associated MG (TAMG) patients for which adjacent tissue was available (n = 44). Data on graph correspond to mean±SEM. The significant *P-values* (in red) refer to t-tests for paired samples.

4.2.2 Correlation of methylation levels among tissues

Interesting correlations between the *MTHFR* methylation levels among blood, tumor tissue, and thymic tissue adjacent to the tumor were observed (Figure 7A–D). In particular, a positive correlation of *MTHFR* methylation levels between tumor tissue and blood was observed in the whole population (n = 69, $P = 0.004$, $r = 0.34$; Figure 7A) and in the subgroup for which the adjacent thymic tissue was available (n = 44, $P = 0.003$, $r = 0.44$; Figure 7B). Moreover, correlations between blood and thymic tissue adjacent to the tumor (n = 44, $P = 0.007$, $r = 0.40$; Figure 7C) and between tumor tissue and adjacent thymic tissue (n = 44, $P = 0.002$, $r = 0.45$; Figure 7D) were observed.

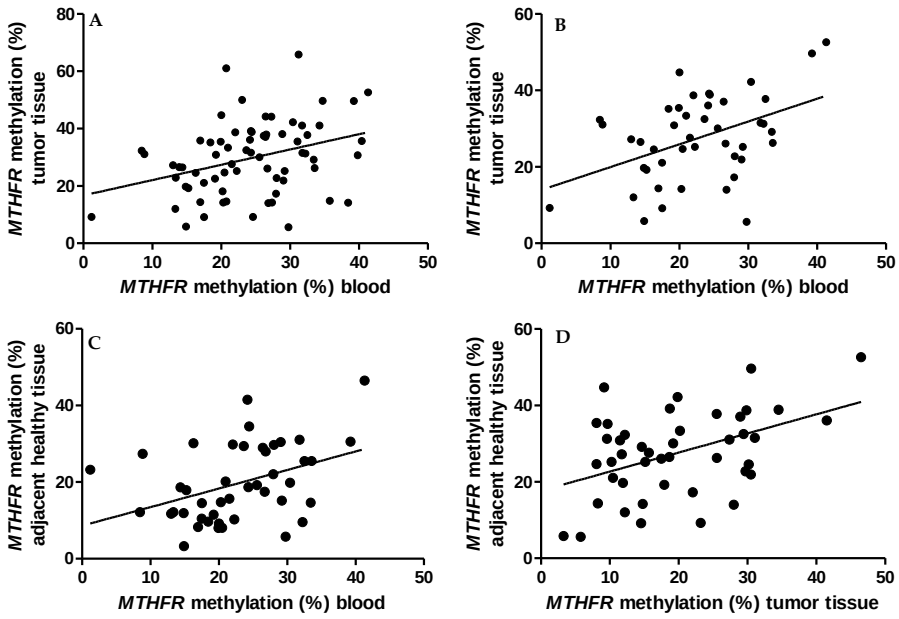

Figure 7. Correlation of *MTHFR* methylation levels between tumor tissue and blood (**A**) in the total population ($r = 0.34$, $P = 0.004$), and (**B**) in the subgroup of 44 patients ($r = 0.44$, $P = 0.003$); (**C**) between adjacent tissue and blood ($r = 0.40$, $P = 0.007$) and (**D**) between tumor and adjacent thymic tissue ($r = 0.45$, $P = 0.002$) in the subgroup of 44 individuals with available thymic tissue adjacent to the tumor. Linear regression analysis was applied (r = Pearson's correlation coefficient).

Regarding *AIRE*, a positive correlation of *AIRE* methylation levels between tumor tissue and blood was observed in the whole population (n = 69, $P = 0.004$, $r = 0.36$; Figure 8A) but not in the subgroup for which the adjacent thymic tissue was available. Moreover, correlation between blood and thymic tissue adjacent to the tumor (n = 44, $P = 0.001$, $r = 0.46$; Figure 8B) was observed.

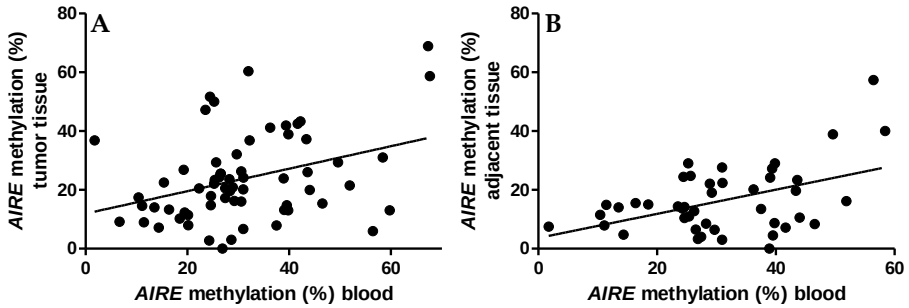

Figure 8. Correlation of *AIRE* methylation levels between (**A**) tumor tissue and blood in the total population (n = 69; *P* = 0.004, *r* = 0.36), and (**B**) between adjacent tissue and blood in the subgroup of 44 individuals with available thymic tissue adjacent to the tumor (n = 44; *P* = 0.001, *r* = 0.46). Linear regression analysis was applied (*r* = Pearson's correlation coefficient).

4.3 Gene expression analysis

Since DNA hypermethylation has been usually associated with gene silencing, during the period (three months) spent in Paris at Center of Research in Myology (Sorbonne Universités, Pierre and Marie Curie University - Paris 6), it was sought to determine whether the methylation levels of some studied gene, such as *MTHFR*, *DNMT3A*, and *AIRE*, correlate with the expression of the relative gene. To determine *MTHFR*, *DNMT3A*, and *AIRE* mRNA levels, we performed a reverse transcription-PCR analysis using tissue from thymoma associate myasthenia gravis patients for which fresh tissue was available.

Correlating DNA methylation levels with gene expression, in the analyzed subgroup, a significant negative correlation between *MTHFR* expression and *MTHFR* methylation in tumor tissue was found (n = 16, *P* = 0.02, r = -0.56; Figure 9). For other genes no significant correlation were noticed (data not shown), let us suppose that other epigenetic mechanisms could influence their expression or that some compensatory mechanisms exist in cancer.

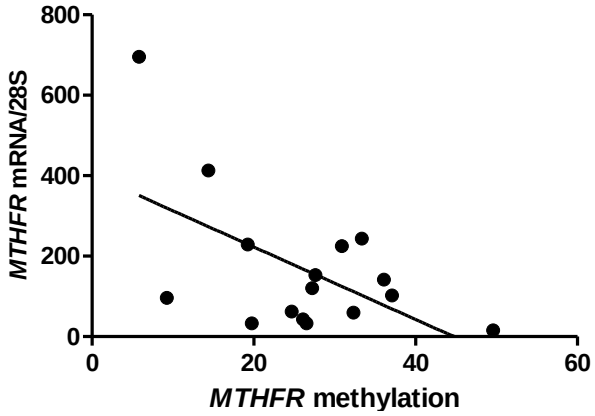

Figure 9. Correlation between *MTHFR* methylation and *MTHFR* expression in tumor tissue (n = 16, *P* = 0.02, *r* = -0.56). Liner regression analysis was applied (*r* = Pearson's correlation coefficient).

4.4 Global methylation analysis

Cancer was described as a pathology characterized by gene specific hypermethylation and global hypomethylation. So in order to assess the global methylation in thymoma-associated myasthenia gravis, the methylation levels of LINE-1 sequences, a highly repeated and widely interspersed human retrotransposon commonly used as a surrogate for global demethylation in tumors, were examined (Chalitchagorn et al., 2004; Estecio et al., 2007). Decreased methylation levels in tumor tissue respect to blood in all TAMG were observed ($P < 0.0001$; Figure 10A). In the subgroup for which the adjacent thymic tissue was available, it was found that the average level of LINE-1 methylation in blood samples and adjacent thymic tissues was 98% and 94%, respectively, whereas it was 83% in thymoma tissues ($P < 0.0001$; Figure 10B), an average loss of 10% of methylation in the tumor, consistent with global demethylation of the tumor cell genome (Chalitchagorn et al., 2004).

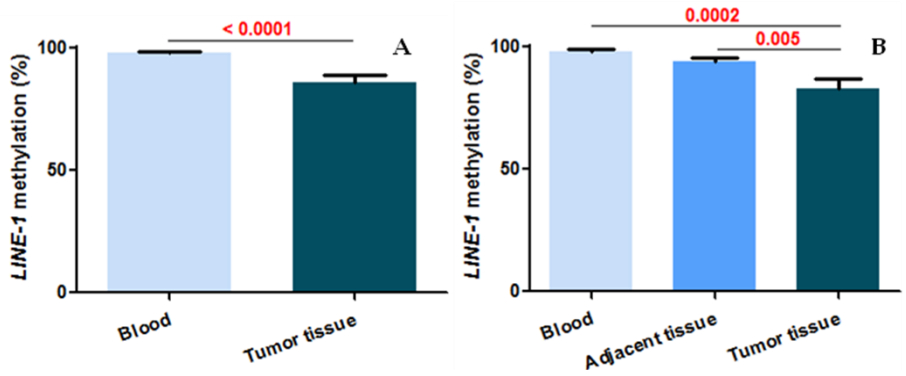

Figure 10. LINE-1 methylation analysis (**A**) in blood and tumor tissue of thymoma-associated MG (TAMG) patients (n = 69) and (**B**) in adjacent thymic tissue of TAMG patients for whom adjacent tissue was available (n = 44). Data on graph correspond to mean±SEM and the significant *P-value* (in red) refers to t-tests for paired samples.

4.5 Correlation among methylation levels in tissues and clinicopathological features

For each of the studied genes the correlation between promoter methylation and both physical and clinicopathological features of the TAMG patients, such as age, gender, histology, Masaoka stage classification, MG onset, and Osserman MG classification in all the sixty-nine TAMG specimens was analyzed (Table 13).

Linear regression analysis revealed that only the h*MLH1* gene shows a positive correlation with age at sampling in blood (n = 69, *P* = 0,009; *r* = 0,31; Figure 11), in particular h*MLH1* methylation levels increase with age, even if they are low. Moreover correlating the clinicopathological features with methylation levels, a correlation between global DNA methylation in tumor tissue, and thymoma histology ($P < 0.0001$), Masaoka classification ($P = 0.03$), and MG Osserman classification ($P = 0.02$), described below by graphics, was observed.

Genes	**Age**	**Gender**	**Histology**	**Masaoka classification**	**MG onset**	**Osserman classification**
***MTHFR* blood**	*P*=0.65; r=-0.05	*P*=0.93	*P*=0.79	*P*=0.59	*P*=0.25	*P*=0.35
***MTHFR* tumor**	*P*=0.98; r=0.001	*P*=0.95	*P*=0.08	*P*=0.68	*P*=0.37	*P*=0.58
***DNMT3A* blood**	*P*=0.17; r=-0.16	*P*=0.96	*P*=0.18	*P*=0.29	*P*=0.82	*P*=0.84
***DNMT3A* tumor**	*P*=0.28; r=0.13	*P*=0.77	*P*=0.32	*P*=0.45	*P*=0.72	*P*=0.81
***DNMT3B* blood**	*P*=0.70; r=-0.05	*P*=0.38	*P*=0.52	*P*=0.97	*P*=0.75	*P*=0.91
***DNMT3B* tumor**	*P*=0.11; r=0.19	*P*=0.68	*P*=0.63	*P*=0.83	*P*=0.80	*P*=0.56
***DNMT1* blood**	*P*=0.72; r=-0.04	*P*=0.85	*P*=0.53	*P*=0.24	*P*=0.62	*P*=0.48
***DNMT1* tumor**	*P*=0.94; r=0.008	*P*=0.57	*P*=0.56	*P*=0.43	*P*=0.14	*P*=0.30
***MGMT* blood**	*P*=0.99; r=0.001	*P*=0.26	*P*=0.78	*P*=0.75	*P*=0.64	*P*=0.90
***MGMT* tumor**	*P*=0.61; r=0.06	*P*=0.89	*P*=0.91	*P*=0.39	*P*=0.67	*P*=0.46
h*MLH1* blood	***P*=0.009**; **r=0.31**	*P*=0.58	*P*=0.78	*P*=0.66	*P*=0.98	*P*=0.76
h*MLH1* tumor	*P*=0.37; r=0.11	*P*=0.34	*P*=0.33	*P*=0.74	*P*=0.08	*P*=0.28
***CDKN2A* blood**	*P*=0.24; r=-0.14	*P*=0.94	*P*=0.22	*P*=0.06	*P*=0.92	*P*=0.72

***CDKN2A* tumor**	*P*=0.47; r=0.08	*P*=0.29	*P*=0.36	*P*=0.47	*P*=0.88	*P*=0.19
***RASSF1A* blood**	*P*=0.08; r=-0.20	*P*=0.69	*P*=0.92	*P*=0.29	*P*=0.84	*P*=0.44
***RASSF1A* tumor**	*P*=0.34; r=-0.11	*P*=0.78	*P*=0.35	*P*=0.62	*P*=0.87	*P*=0.11
***AIRE* blood**	*P*=0.09; r=0.20	*P*=0.57	*P*=0.29	*P*=0.44	*P*=0.02	*P*=0.36
***AIRE* tumor**	*P*=0.91; r=0.01	*P*=0.61	*P*=0.49	*P*=0.26	*P*=0.14	*P*=0.39
***LINE-1* blood**	*P*=0.47; r=0.08	*P*=0.96	*P*=0.62	*P*=0.11	*P*=0.07	*P*=0.19
***LINE-1* tumor**	*P*=0.06; r=-0.23	*P*=0.96	***P*<0.0001**	***P*=0.03**	*P*=0.93	***P*=0.02**

Table 13. Correlation among methylation levels of the studied genes and age, gender, thymic histology, Masaoka classification, MG onset, MG Osserman classification in blood and tumor tissue of all TAMG patients (n = 69). The effect of clinical and pathological features on mean methylation levels was assessed by means of analysis of variance (ANOVA), including age at sampling and gender as covariates. Linear regression analysis was performed to search for a correlation between age and methylation levels in the blood and tumor tissue (*r* = Pearson's correlation coefficient).

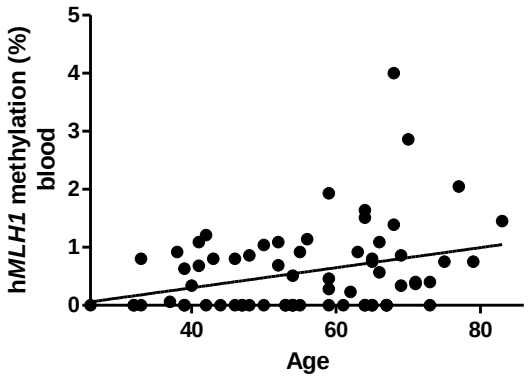

Figure 11. Correlation between age at sampling and h*MLH1* methylation levels in blood (n = 69, *P* = 0,009; *r* = 0,319. Linear regression analysis was applied classification (*r* = Pearson's correlation coefficient).

As indicated above, LINE-1 methylation levels in tumor tissue correlated with thymoma histology, in particular type B (B1, B2, and B3) thymomas showed higher methylation levels respect to type A and AB thymomas ($P < 0.0001$; Figure 12), and with thymoma Masaoka stage classification, with lower methylation levels in stage IIa respect to stages IIb, III, and IVa ($P = 0.03$; Figure 13).

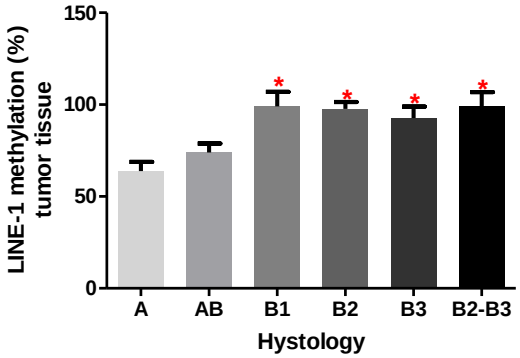

Figure 12. Correlation between LINE-1 methylation and thymoma histology in tumor tissue of all TAMG patients (n = 69). Data on graph correspond to mean+SEM. *P-value* refers to ANOVA test including age at sampling and gender as covariates.

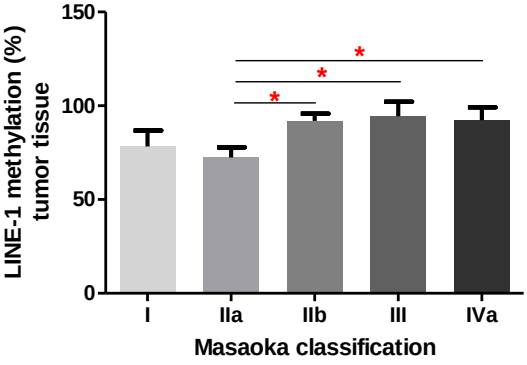

Figure 13. Correlation between thymoma Masaoka stage classification and LINE-1 methylation in tumor tissue of all TAMG patients (n = 69). Data on graph correspond to mean+SEM. *P-value* refers to ANOVA test including age at sampling and gender as covariates.

Moreover, LINE-1 methylation in tumor tissue correlates also with Osserman classification of myasthenia gravis, in particular stage IIB shows higher methylation levels than stages IIA and III ($P = 0.02$; Figure 14).

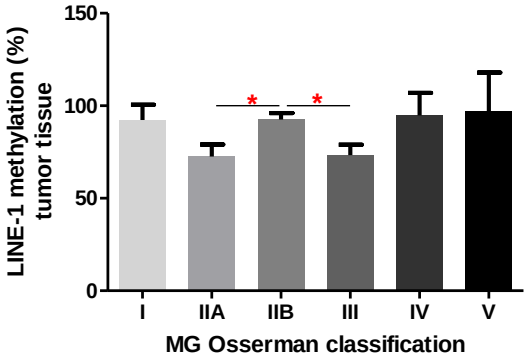

Figure 14. Correlation between MG Osserman classification and LINE-1 methylation in tumor tissue of all TAMG patients (n = 69). Data on graph correspond to mean+SEM. *P-value* refers to ANOVA test including age at sampling and gender as covariates.

4.6 Correlation among methylation levels and one-carbon metabolism genes

Another aim of the study was to understand if polymorphisms in folate metabolism genes could influence the methylation status of studied genes. So in the population composed by sixty-nine TAMG patients, the correlation among the methylation levels of the studied genes and analyzed polymorphisms was evaluated. All *P-values*, obtained by ANOVA analysis including age at sampling and gender as covariates, were referred in Table 14.

Genes	***DNMT3B* -579G>T**	***DNMT3B* -149C>T**	***MTHFR* 677C>T**	***TYMS* 28bp**	***MTRR* 66A>G**	***MTR* 2756A>G**
***MTHFR* blood**	*P*=0.73	*P*=0.37	*P*=0.53	*P*=0.85	*P*=0.49	*P*=0.91
***MTHFR* tumor**	*P*=0.11	*P*=0.27	*P*=0.69	*P*=0.60	***P*=0.02**	*P*=0.40
***DNMT3A* blood**	*P*=0.36	*P*=0.31	*P*=0.78	*P*=0.73	*P*=0.24	*P*=0.90
***DNMT3A* tumor**	*P*=0.74	*P*=0.45	*P*=0.17	*P*=0.06	*P*=0.89	*P*=0.41
***DNMT3B* blood**	***P*=0.01**	***P*=0.007**	*P*=0.20	*P*=0.29	*P*=0.95	*P*=0.76
***DNMT3B* tumor**	***P*=0.02**	***P*=0.001**	*P*=0.32	*P*=0.23	*P*=0.85	*P*=0.18
***DNMT1* blood**	*P*=0.85	*P*=0.93	*P*=0.48	*P*=0.78	***P*=0.03**	*P*=0.73
***DNMT1* tumor**	*P*=0.86	*P*=0.71	*P*=0.69	*P*=0.90	*P*=0.36	*P*=0.46
***MGMT* blood**	*P*=0.28	*P*=0.56	*P*=0.68	*P*=0.38	*P*=0.14	*P*=0.47
***MGMT* tumor**	*P*=0.39	*P*=0.22	*P*=0.56	*P*=0.71	*P*=0.39	*P*=0.82
h*MLH1* blood	*P*=0.23	*P*=0.38	*P*=0.30	*P*=0.65	*P*=0.48	*P*=0.41
h*MLH1* tumor	*P*=0.05	***P*=0.01**	*P*=0.70	*P*=0.25	***P*=0.0004**	*P*=0.45
***CDKN2A* blood**	*P*=0.21	*P*=0.82	*P*=0.15	*P*=0.52	*P*=0.28	*P*=0.98
***CDKN2A* tumor**	*P*=0.48	*P*=0.59	*P*=0.63	*P*=0.69	*P*=0.75	*P*=0.73
***RASSF1A* blood**	***P*=0.02**	*P*=0.42	*P*=0.83	*P*=0.66	*P*=0.29	*P*=0.08
***RASSF1A* tumor**	***P*=0.04**	***P*=0.004**	*P*=0.09	*P*=0.77	*P*=0.72	*P*=0.07
***AIRE* blood**	*P*=0.38	*P*=0.17	*P*=0.34	*P*=0.43	*P*=0.06	*P*=0.06
***AIRE* tumor**	*P*=0.16	*P*=0.76	*P*=0.46	*P*=0.52	*P*=0.59	*P*=0.06
***LINE-1* blood**	*P*=0.55	*P*=0.58	*P*=0.47	*P*=0.28	*P*=0.19	*P*=0.76
***LINE-1* tumor**	*P*=0.25	*P*=0.55	*P*=0.33	*P*=0.38	*P*=0.60	*P*=0.07

Table 14. Correlation among the methylation levels of the studied genes and analyzed polymorphisms. *P-value* refers to ANOVA test including age at sampling and gender as covariates.

We observed a statistically significant correlation between *MTHFR* methylation in tumor tissue and *MTRR* 2756A>G polymorphism, in particular *MTRR* 2756AG genotype shows lower methylation levels (P = 0.02; Figure 15).

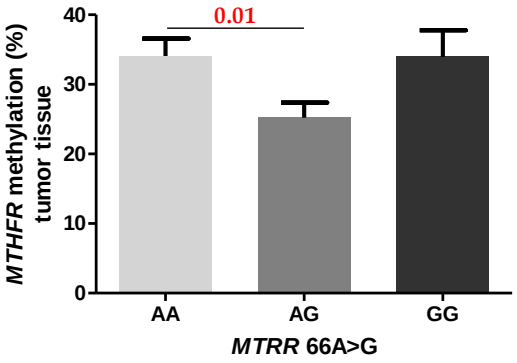

Figure 15. Correlation between *MTHFR* methylation levels in tumor tissue and *MTRR* 66A>G polymorphism. *P-value* refers to ANOVA test including age at sampling and gender as covariates.

DNMT3B methylation levels correlate with *DNMT3B* -579G>T and *DNMT3B* -149C>T promoter polymorphisms both in blood and in tumor tissue. Patients with mutant genotype *DNMT3B* -579TT show higher *DNMT3B* methylation levels both in blood (P = 0.01; Figure 16A) and in tumor (P = 0.02; Figure 16B). Also *DNMT3B* -149TT mutant genotype was associated with higher *DNMT3B* methylation levels both in blood (P = 0.007; Figure 17A) and in tumor tissue (P = 0.001; Figure 17B).

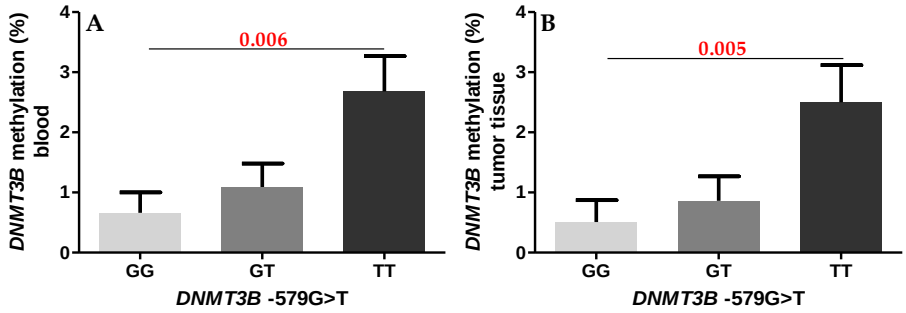

Figure 16. Correlation between *DNMT3B* methylation levels in tumor tissue and *DNMT3B* -579G>T polymorphism (**A**) in blood and (**B**) in tumor tissue. *P-value* refers to ANOVA test including age at sampling and gender as covariates.

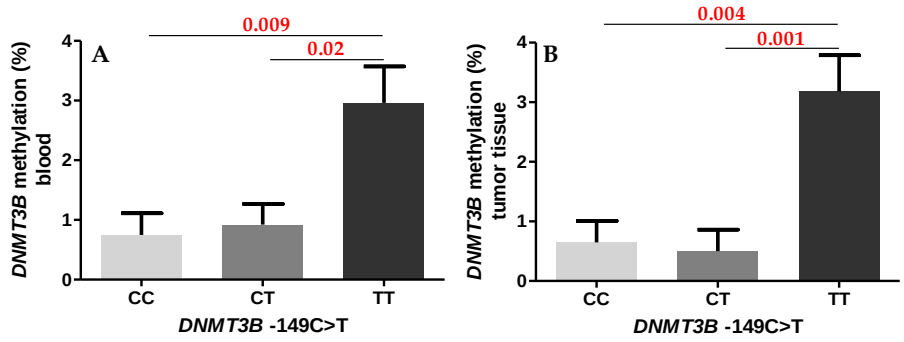

Figure 17. Correlation between *DNMT3B* methylation levels in tumor tissue and *DNMT3B* -149C>T polymorphism (**A**) in blood and (**B**) in tumor tissue. *P-value* refers to ANOVA test including age at sampling and gender as covariates.

Blood *DNMT1* methylation correlates with *MTRR* 66A>G genotype, in particular *MTRR* 66GG mutant genotype shows lower methylation levels respect to wild type ($P = 0.03$; Figure 18).

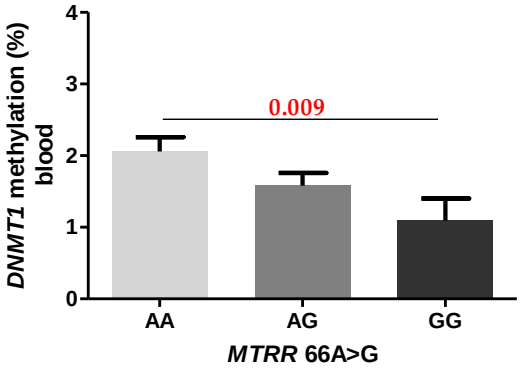

Figure 18. Correlation between *DNMT1* methylation levels in tumor tissue and *MTRR* 66A>G polymorphism. *P-value* refers to ANOVA test including age at sampling and gender as covariates.

h*MLH1* methylation in tumor tissue correlates significantly with *DNMT3B* -149C>T ($P = 0.01$; Figure 19A) and *MTRR* 66A>G ($P = 0.004$; Figure 19B) polymorphisms. Both *DNMT3B* -149TT and *MTRR* 66GG genotypes show higher methylation levels respect to wild type and heterozygous genotypes.

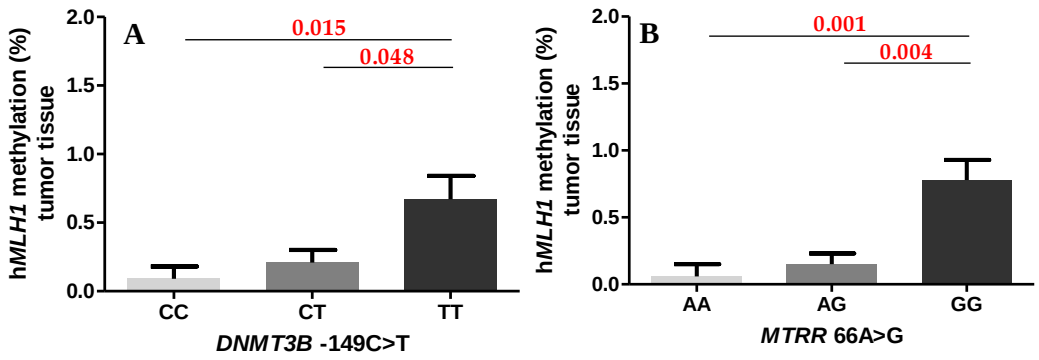

Figure 19. Correlation between h*MLH1* methylation levels in tumor tissue and (**A**) *DNMT3B* -149C>T and (**B**) *MTRR* 66A>G polymorphism. *P-value* refers to ANOVA test including age at sampling and gender as covariates.

Chapter 5: Discussion

In the present study, in order to shed light on the contribution of DNA methylation to the development of thymoma associated myasthenia gravis (TAMG), we investigated the methylation levels of genes involved in DNA methylation reactions (*DNMT1*, *DNMT3A*, *DNMT3B*), in one-carbon metabolism (*MTHFR*), in autoimmune regulation (*AIRE*) and key cancer genes, such as genes of DNA repair (*MGMT*, h*MLH1*), and cell cycle (*CDKN2A*, *RASSF1A*), in blood and tumor tissue DNA from sixty-nine thymoma associated myasthenia gravis patients; for forty-four of them also thymic epithelial tissue adjacent to the tumor one was available. We observed that *MTHFR*, *DNMT3A* and *AIRE* promoters show different methylation levels between blood and tumor tissue in the total population composed of sixty-nine patients, in particular *MTHFR* and *DNMT3A* show higher methylation levels in tumor tissue respect to blood, while *AIRE* show an opposite trend. In the subgroup of 44 patients for whom adjacent thymic specimen was available, different *MTHFR* methylation levels between the adjacent thymic tissue and the tumor one were noticed, in particular tumor tissue showed on average higher methylation levels respect to thymic adjacent one. For *DNMT3A* higher methylation levels in healthy tissue adjacent to tumor with respect to blood were observed. Indeed *AIRE* shows lower methylation levels in tumor tissue respect to blood. All the other studied genes are largely hypometylated both in blood and in tumor tissue, and the mean methylation levels was no higher than 2%. Comparing the methylation levels of the studied genes in different tissues, statistically significant correlations of *MTHFR* promoter methylation levels between blood and tumor tissue, adjacent thymic tissue and blood, and tumor tissue and adjacent tissue were observed; also *AIRE* shows a positive correlation of methylation levels between blood and tumor tissue in all TAMG, and between adjacent thymic tissue and blood in the subgroup.

MTHFR is one of the major enzyme in folate metabolism since it catalyzes the irreversible conversion of 5,10-methylene THF (the methyl donor in the conversion of dUMP to dTMP) into 5-methyl THF, which remethylates homocysteine to methionine, necessary for the formation of S-adenosylmethionine, the universal donor of methyl groups; therefore, this key protein controls whether folate is partitioned towards DNA precursor synthesis or DNA methylation, and an alteration of this enzyme can interfere with the provision of methyl

groups necessary for DNA methylation reactions. It is revealing that the *MTHFR* gene is regulated by promoter methylation and an increased *MTHFR* promoter methylation has been observed in several human diseases, such as cardiovascular and renal diseases (Ghattas et al., 2014; Wei et al., 2015), male infertility (Khazamipour et al., 2009; Wu et al., 2010; Rotondo et al., 2012), and preeclampsia (Ge et al., 2015). Moreover, it is also emerging that *MTHFR* hypermethylation could be involved in cancer formation; in fact, a correlation between *MTHFR* hypermethylation and lung cancer or cervical cancer lesions was observed (Vaissiere et al., 2009; Botezatu et al., 2013). In our cohort we observed higher *MTHFR* methylation levels in tumor tissue with respect to blood and adjacent thymic tissue, and we found a strong correlation between methylation levels in blood and tumor tissue. To our knowledge, this is the first time that *MTHFR* methylation has been analyzed in TAMG. We studied a CpG island previously associated with gene silencing, in fact, in a small subgroup of thymoma tissue an inverse correlation between the methylation levels of this region and gene expression levels was observed, suggesting that hypermethylation of the *MTHFR* gene at the studied CpG sites may have a functional significance and may result in partial or complete silencing of the gene; this data was supported by literature data in lung cancer in which was observed the same negative correlation (Vaissiere et al., 2009). *MTHFR* hypermethylation can increase the availability of 5,10-methylene THF for the synthesis of thymidine and purine, causing the hyper-proliferation of cancerous cells. In this regard, it was suggested that *MTHFR* hypermethylation might confer a growth advantage to cancer cells and contribute to the cancer phenotype in tumors of the upper aero-digestive tract (Mani et al., 2012). Moreover, *MTHFR* promoter methylation levels have been correlated with cancer risk factors and with markers of impaired folate metabolism, including tobacco smoking, low circulating folates and vitamin B12, high homocysteine levels, and increased chromosome instability (Vaissiere et al., 2009; Wei et al., 2015; Tannorella et al., 2015; Coppedè et al., 2016).

The present study revealed a clear involvement of *MTHFR* promoter methylation in TAMG, particularly the higher methylation levels in tumor tissue with respect to other tissues. These findings leads us to suppose that it could be involved in mechanisms associated with the development and progression of cancer, especially in the activation of proto-oncogenes. Obviously other studies need to further explore the significance of *MTHFR* methylation in TAMG, in order to better understand its pathogenic role in the onset of

thymomas. The *MTHFR* protein is also required for the synthesis of DNA precursors, and impairments of this protein could contribute to cancer development by increasing the rate of point mutations and chromosome instability in rapidly dividing cells (Coppedè, 2014). We observed a correlation among *MTHFR* promoter methylation levels in blood, thymic epithelial cells adjacent to the cancer, and thymoma cells that, albeit weak, was statistically significant. Such a correlation could suggest that *MTHFR* methylation levels in blood might reflect those in other tissues, and might represent a peripheral biomarker of the disease. In this regard, it was observed that females with increased *BRCA1* blood methylation have a 3.5-fold increased risk for early-onset breast cancer with histological features commonly seen in tumors arising in women with germline *BRCA1* mutations (Wong et al., 2011). Similarly, glutathione S-transferase 1 (GSTP1) promoter methylation in peripheral DNA is regarded as a potential prognostic marker of prostate cancer (Mahon et al., 2014). However, as far as *MTHFR* promoter methylation in the blood is concerned, it should be noted that rather than being a specific marker of a given disease, it could represent a more general biomarker of increased genomic instability. For example, some studies suggest a correlation between hyperhomocysteinemia and *MTHFR* promoter methylation (Tannorella et al., 2015); others have linked *MTHFR* promoter methylation in blood cells with markers of chromosome damage, such as an increased frequency of micronuclei (Coppedè et al., 2016) or alterations of LINE-1 methylation and stability (Vaissiere et al., 2009), and there is also indication that *MTHFR* promoter methylation in blood DNA might reflect dietary B-group vitamin deficiency (Wei et al., 2015; Grossi et al., 2016) or environmental exposure to cancerogenic agents, such as those deriving from tobacco smoking (Vaissiere et al., 2009). Collectively, those studies suggest that increased *MTHFR* promoter methylation in blood cells might be a more general marker of impaired one-carbon metabolism and genome instability, rather than a specific disease biomarker. An interesting question is how an increased *MTHFR* promoter methylation could be linked to the regulation of autoimmune-related genes in TAMG patients. By the analysis of *AIRE* promoter methylation, it was observed that *AIRE* is hypomethylated in tumor tissue, and also in adjacent thymic tissue, respect to blood. Correlating *AIRE* expression and *AIRE* promoter methylation, no significant correlation was observed. Also in literature data it was observed that the autoimmune regulator gene (AIRE) is hypomethylated in thymomas, and that both DNA and histone tail methylation marks regulate *AIRE* expression (Kont et al., 2011). *AIRE* is a key gene in safe-guarding central

tolerance to a wide variety of tissue-specific antigens and its deregulation could cause a disruption of their expression. It is emerging that transcriptional activation of the *AIRE* promoter could be achieved through separate elements, other than DNA methylation, which can function independently of each other or cooperate in tissue-specific activation or inhibition. mTECs may have cell type specific factors that activate *AIRE* expression by recruiting RNA polymerase to the promoter or enhancers, which locate upstream or downstream of the gene and bind activator proteins (Kont et al., 2011). *AIRE* expression is also regulated by conserved non-coding sequences upstream the gene, activated by nuclear factor-kβ (LaFlam et al., 2015; Haljasorg et al., 2015) and its transcription seems to undergo spatio-temporal regulation, since it is ubiquitously transcribed during the earliest stages of embryogenesis, during which it is restricted to mTECs, extrathymic AIRE-expressing cells, thymic B cells, and becomes upregulated in mature mTEC (Anderson and Su, 2016). *AIRE* expression is also regulated by a post-transcriptional mechanisms, in particular at protein level by the bifunctional arginine demethylase and lysyl-hydroxylase JMJD6, since its loss causes intron 2 retention and a subsequent decrease in *AIRE* protein expression (Yanagihara et al., 2015). Therefore, *AIRE* expression is regulated at many levels, so it is quite difficult to find a functional significance of hypomethylation found in thymic and thymoma tissue, as well as it is difficult to find a strong correlation between *AIRE* promoter methylation and its expression. In a recent study, it is emerged that *AIRE* expression could be influenced by SNPs in the promoter region. *AIRE* -230Y polymorphism is located in a conserved region of the promoter and is adjacent to a predicted WT1 transcription factor binding site, suggesting that *AIRE* -230Y could affect *AIRE* expression. Moreover, this SNP abolishes a CpG methylation site affecting the methylation status of the *AIRE* promoter, in particular this site is adjacent to a CpG site that was found to be differentially methylated in different cell lines. Lower expression was seen in the alleles of *AIRE*-655G*AIRE*-230T haplotype that abolished a CpG site (Lovewell et al., 2015). We correlate *MTHFR* methylation with *AIRE* methylation, in order to understand if increased *MTHFR* promoter methylation could influence *AIRE* methylation but we do not find any significant correlation (data not shown) so we speculated that *MTHFR* methylation could contribute to histone tail methylation changes or DNA methylation of other *AIRE* regulator regions, upstream of the promoter region. However an important issue to take into account is that not always an increased promoter methylation of *MTHFR* causes the demethylation of all the other genes in the same tissue. In lung cancer a

high frequency of aberrant hypermethylation of *MTHFR* and also of *RASSF1A* and *CDKN2A* when compared with control blood samples was revealed (Vaissiere et al., 2009). Therefore, it is not surprising to find different genes which are hypermethylated in cancer together with *MTHFR*. Similarly, in semen DNA of infertile men promoter hypermethylation of both *MTHFR* and *SNRPN* genes was observed (Botezatu et al., 2014). Furthermore, studying the same genes in blood DNA of patients with Alzheimer's disease, recently it was demonstrated that increased *MTHFR* promoter methylation correlates with increased methylation levels of both *de novo* DNMTs, and all these changes were linked to global levels of one-carbon metabolites (Grossi et al., 2016). The relationship between *MTHFR* hypermethylation and hypomethylation of other genes is not so immediate, since in cancer the mechanism remains elusive.

Regarding the *DNMTs*, the writers of DNA methylation reactions, in this study only *DNMT3A* showed significant increased methylation levels in tumor tissue, comparable to those found in adjacent thymic tissue, respect to blood that results completely demethylated; the other two *DNMTs* genes, *DNMT1* and *DNMT3B*, resulted hypomethylated, and even though *DNMT1* shows higher methylation levels in blood respect to tumor tissue, the mean methylation levels were no higher than 2%, so it is not possible to define a possible involvement of them in thymoma associated myasthenia gravis. The methylation of these genes was not yet investigated in thymomas but a previous study showed that they were overexpressed in the advanced stages of thymic epithelial tumors with respect to early stages (Chen et al., 2009). A double role of *de novo* DNMTs in tumor stages, as oncogenes and tumor suppressor genes, was proposed. In healthy tissues, tumor suppressor genes are unmethylated and oncogenes are methylated. In early tumor stages, DNMTs seem to lead to methylation-associated repression of tumor suppressor genes and to promote tumor initiation, while in advanced tumor stages the downregulation of *de novo* DNMTs seems to be associated with promoter DNA hypomethylation of specific oncogenes and, consequently, would promote tumorigenesis (Fernandez et al., 2012). Concerning the *DNMT3A* gene, it shows higher methylation levels in both cancer tissue and adjacent thymic tissue, where almost the same levels, have been found with respect to blood, suggesting that its methylation is not connected exclusively with tumor events but already shows alterations in healthy tissue. Aberrant *DNMT3A* methylation was also observed in other cancers, such as in acute myeloid leukaemia (Jost et al., 2014) and in breast cancer, where *DNMT3A* expression

is associated with advanced stages (Santos et al., 2015). Studies performed in mice with conditional knockout of *Dnmt3a* revealed a role for DNA methylation in mediating the self-renewal and differentiation of normal hematopoietic stem cells and leukaemia stem cells (Mayle et al., 2015). A functional role of this gene in thymic epithelial tumors emerged from studies that focused on mutation analysis, revealing that *DNMT3A* is one the most frequently mutated genes in thymic carcinoma (Wang et al., 2014), and that the mutation p.G728D is associated with B3 thymomas (Belani et al., 2014). Also for *DNMT3A* we did not find a correlation between gene promoter methylation and gene expression, probably due to the small number of analysed specimens but also due to different levels of gene regulation. It was observed that the overexpression of UHRF1/2 factors, key epigenetic regulators essential for DNA maintenance methylation, in cancers could inactivate or suppress DNMT3A activity in cancers, which may in turn promote tumorigenesis through consequent DNA hypomethylation (Jia et al., 2016).

For about the other studied genes involved in DNA repair, *MGMT* and h*MLH1*, and cell cycle, *CDKN2A* and *RASSF1A*, they are largely hypomethylated in our TAMG population. Only h*MLH1* and *MGMT* showed higher methylation levels in tumor respect to blood but their methylation levels were very low, no higher than 2%. The methylation status of these key cancer genes has been widely studied in other types of cancer, and usually increased methylation levels correlating with cancer were found. *MGMT* encodes O-6-methylguanine-DNA methyltransferase, a DNA repair protein that removes mutagenic and cytotoxic adducts from O^6-guanine in DNA, the major carcinogenic lesion in DNA induced by alkylating mutagens. Alkylation of DNA at the O^6 position of guanine is an important step in the appearance of mutations in cancer, primarily due to the tendency of the O^6-methylguanine to pair with thymine during replication, resulting in G:C to A:T transition. O^6-alkylguanine DNA adduct may also cross-link with the complementary cytosine residues, blocking DNA replication. MGMT protects cells against these lesions, transferring the alkyl group from the O^6-guanine in DNA to an active cysteine within its own sequence in a reaction that inactivates one MGMT molecule for each lesion repaired (Jacinto and Esteller, 2007). *MGMT* promoter hypermethylation, causing the gene silencing, can lead to an important mutator pathway in human cancer because the O^6-methylguanine adducts are not removed, driving G:C to A:T transitions. Also h*MLH1* gene is involved in DNA repair, in fact, it is a member of the mismatch repair genes. The protein repairs mistakes made during

DNA replication in preparation for cell division, replacing the section of DNA that contains mistakes with a new corrected DNA sequence. *RASSF1A* and *CDKN2A* are two genes involved in cell cycle and act as tumor suppressor genes, in particular, *RASSF1A* is functionally involved in different cell processes including cell cycle control, microtubule stabilization, cellular adhesion and mortality, as well as apoptosis. It interacts with CDC20, an activator of the anaphase-promoting complex (APCCdc20) that initiates chromatid separation and entrance into anaphase. The APCCdc20 protein complex targets securin for its destruction, enabling sister chromatid separation. It also targets S and M-phase (S/M) cyclins for destruction, which inactivates S/M cyclin-dependent kinases (Cdks) and allows the cell to exit from mitosis. *CDNK2A* gene generates several transcript variants which differ in their first exons, one of which contains an alternative open reading frame that specifies a protein that functions as a stabilizer of the tumor suppressor protein TP53 because it can interact with, and sequester, the E3 ubiquitin-protein ligase MDM2, a protein responsible for the degradation of TP53. It has an important role in cell cycle inducing G2 arrest and apoptosis in a TP53-independent manner by preventing the activation of cyclin B1/CDC2 complexes. It acts as tumor suppressor inducing cell cycle arrest in G1 and G2 phases. In thymic epithelial tumors, promoter methylation of the *CDKN2A* gene was reported in up to 11% of thymomas and 25% of thymic carcinoma (Hirabayashi et al., 1997), and immunohistochemical analysis of TETs demonstrated copy number loss of *CDKN2A* accompanied by lack of expression of its related protein and identified tumors with poor prognosis (Petrini et al., 2012). The methylation frequency of *CDKN2A* and *MGMT*, promoters was significantly lower in thymomas (29%) than in thymic carcinomas (86%), and the frequency of tumors with methylation of multiple genes in thymic carcinoma was higher than in thymoma (60% vs 20%). In particular, *MGMT* methylation was detected only in 23% of thymoma and in 83% of thymic carcinoma (Hirose et al., 2009). Similar data was observed in another study, in which *MGMT* methylation (74% vs 29%) and loss of its protein expression (87% vs 23%) have been reported to be significantly more frequent in thymic carcinoma than in thymoma and in advanced of thymic epithelial tumors (stages III/IV) compared with early stage tumors (stages I/II) (Mokhtar et al., 2014). By these data emerged that the analyzed genes are deregulated more in thymic carcinoma that in thymomas, therefore we could hypothesize that in thymoma associated myasthenia gravis they are active and are free to act as DNA repair or tumor suppressor genes. The cited studies do not

analyze the specific methylation levels of genes but use a technique that indicates if the gene is methylated or not, so the differences between our findings and those of papers in literature could be due to different techniques or to the different origin of populations object of the studies. In fact all studies were performed on Asiatic population that could have different predisposition respect to Caucasian population.

Cancer was described as a pathology characterized by gene specific hypermethylation and global hypomethylation. Gene-specific hypermethylation can lead to transcriptional silencing, for examples of tumor suppressor genes, and global hypomethylation can contribute to chromosomal instability, reactivation of transposable elements, and loss of imprinting. Both, hypo- and hypermethylation may facilitate tumorigenesis. So in order to assess the global methylation in thymoma-associated myasthenia gravis, we examined the methylation levels of LINE-1 sequences, a highly repeated and widely interspersed human retrotransposon commonly used as a surrogate for global demethylation in tumors (Chalitchagorn et al., 2004; Estecio et al., 2007). In all TAMG, LINE-1 were hypomethylated in tumor tissue respect to blood and in the subgroup for which the adjacent thymic tissue was available; we found that the average level of LINE-1 methylation in blood samples and adjacent thymic tissues was 98% and 94%, respectively, whereas it was 83% in thymoma tissues, an average loss of 10% of methylation in the tumor, consistent with global demethylation of the tumor cell genome (Chalitchagorn et al., 2004). Moreover we found a correlation between tumor LINE-1 methylation and clinicopathological features, such as histology, Masaoka stage, and MG Osserman classification, in particular type B (B1, B2, and B3) thymomas show higher methylation levels respect to type A and AB thymomas, thymoma stage IIa shows lower methylation levels respect to stages IIb, III, and Iva; regarding MG Osserman classification, stage IIB shows higher methylation levels than stages IIA and III. LINE-1 are active in the germ line and during embryogenesis, yet they are epigenetically suppressed in somatic cells. However, in line with LINE-1 hypomethylation during tumorigenesis, LINE-1 can be reactivated and may have a role in genome instability, participating in the pathological processes of cancer initiation and progression (Xiao et al., 2015). Tumor LINE-1 hypomethylation is intensively studied and has been observed in almost all human cancer types (Portela and Esteller, 2010; Barchitta et al., 2014). In normal cells, DNA methylation plays important roles in X-chromosome inactivation, genomic imprinting, and repression of repetitive elements, such as retrotransposons and endogenous

retroviruses. Therefore, global hypomethylation may associate with tumor malignancy through a variety of mechanisms, that include activation of proto-oncogenes, endogenous retroviruses, or transposable elements, transcriptional dysregulation and chromosomal instability (Eden et al., 2003; Barchitta et al. ,2014). Also in other type of cancer LINE-1 hypomethylation correlates with a worse prognosis, for example in ovarian cancer, where LINE-1 hypomethylation correlated with worse prognosis and survival and in a well genetically and clinically characterized group of ovarian cancers (Notaro et al., 2016). In colon cancer was observed that patients with LINE-1 hypomethylated tumors had a significantly worse overall survival compared to patients with a higher level of LINE-1 tumor DNA methylation (Swets et al., 2016). Moreover, in colorectal cancer, tumor LINE-1 methylation levels correlate with tumor stage; a decrease in LINE-1 methylation, resulting in hypomethylation, was associated with more advanced disease stages (Baba et al., 2010; Sunami et al., 2011). More recently was observed also a correlation between plasma cell free DNA (cfDNA) and LINE-1 hypomethylation index, in particular colorectal cancer patients with large tumors, advanced stage, and distant metastasis had statistically significantly higher cfDNA LINE-1 hypomethylation index than healthy donors, suggesting that it could be used as prognostic biomarker (Nagai et al., 2017).

By correlating the clinicopathological features with the methylation status of all other genes, an interesting association between aging and h*MLH1* increased methylation in blood was observed. Gene specific methylation of h*MLH1* has been just associated with aging in some types of cancer (Menigatti et al., 2009; Tserga et al., 2012). This gene is involved in DNA repair processes and its methylation might reflect an age related decline of DNA repair capabilities. It is well known that global hypomethylation and CpG island hypermethylation are accumulated during aging and might contribute to tumor progression (Fraga and Esteller, 2007). During the last years an increasing number of genes has been reported to be affected by methylation changes during aging, that might depend on different environmental or genetic factors acting during senescence (Calvanese et al., 2009). For example during aging the increase of reactive oxygen species (ROS) causes an enhancement of oxidative stress damaging tissue; DNMTs and HDACs complexes may be recruited at damage of DNA, causing a delocalization of epigenetic key enzymes that leads to global or local epigenetic alterations of cancer cells (O'Hagan et al., 2011). External factors are known to contribute to epigenetic alterations during aging. For instance, several studies have shown

that the DNA methylome could be directly altered by diet, xenobiotic chemicals, and exogenous stimuli, such as inflammation and viral/bacterial infection (Berdasco and Esteller, 2012). It can be assumed an interaction of genetically determined and environmentally induced factors affecting epigenetic patterns which in turn are correlated with the differences in inter-individual susceptibility to functional decline and vulnerability to diseases in the elderly people. Whether the aging phenotypes induce the epigenetic changes or vice versa remain an open question that needs further investigations (D'Aquila et al., 2013).

By comparing genotype and allele frequencies between myasthenic patients with and without thymoma, we observed a significant decrease in *TYMS* 28bp 2R allele frequency in patients with thymoma with respect to normal thymus. Moreover, the *TYMS* 28bp 2R2R was associated with decreased risk to develop a thymoma among AChR+ MG patients. Unfortunately, adjusting the OR for age and gender the statistical significance became borderline. No other statistically significant differences for other genes were found. Thymidylate synthetase is a key enzyme in the folate metabolism, in fact it catalyzes the deoxyuridine monophosphate conversion for deoxythymidine monophosphate process of DNA synthesis. The conversion is essential for the production of thymine, a nucleotide required for DNA repair and synthesis. The enzyme has become of interest as a target for cancer chemotherapeutic agents, in fact, it is considered to be the primary site of action for 5-fluorouracil, 5-fluoro-2-prime-deoxyuridine, and some folate analogs. In thymoma thymidilate synthase mRNA levels were significantly higher in the tumor tissue specimens than in the normal adjacent thymus ones (Sasaki et al., 2003). Among tumor tissue, *TYMS* mRNA expression levels were significantly higher in thymomas compared to thymic carcinoma; specifically TYMS levels were significantly decreased from B1-type thymomas to B3-type and carcinoma, and this decrease correlated linearly with protein expression levels in all cases. Very low *TYMS* levels were also detected in A thymomas, while AB thymomas showed intermediate expression levels (Monica et al., 2013). Individuals that are homozygous for the 3R were found to have elevated intra-tumoral *TYMS* mRNA, in fact i*n vitro* 3.6 times less mRNA was shown, and tumor tissue with the 2R/3R genotype has been shown to produce significantly less cellular thymidilate synthase protein than that with a 3R/3R (Horie et al., 1995; Kawakami et al., 1999; Pullarkat et al., 2001). High level TYMS expression is related to an aggressive tumor phenotype and a poor outcome in a variety of malignant tumors. Polymorphisms in *TYMS* influencing enzyme activity could affect plasma

folate levels and, thereby indirectly, plasma homocysteine levels, since TYMS competes with MTHFR for the availability of 5,10-methylenetetrahydrofolate (5,10-MTHF). Increased enzyme activity due to the presence of 3R/3R genotype could remove substrate to *MTHFR* resulting in increased homocysteine levels; this was demonstrated by a study in which *TYMS* 3R/3R genotype was associated with reduced plasma folate and, among individuals with low dietary folate intake, with elevated plasma homocysteine levels, suggesting that *TYMS* and *MTHFR* compete for limited supplies of folate required for the remethylation of homocysteine (Trinh et al., 2002). In acute lymphoblastic leukemia the *TYMS* 2R2R genotype was associated with a lower total leukocyte count, smaller percentage of blasts, and more adequate platelet count compared to 2R3R and 3R3R genotypes, suggesting that *TYMS* 5'-UTR 28bp tandem repeat reduces risk also for acute lymphoblastic leukemia (Dunna et al., 2014). We could suppose that in thymoma a decreased activity of *TYMS* decelerate the cancer process, reducing the proliferation of rapidly diving cancer cells; anyway to address the real contribute of *TYMS* in TAMG, a larger population need to be analyzed.

Polymorphisms of gene involved in one-carbon metabolism could affect DNA methylation, in fact we found a statistically significant correlations between the *MTRR* 66A>G polymorphism and *MTHFR* and h*MLH1* promoter methylation in tumor tissue and *DNMT1* in blood, between the *DNMT3B* -579TT genotype and higher promoter methylation levels of *DNMT3B* and h*MLH1* in tumor tissue and blood, the same happens for *DNMT3B* -149TT genotype related to *DNMT3B* methylation in thymoma tissue and blood.

Some studies analyzed the contribution of one-carbon metabolism gene polymorphisms on cancer risk, although showing different and contrasting genetic-epigenetic interactions. We observed that *MTRR* 66AG genotype shows significant lower *MTHFR* methylation respect to wild-type genotype in thymoma tissue, and that *MTRR* 66GG genotype is associated to lower *DNMT1* methylation respect to wild-type genotype in blood. An inverse tendency was observed correlating *MTRR* 66A>G with h*MLH1* promoter methylation, in particular the *MTRR* 66GG mutant genotype shows higher methylation levels respect to wild-type and heterozygous genotypes. MTRR is a crucial enzyme of the homocysteine/methionine metabolic pathway required for conversion of the inactive form of MTR to its active form, and the *MTRR* 66 A>G polymorphism is located in the FMN-binding domain of the MTRR enzyme that is suggested to interact with MTR. This polymorphism is reported to have decreased enzyme affinity for MTR, and it is associated to reduced enzymatic activity, that

result in the reduction of DNA methylation (Olteanu et al., 2002). The reduced *MTHFR* and *MTR*/*MTRR* efficiency may affect SAM production and therefore DNA methylation, on the other hand these reduced activity might favor DNA synthesis and repair, thus preventing DNA breaks. A study observed that variation in both maternal and infant genotype at the *MTRR* locus resulted in changes in infant methylation, providing evidence that aberrations at this point of one carbon metabolism might affect the capacity to methylate DNA (McKay et al., 2012). Epidemiological and case-control studies have reported the *MTRR* 66 A>G polymorphism to be associated with several cancers (Han et al., 2012). More recently, higher LINE-1 methylation (about 3%) in the breasts of women carrying at least one variant allele of the *MTR* 2756A>G and *MTRR* 66 A>G polymorphism compared to those carrying the more common genotypes of these SNPs was found, suggesting a protective effect in breast cancer associated with carrying the variant G alleles of these SNP (Llanos et al., 2015). *MTRR* 66GG genotype was also associated with decreased leukemia risk in the Caucasian population, in children and for acute lymphoblastic leukemia; in contrast, increased risk was observed in the Asian population and for acute myeloid leukemia (Fang et al., 2014). In a study *MTHFR*, *MTR*, and *DNMT3B* polymorphisms were found associated with colorectal cancer risk and rare variants of *MTR* and *MTRR* may reduce promoter hypermethylation (de Vogel et al., 2009). So we could speculate an effect of this SNP on *MTHFR*, *DNMT1* and h*MLH1* promoter methylation in thymoma associated myasthenia gravis, as a reduced MTRR activity causes a reduction of SAM availability necessary for DNA methylation reaction, even if we need to be careful for *DNMT1* and h*MLH1* because their methylation level we found are very low, no higher than 2% for *DNMT1* and no higher than 1% for h*MLH1*.

DNA methylation is largely depending from the availability of one-carbon nutrients and is mediated by DNA methyltransferases. Among them, DNMT3B, a *de novo* methyltrasferase, play an important role in the generation of aberrant methylation. We found that *DNMT3B* -579 G>T and *DNMT3B* -149 C>T promoter polymorphisms are associated with *DNMT3B* higher methylation levels both in blood and in thymoma tissue, and that *DNMT3B* -149TT genotype is associated to higher h*MLH1* promoter methylation levels in tymoma tissue, even if the methylation levels are no higher than 2% and 1% for *DNMT3B* and h*MLH1*, respectively.

In thymomas, a statistically significant association of the *DNMT3B* -579T allele and the TT homozygous genotype with the risk of thymoma respect to healthy controls, was found

(Coppedè et al., 2013). *DNMT3B* promoter polymorphisms have been reported to be associated with the risk of malignant solid tumors, in fact mutant allele (T) carriers have been shown to have a significant increase cancer risk, even if the results remain controversial, depending on different ethnicity, tumor types, and study designs (Zhu et al., 2012a; Zhang et al., 2015; Zhu et al., 2015; Gao et al., 2016; Khoram-Abadi et al., 2016). Moreover, *DNMT3B* promoter polymorphisms have been associated with global DNA methylation and are increasingly investigated in other complex diseases than cancer (Nam et al., 2010; Fonseca-Silva et al., 2012; Coppedè et al., 2013; Murphy et al., 2013; Gouda et al., 2016). Several studies observed a correlation between *DNMT3B* promoter polymorphisms, DNMT3B protein levels, and DNA methylation levels, suggesting that the increased risk of cancer observed in carriers of those polymorphisms might be due to impairments of DNA methylation in cancer cells (Bao et al., 2011; Zhu et al., 2012a; Naghibalhossaini et al., 2015). Therefore, alterations in folate metabolism genes could deregulate one-carbon metabolism and affect downstream mechanisms, which may initiate and promote the development of cancer.

Conclusions

From the DNA methylation analyses performed in this study, the involvement of an alteration of the methylation pattern, for specific genes (*MTHFR*, *DNMT3A*, *AIRE*), and of a global hypomethylation in thymoma-associated myasthenia gravis has been proven, although we are aware that other studies are necessary to assess a potential pathogenic role of these changes in TAMG. Moreover, a contribution of DNA promoter methylation on gene expression, expecially for *MTHFR*, emerged. The fact that for the other two genes analyzed (*DNMT3A* and *AIRE*), the correlation between methylation and gene expression was not found, let us to suppose that mechanisms other than the methylation of specific CpG islands of the promoter could be involved in the regulation of gene expression. Furthermore it should be considered that cancer is not a homogeneous disease but rather a heterogeneous disorder and that each tumor exhibits a "unique" pattern of epigenetic alterations (a sort of "epigenetic signature"). By these data a small contribute of polymorphisms of genes involved in folate metabolism gene also emerged, leaving space to suppose a potential correlation between thymomas and environmental factors, such as diet. However to date it is quite difficult to clarify the real contribute of this complex pathway on thymoma associated myasthenia gravis pathogenesis.

The major limit of this study is the lack of a comparison between TAMG samples and thymomas obtained from individuals without MG. This aspect needs to be addressed in a forthcoming deepening of the study, as it could help to clarify if the observed methylation changes are TAMG-specific, or rather common in thymomas.

Anyway, it is desirable that the analysis of the epigenetic changes is implemented in cancer, and specifically in thymoma associated to MG, since it is emerging that the epigenetic signatures offer not only the possibility of diagnosing a pathological condition and of providing a classification of cancer subtypes and monitoring over time but also of having a predictive potential for drug treatment efficiencies.

List of abbreviations

5caC: 5-carboxylcytosine
5fC: 5-formylcytosine
5-FU: 5-fluorouracil
5hmC: 5-hydroxymethylcytosine
5mC: 5-methylcytosine
ABCA1: ATP-binding cassette, subfamily a, member 1
ACh: acethylcoline
AChR: acethylcoline receptor
AIRE: autoimmune regulator
ALK: anaplastic lymphoma kinase
ALL: acute lymphoblastic leukemia
APC: adenomatous polyposis coli
APECED: autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
APS1: autoimmune polyglandular syndrome type 1
ASXL1: additional sex combs-like 1
ATF7IP-MBD1: activtating transcription factor 7-interactin protein methyl-CpG-binding domain protein 1
ATM: ataxia-telangiectasia mutated
BAFF: B cel activating factor
BAP1: baculoviral iap repeat-containing protein 3
BCOR: BCL6 corepressor
BER: base excision repair
BIRC3: BRCA1-associated protein 1
Casp 2: contactin-associated pratein 2
CA-SSR-1: non-coding CA simple sequence repeat 1
CCL12: chemokine, cc motif, ligand 1
CDH1: cadherin
CDK: cyclin-dependent kinase
CDKN2A: cyclin-dependent kinase inhibitor 2a
CGI: CpG island
CLC: charcot-leyden crystal protein
CN: copy number
Col2a1: collagen, type ii, alpha-1
Csk: c-src tyrosine kinase
cTEC: cortical thymic epithelial cell
CTLA4: cytotoxic t lymphocyte-associated 4
CXCL13: chemokine, cxc motif, ligand 13
CYLD: CYLD gene
DAPK: death-associated protein kinase 1

DHF: dihydrofolate
DNMT: DNA methyltrasferase
dTMP: deoxythymidine monophosphate
dUMP: deoxyuridine monophosphate
EBV: epstein barr virus
EGFR: epidermal growth factor receptor
EOMG: early-onset myasthenia gravis
ERBB4: v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4
ETF1: eukaryotic translation termination factor 1
Fabp9: fatty acid-binding protein 9
FBN3: fibrillin 3
FEZF2: fez family zinc finger protein 2
FGFR3: fibroblast growth factor receptor 3
FHIT: fragile histidine triad gene
FOXC1: forkhead box c1
FUS: fused in sarcoma
Gad1: glutamate decarboxylase 1
GNMT: glycine N-methyltrasferase
GTF2I: general transcription factor ii-i
GWAS: genome-wide association study
H3K4: histone 3 lysine 4
Hcy: homecysteine
HDAC: histone deacetylase
HLA: human leukocyte antigen
hMLH1: MutL homolog 1
HPP1: hyperplastic polyposis gene 1
HPyV7: human polyomavirus 7
HRAS: v-ha-ras harvey rat sarcoma viral oncogene homolog
IFN: interferon
IGF-2: insulin-like growth factor II
IL4: interleukin 4
IRF: IFN regulatory factor
KIT: v-kit hardy-zuckerman 4 feline sarcoma viral oncogene homolog
Krt10: keratin 10
lcnRNA: long non-coding RNA
Lgi 1: leucin-rich glioma-inactived protein 1
LINE1: long interspersed nuclear element 1
LOMG: late-onset myasthenia gravis
LRP4: lipoprotein-related protein 4
LTAg: large T antigen
Maoa: monoamine oxidase a

MBD: methyl-CpG-binding domain
Mbp: myelin basic protein
MG: myasthenia gravis
MGMT: O(6)-methylguanine-DNA methyltransferase
MHC: major histocompatibility complex
miRNA: microRNA
mTEC: medullary thymic epithelial cell
MTHFR: methylentetrahydrofolate reductase
MTR: methionine synthase
MTRR: methionine synthase reductase
Muc1: mucin 1
MuSK: muscle-specific kinase
MYC: v-myc avian myelocytomatosis viral oncogene homolog
NDR: nucleosome-depleted regions
NF-KB: nuclear factor kappa-b
NK: natural killer
NMJ: neuromuscular junction
NRAS: neuroblastoma ras viral oncogene homolog
OGG1: 8-oxoguanine dna glycosylase
PBMC: peripheral blood mononuclear cell
PBRM1: polybromo 1
PCFT: proton-coupled folate
PHF15: phd finger protein 15
piRNA: PIWI interacting RNA
PLK3: polo-like kinase 3
PPP1R15A: protein phosphatase 1, regulatory subunit 15aPRCA: pure red cell aplasia
PTEN: phosphatase and tensin homolog
PTPN22: protein tyrosine phosphatise non-receptor 22
RARB: retinoic acid receptor β
RASSF1A: ras association domain family protein 1
RB1: retinoblastoma
RELB: v-rel avian reticuloendotheliosis viral oncogene homolog b
Resp18: regulated endocrine-specific protein 18
RFC: reduced folate carrier
SAH: S-adenosylhomocysteine
SAM: S-adenosylmethionine
SETD2: SET domain-containing protein 2
SFXN3: sideroflexin 3
siRNA: short interfering RNA
SLE: systemic lupes erythematosus

SMARCA4: swi/snf-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4
SNP: single nucleotide polymorphism
SRGAP1: slit-robo rho GTPase-activating protein 1
STK11: serine/threonine protein kinase 11
TAMG: thymoma associated myasthenia gravis
TDG: thymine DNA glycosylate
TEC: thymic epithelial cell
TET: thymic epithelial tumor
TET2: ten-eveven traslocation protein 2
THF: tetrahydrofolate
Timd2: T-cell immunoglobulin and mucin domains-containing protein 2
TLR: toll-like receptor
TNFRSF11A: tumor necrosis factor receptor superfamily, member 11a
TNFα: tumor necrosis factor α
TNIP1: TNFAIP3-interacting protein 1
TP53: tumor protein p53
TRA: tissue restricted-antigen
Treg: regulatory T cell
TSLP: thymic stromal lymphopoietin
TSS: transcription start site
TYMS: thymidilate synthase
UTR: untranslated region
VAV1: VAV1 oncogene
VGKC: voltage-gated potassium channel
VNTR: variable number of tandem repetitions
WDR70: wd repeat-containing protein 70
WHO: world health organization
WT1: Wilms tumor 1
ZnF: zinc finger

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1. Lopomo A, Ricciardi R, Maestri M, De Rosa A, Melfi F, Lucchi M, Mussi A, Coppedè F, Migliore L. Gene-Specific Methylation Analysis in Thymomas of Patients with Myasthenia Gravis. Int J Mol Sci 2016 Dec 16;17(12). pii: E2121.

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2. Lopomo A, Ricciardi R, Maestri M, De Rosa A, Grossi E, Coppedè F, Migliore L. "Application of Artificial Neural Networks for revealing risk factors linked to thymic pathology in Myasthenia Gravis" European Society of Human Genetics (ESHG), Barcelona (Spain) May 21-24, 2016.

3. Coppedè F, Lopomo A, Migliore L, Spisni R. "*CDKN2A* Hypermethylation and Synchronous Bilateral Colorectal Cancer" Digestive Disease Week (San Diego, CA) May 21-24, 2016.

4. Lopomo A, Denaro M, Coppedè F, De Rosa A, Maestri M, Lucchi M, Mussi A, Ricciardi R, Migliore. "Epigenetic alterations in thymoma-associated Myasthenia Gravis". XVIII Congresso SIGU Rimini (Italy) October 21-24, 2015.

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Communication

Gene-Specific Methylation Analysis in Thymomas of Patients with Myasthenia Gravis

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Abstract: Thymomas are uncommon neoplasms that arise from epithelial cells of the thymus and are often associated with myasthenia gravis (MG), an autoimmune disease characterized by autoantibodies directed to different targets at the neuromuscular junction. Little is known, however, concerning epigenetic changes occurring in thymomas from MG individuals. To further address this issue, we analyzed DNA methylation levels of genes involved in one-carbon metabolism (*MTHFR*) and DNA methylation (*DNMT1*, *DNMT3A*, and *DNMT3B*) in blood, tumor tissue, and healthy thymic epithelial cells from MG patients that underwent a surgical resection of a thymic neoplasm. For the analyses we applied the methylation-sensitive high-resolution melting technique. Both *MTHFR* and *DNMT3A* promoters showed significantly higher methylation in tumor tissue with respect to blood, and *MTHFR* also showed significantly higher methylation levels in tumor tissue respect to healthy adjacent thymic epithelial cells. Both *DNMT1* and *DNMT3B* promoter regions were mostly hypomethylated in all the investigated tissues. The present study suggests that *MTHFR* methylation is increased in thymomas obtained from MG patients; furthermore, some degrees of methylation of the *DNMT3A* gene were observed in thymic tissue with respect to blood.

Keywords: thymoma; myasthenia gravis; cancer; epigenetics; DNA methylation; *MTHFR*; *DNMT3A*

1. Introduction

Thymomas are uncommon neoplasms that arise from epithelial cells of the thymus and are often accompanied by non-neoplastic lymphocytic proliferation. On the basis of the morphology of epithelial cells and the lymphocyte to epithelial cell ratio, they are classified into five histological types, A, AB, B1, B2, and B3 [1]. The most widely used system for staging thymomas is the Masaoka–Koga staging system; according to this, thymomas are classified in stage I comprising encapsulated tumors, stages II and III showing direct local invasion, and stage IV showing metastatic spread [2]. Thymomas are associated with a wide variety of autoimmune diseases, and among these about 30%–40% of thymomas are associated with myasthenia gravis (MG), an

autoimmune disease characterized by autoantibodies directed to different targets at the neuromuscular junction, such as acetylcholine receptor (AChR), muscle specific kinase (MuSK), and agrin-receptor low-density lipoprotein receptor related-protein 4 (LRP4) [3]. Almost all thymoma-associated MG (TAMG) patients have antibodies to the AChR; very rare exceptions have been seen in anti-MuSK+ or in double seronegative patients, while anti-LRP4 autoantibodies have not been reported [4]. The molecular events that characterize thymic neoplasms, including point mutations, chromosomal aberrations, and epigenetic modifications, such as changes in DNA methylation, have been described in the last few years [5]. Aberrant DNA methylation is the most widespread epigenetic alteration in carcinogenesis, and consists of the addition of a methyl group to cytosines, mainly in a CpG dinucleotide context, leading to gene silencing when occurring in the promoter region of a gene [6]. The reversibility of epigenetic changes, unlike genetic modifications, makes them a therapeutic target since, for instance, demethylating drugs can re-express genes silenced by methylation [7].

Concerning thymomas, the silencing of tumor suppressor genes, such as *FHIT*, *MLH1*, and *E-cad*, by DNA promoter methylation, has been described [8]. Aberrant methylation of other genes such as *MGMT*, *CDKN2A*, *HPP1*, and *DAP-K*, associated with the loss of protein expression, was observed [9]. Interestingly, promoter methylation of the *CDKN2A* gene was reported in up to 11% of thymomas and 25% of thymic carcinoma [10], while aberrant *MGMT* methylation and loss of its protein expression was more frequent in thymic carcinoma than in thymoma [11].

Folate metabolism plays an important role in the methylation process as it provides one-carbon units for both purine and pyrimidine base synthesis or for the formation of S-adenosylmethionine (SAM), the universal donor of methyl groups, thus playing an important role in DNA or RNA synthesis and repair, and in DNA methylation processes [12]. A key role in this metabolism is provided by methylenetetrahydrofolate reductase (MTHFR), the enzyme that catalyzes the reaction, which directs the provision of methyl groups donated from folate cofactors towards the remethylation of homocysteine to methionine; this last produces SAM, which is then used by DNA methyltransferases (DNMTs) that transfer the methyl group from SAM to the DNA. As a consequence, folate deficiency can disrupt DNA integrity and promote carcinogenesis [13]. DNMT1 is primarily involved in the maintenance of DNA methylation patterns during development and cell division, whereas DNMT3A and DNMT3B are the de novo methyltransferases that establish DNA methylation patterns during early development [14]. Given supportive evidence from the literature of the contributions of aberrant methylation and expression of *MTHFR* and *DNMTs*, such as *DNMT1*, *DNMT3A*, and *DNMT3B*, in other types of cancer, such as those of the breast, lung, and brain [15–17], we performed the present study to evaluate the methylation levels of these genes in thymomas of patients with MG.

2. Results

2.1. Methylation Levels among Tissues

Table 1 shows the mean methylation levels of the *MTHFR*, *DNMT3A*, *DNMT3B*, and *DNMT1* genes in blood and tumor tissue DNA of all TAMG patients. *MTHFR* shows higher methylation levels in tumor tissue respect to blood (p = 0.007), and *DNMT3A* shows about 10% promoter methylation in tumor tissue but is completely demethylated in blood ($p < 0.0001$). *DNMT3B* and *DNMT1* studied regions were largely hypomethylated both in blood and tumor tissue DNA.

Figure 1 shows the mean methylation levels of the studied genes in blood, tumor tissue, and thymic epithelial tissue adjacent to the cancer, in the 44 patients for whom adjacent tissue was available. *MTHFR* shows higher methylation levels in tumor tissue with respect to blood ($p = 0.0053$) and to adjacent tissue ($p < 0.001$). *DNMT3A* shows higher methylation levels in tumor tissue and adjacent thymic tissue with respect to blood that results in them being completely demethylated ($p < 0.001$), but no significant difference was observed between thymomas and adjacent thymic tissue. The other two genes, *DNMT3B* and *DNMT1*, show very low methylation levels, no more than 2%, and no statistically significant difference was observed among the studied tissues. Age, gender, and

clinico-pathological features had no effect on the mean methylation levels of the studied genes (Table S1).

Table 1. Methylation levels (%) of the studied genes in blood and tumor tissue of total thymoma-associated MG (TAMG) patients (n = 69). Blood and tumor tissues were taken from the same donors, and the p-value refers to a t-test for paired samples.

Gene	Blood (Mean ± SEM)	Tumor Tissue (Mean ± SEM)	p-Value
MTHFR	24.28 ± 0.99	29.66 ± 1.55	0.007
DNMT3A	0.35 ± 0.08	9.78 ± 0.91	<0.0001
DNMT3B	1.14 ± 0.24	0.95 ± 0.26	0.408
DNMT1	1.67 ± 0.12	1.43 ± 0.13	0.041

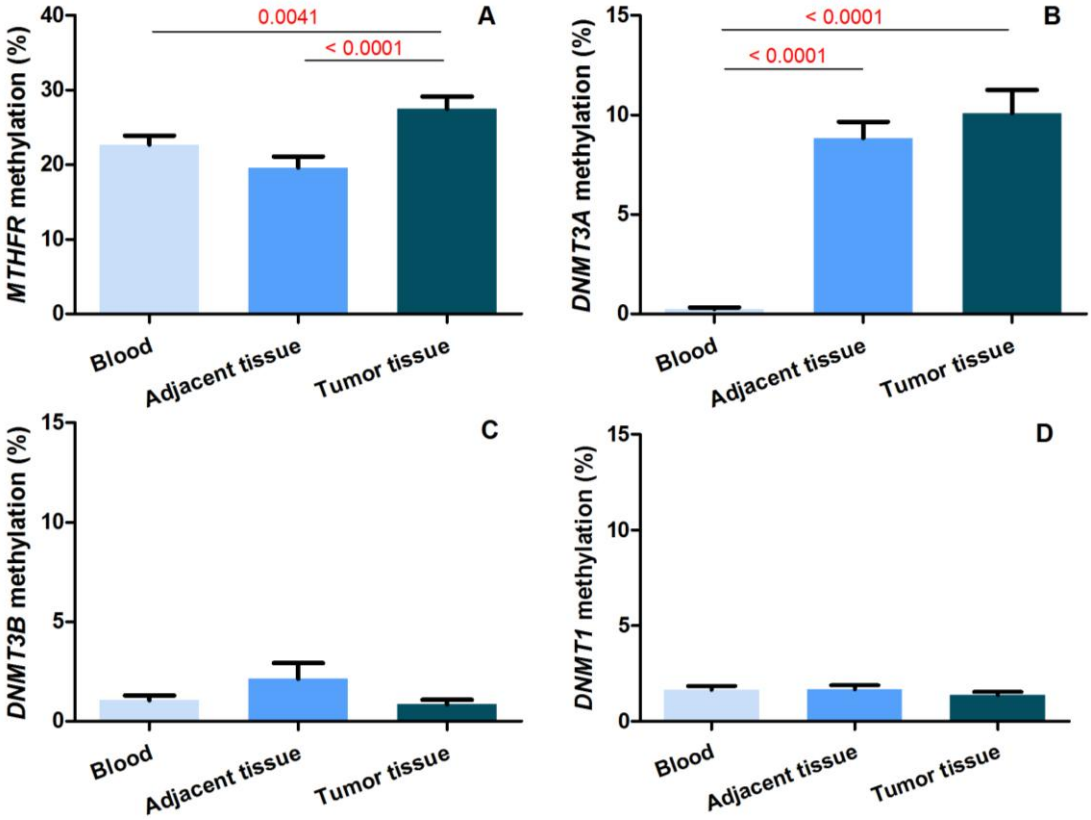

Figure 1. Methylation levels (%) of the studied genes ((**A**) *MTHFR*, (**B**) *DNMT3A*, (**C**) *DNMT3B*, and (**D**) *DNMT1*) in blood, tumor tissue and adjacent thymic tissue of thymoma-associated MG (TAMG) patients for whom adjacent tissue was available (n = 44). The significant p-values (in red) refer to t-tests for paired samples.

2.2. Correlation of Methylation Levels among Tissues

Interesting correlations between the *MTHFR* methylation levels among blood, tumor tissue, and thymic tissue adjacent to the tumor were observed (Figure 2A–C). In particular, correlation of *MTHFR* methylation levels between tumor tissue and blood was observed in the whole population (Figure 2A; n = 69; p = 0.004, r = 0.34) Moreover, correlations between blood and thymic tissue adjacent to the tumor (Figure 2B; n = 44; p = 0.007, r = 0.40) and between tumor tissue and adjacent thymic tissue (Figure 2C; n = 44; p = 0.002, r = 0.45) were observed.

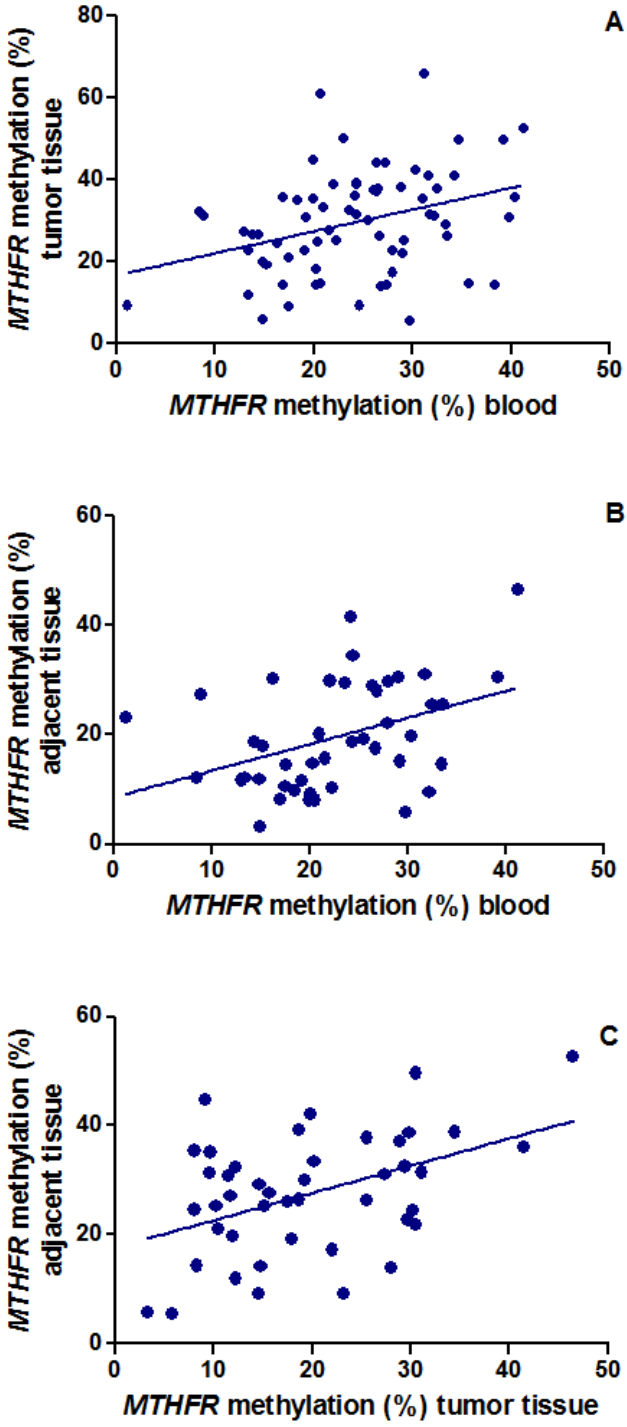

Figure 2. Correlation of *MTHFR* methylation levels (**A**) between tumor tissue and blood in the total population (r = 0.34; p = 0.004); (**B**) between adjacent tissue and blood (r = 0.40; p = 0.007) and (**C**) between tumor and adjacent thymic tissue (r = 0.45; p = 0.002) in the subgroup of 44 individuals with available thymic tissue adjacent to the tumor; r = Pearson's correlation coefficient.

3. Discussion

In the present study we investigated the methylation levels of genes involved in DNA methylation reactions, such as *DNMT1*, *DNMT3A*, and *DNMT3B*, and in one-carbon metabolism, *MTHFR*, in blood and tumor tissue DNA from 69 thymoma-associated myasthenia gravis patients; for 44 of them we also had thymic epithelial tissue adjacent to the tumor. We observed that *MTHFR* and *DNMT3A* promoters show different methylation levels between blood and tumor tissue; this

data was observed in the total population composed of 69 patients as well as in the smaller subgroup of 44 patients for whom healthy thymic specimens were available. In the subgroup of 44 patients, different *MTHFR* methylation levels between the adjacent thymic tissue and the tumor tissue were noticed; in particular, tumor tissue showed on average higher methylation levels with respect to adjacent healthy tissue. For *DNMT3A*, higher methylation levels in the healthy tissue adjacent to a tumor were observed with respect to blood. Comparing the methylation levels of the studied genes in different tissues, statistically significant correlations of *MTHFR* promoter methylation levels between blood and tumor tissue, adjacent thymic tissue and blood, and tumor tissue and adjacent tissue were observed.

MTHFR is one of the major enzymes in folate metabolism since it catalyzes the irreversible conversion of 5,10-methylene THF (the methyl donor in the conversion of dUMP to dTMP) into 5-methyl THF, which remethylates homocysteine to methionine, necessary for the formation of S-adenosylmethionine, the universal donor of methyl groups; therefore, this key protein controls whether folate is partitioned towards DNA precursor synthesis or DNA methylation, and an alteration of this enzyme can interfere with the provision of methyl groups necessary for DNA methylation reactions. It is revealing that the *MTHFR* gene is regulated by promoter methylation and an increased *MTHFR* promoter methylation has been observed in several human diseases, such as cardiovascular and renal diseases [18–19], male infertility [20–22], and preeclampsia [23]. Moreover, it is also emerging that *MTHFR* hypermethylation could be involved in cancer formation; in fact, a correlation between *MTHFR* hypermethylation and lung cancer or cervical cancer lesions was observed [15,24].

In our cohort we observed higher *MTHFR* methylation levels in tumor tissue with respect to blood and adjacent thymic tissue, and we found a strong correlation between methylation levels in blood and tumor tissue. To our knowledge, this is the first time that *MTHFR* methylation has been analyzed in TAMG. We studied a CpG island previously associated with gene silencing; in fact, an inverse correlation was observed between the methylation levels of this region and gene expression levels, suggesting that hypermethylation of the *MTHFR* gene at the studied CpG sites may have a functional significance and may result in partial or complete silencing of the gene [15]. The silencing of *MTHFR* could cause a significant decrease in the global 5-methylcytosine content, leading to the activation of proto-oncogenes, as well as to global hypomethylation, as demonstrated in lung cancer by Vaissière and coworkers [15]. Furthermore, this can increase the availability of 5,10-methylene THF for the synthesis of thymidine and purine, causing the hyper-proliferation of cancerous cells. In this regard, it was suggested that *MTHFR* hypermethylation might confer a growth advantage to cancer cells and contribute to the cancer phenotype in tumors of the upper aero-digestive tract [25]. Moreover, *MTHFR* promoter methylation levels have been correlated with cancer risk factors and with markers of impaired folate metabolism, including tobacco smoking, low circulating folates and vitamin B12, high homocysteine levels, and increased chromosome instability [15,18,26,27].

This study revealed a clear involvement of *MTHFR* promoter methylation in TAMG, particularly the higher methylation levels in tumor tissue with respect to other tissues. These finding leads us to suppose that it could be involved in mechanisms associated with the development and progression of cancer, especially in the activation of proto-oncogenes. Obviously other studies need to further explore the significance of *MTHFR* methylation in TAMG, in order to better understand its pathogenic role in the onset of thymomas. The MTHFR protein is also required for the synthesis of DNA precursors, and impairments of this protein could contribute to cancer development by increasing the rate of point mutations and chromosome instability in rapidly dividing cells [12]. We observed a correlation among *MTHFR* promoter methylation levels in blood, thymic epithelial cells adjacent to the cancer, and thymoma cells that, albeit weak, was statistically significant. Such a correlation could suggest that *MTHFR* methylation levels in blood might reflect those in other tissues, and might represent a peripheral biomarker of the disease. In this regard, it was observed that females with increased *BRCA1* blood methylation have a 3.5-fold increased risk for early-onset breast cancer with histological features commonly seen in tumors arising in women with germline *BRCA1* mutations [28]. Similarly, glutathione S-transferase 1 (*GSTP1*) promoter methylation in

peripheral DNA is regarded as a potential prognostic marker of prostate cancer [29]. However, as far as *MTHFR* promoter methylation in the blood is concerned, it should be noted that rather than being a specific marker of a given disease, it could represent a more general biomarker of increased genomic instability. For example, some studies suggest a correlation between hyperhomocysteinemia and *MTHFR* promoter methylation [26]; others have linked *MTHFR* promoter methylation in blood cells with markers of chromosome damage, such as an increased frequency of micronuclei [27] or alterations of LINE-1 methylation and stability [15], and there is also indication that *MTHFR* promoter methylation in blood DNA might reflect dietary B-group vitamin deficiency [18,30] or environmental exposure to cancerous agents, such as those deriving from tobacco smoking [15]. Collectively, those studies suggest that increased *MTHFR* promoter methylation in blood cells might be a more general marker of impaired one-carbon metabolism and genome instability, rather than a specific disease biomarker.

Another interesting question that still needs to be addressed is how an increased *MTHFR* promoter methylation could be linked to the regulation of autoimmune-related genes in TAMG patients. For example, it is known that the autoimmune regulator (*AIRE*) gene is hypomethylated in thymomas, and that both DNA and histone tail methylation marks regulate *AIRE* expression [31]. Therefore, we speculate that *MTHFR* methylation contributes to both DNA and histone tail methylation changes; however, this must be demonstrated in subsequent studies.

Regarding the DNMTs, the writers of DNA methylation reactions, in this study only *DNMT3A* showed increased methylation levels in tumor tissue, comparable to those found in adjacent thymic tissue, with respect to blood; the other two DNMTs genes, *DNMT1* and *DNMT3B*, resulted hypomethylated. The methylation of these genes was not yet investigated in thymomas but a previous study showed that they were overexpressed in the advanced stages of thymic epithelial tumors with respect to early stages [14].

A double role of de novo DNMTs in tumor stages, as oncogenes and tumor suppressor genes, was proposed. In healthy tissues, tumor suppressor genes are unmethylated and oncogenes are methylated. In early tumor stages, DNMTs seem to lead to methylation-associated repression of tumor suppressor genes and to promote tumor initiation, while in advanced tumor stages the downregulation of de novo DNMTs seems to be associated with promoter DNA hypomethylation of specific oncogenes and, consequently, would promote tumorigenesis [32].

In our cohort, *DNMT1* and *DNMT3B* do not show different methylation levels among different tissues, so we cannot hypothesize the involvement of the methylation of these genes in thymoma-associated myasthenia gravis pathogenesis.

Concerning the *DNMT3A* gene, it shows higher methylation levels in both cancer tissue and adjacent thymic tissue, where almost the same levels have been found with respect to blood, suggesting that its methylation is not connected exclusively with tumor events but already shows alterations in healthy tissue. Aberrant *DNMT3A* methylation was also observed in other cancers, such as in acute myeloid leukemia [16] and in breast cancer, where *DNMT3A* expression is associated with advanced stages [17]. Studies performed in mice with conditional knockout of Dnmt3a revealed a role for DNA methylation in mediating the self-renewal and differentiation of normal hematopoietic stem cells and leukemia stem cells [33]. A functional role of this gene in thymic epithelial tumors emerged from studies that focused on mutation analysis, revealing that *DNMT3A* is one the most frequently mutated genes in thymic carcinoma [34], and that the mutation p.G728D is associated with B3 thymomas [35]. Similarly, a SNP in the promoter of *DNMT3B*, namely −579G>T, was associated with TAMG [36], overall suggesting a contribution of de novo DNMTs in thymomas.

From the DNA promoter methylation analyses performed in this study, the involvement of *MTHFR* and *DNMT3A* methylation in thymoma-associated myasthenia gravis has been proven, even if other studies are needed to assess a potential pathogenic role of these genes in TAMG. Other limitations of the present study that need to be addressed in subsequent investigations include the analysis of gene expression levels of the studied genes in TAMG samples and a comparison study between TAMG samples and thymomas obtained from individuals without MG, which could help

us to clarify whether the observed methylation changes are TAMG-specific or rather are common in thymomas.

4. Materials and Methods

4.1. Study Population

A total of 69 AChR positive patients (AChR+) with thymoma (TAMG) were recruited at the Myasthenia Clinic (Department of Neuroscience and Cardiac and Thoracic Department, Pisa University Hospital). The diagnosis of myasthenia gravis was made on the clinical symptoms of the patient together with the detection of positivity of AChR antibodies. Clinical stages of MG were assessed according to the Osserman classification. Demographic characteristics of the population are shown in Table 2. DNA samples were obtained from both the surgically resected tumor tissue of 69 patients (Table 2) and from the adjacent normal tissue available from 44 of them. An aliquot of blood in EDTA tubes was also collected from each patient and stored at −20 °C until assayed. Each patient gave informed written consent for genotype analysis before blood drawing. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Pisa University Hospital (Protocol number 21302/2015).

4.2. Extraction of Genomic DNA

Genomic blood and tissue DNA was extracted using QIAmp DNA Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instruction. The extracted DNA was quantified using a Nano Drop ND 2000c spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, DE, USA).

4.3. Bisulfite Modification

Two hundred nanograms of DNA from each sample were treated with sodium bisulfite using the EpiTectH Bisulfite Kit (Qiagen) according to the manufacturer's protocol. Sodium bisulfite treatment converts all unmethylated cytosines into uracil, while methylated cytosines are left unchanged.

4.4. Methylation-Sensitive–High-Resolution Melting (MS-HRM) Analysis

Promoter methylation was assessed by means of methylation-sensitive–high-resolution melting (MS-HRM) analysis in a CFX96 Real-Time PCR detection system (Bio-Rad, Milan, Italy). For the MS-HRM analysis we developed protocols according to the literature [37–38]. All analyses were run according to the following conditions: one cycle of 95 °C for 12 min, 60 cycles of 95 °C for 30 s, T_a for 30 s and 72 °C for 15 s; followed by an HRM step of 95 °C for 10 s and 50 °C for 1 min, 65 °C for 15 s, and continuous acquisition to 95 °C at one acquisition per 0.2 °C. PCR was performed in a final volume of 25 μL, containing 12.5 μL of master mix (Qiagen), 10 pmol of each primer, and 10 ng of bisulfite-modified DNA template. Each reaction was performed in duplicate. We analyzed 10% of the samples independently on separate occasions to verify the inter-assay variability and observed good reproducibility. Table 3 shows the conditions used for each gene, such as primers, annealing temperature, CpG sites, and amplicon length; for *MTHFR* and *DNMT3A* we used primers derived from the literature [15–16], while *DNMT1* and *DNMT3B* primers were designed by us using the Meth-primer program (available at http://www.urogene.org/methprimer). Fully methylated and unmethylated DNA (EpiTectH methylated and unmethylated human control DNA, bisulfite converted, Qiagen) were mixed to obtain the following ratios of methylation: 0%, 12.5%, 25%, 50%, 75%, and 100%. Standard curves with known methylation ratios were included in each assay and were used to deduce the methylation ratio of each blood, adjacent healthy tissue, and tumor tissue. Validation of the MS-HRM assays was performed by means of pyrosequencing, as detailed elsewhere [26]. In order to obtain single methylation percentage values from MS-HRM assays, we applied an interpolation method developed and described by us, which allowed us to obtain precise HRM methylation values comparable to those obtained by pyrosequencing [37].

Table 2. Demographic and clinico-pathological characteristics of the studied population (TAMG: thymoma-associated myasthenia gravis, MG: myasthenia gravis, F: females, M: males, NS: no specified tumor histology).

No. TAMG Patients	Age ± SD	Gender (%)	MG Onset (%)	MG Osserman Classification (%)	Thymoma Histology (%)	Masaoka Stage (%)
69	55.5 ± 13.2	F: 37 (54) M: 32 (46)	<50 years: 26 (38) ≥50 years: 43 (62)	I: 7 (10.0) IIA: 11 (16.0) IIB: 34 (49.5) III: 13 (19.0) IV: 3 (4.0) V: 1 (1.5) -	A: 13 (18.8) AB: 13 (18.8) B1: 5 (7.2) B2: 23 (33.4) B3: 8 (11.6) B2-B3: 5 (7.2) NS: 2 (3.0)	I: 7 (10) IIa: 15 (22) IIb: 29 (42) III: 7 (10) IVa: 9 (13) NS: 2 (3) -

Table 3. Primer sequences and annealing temperatures (T_a) used during MS-HRM analysis, amplicon length, number of CpG sites for each gene, accession number, and amplified region (* DMR2: differentially methylated region = upstream promoter region involved in the regulation of transcript 2 of *DNMT3A* gene, details are provided in [16]).

Gene	Primer Sequences	T_a	Amplicon Length	CpG Sites	Accession Number	Amplified Region
MTHFR	F 5′-TTTTAATTTTTGTTTGGAGGGTAGT-3′ R 5′-AAAAAAACCACTTATCACCAAATTC-3′	54°	155 bp	7	NM_005957.4	from +30 to +184 bp
DNMT1	F 5′-GGTATCGTGTTTATTTTTTAGTAA-3′ R 5′-ACGAAACCAACCATACCCAA-3′	52°	114 bp	9	NG_028016.3	from −106 to +8 bp
DNMT3A	F 5′-GGTTTGGGTTTATTGTAGGAAGGTTATTAAGGT-3′ R 5′-AATCCAAAACCCCCCTATCACGAAA-3′	58°	199 bp	7	NM_153759.3	DMR2 *
DNMT3B	F 5′-TGGTGTTGTGTGATTATAGTGG-3′ R 5′-TCACCCTAAAAAATCAAAAACC-3′	55°	174 bp	6	NG_007290.1	from −397 to −223 bp

4.5. Statistical Analysis

Differences in mean methylation levels among the three groups were evaluated with a Student's t-test for paired samples. The effect of clinical and pathological features, such as onset of MG (early or late), MG Osserman classification, and thymoma histology, on mean methylation levels was assessed by means of analysis of variance (ANOVA), including age at sampling and gender as covariates. Linear regression analysis was performed to search for a correlation between age and methylation levels in the studied tissues, as well as to search for a correlation of *MTHFR* methylation levels among the different tissues. Analyses were performed with the STATGRAPHICS 5.1 Plus software package for Windows. Since we studied four different genes in our cohort, a Bonferroni's correction for multiple comparisons was applied, and the cut-off *p*-value for considering a result to be statistically significant was set at 0.05/4 = 0.0125. The statistical power of the study was evaluated with the clinical tool and calculators for medical professionals, ClinCalc (Available at http://clincalc.com/Stats/Power.aspx). The sample size was chosen to have an a priori power of >80% to detect mean methylation differences of 5% or higher.

5. Conclusions

In conclusion, the present study revealed increased *MTHFR* promoter methylation in thymomas obtained from MG patients and some degrees of methylation of the *DNMT3A* gene in thymic tissue with respect to blood were observed. Other studies supported by gene expression analysis are needed to assess a potential pathogenic role of these genes in TAMG.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/link.

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Author Contributions: Angela Lopomo performed the experiments and wrote the manuscript; Roberta Ricciardi, Michelangelo Maestri, and Anna De Rosa clinically characterized myasthenia gravis patients and provided blood samples; Franca Melfi, Marco Lucchi, and Alfredo Mussi characterized and provided thymic specimens from myasthenia gravis patients; Fabio Coppedè and Lucia Migliore conceived and designed the study, provided reagents, materials, and statistical advice, and critically reviewed the entire manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Material: Gene-Specific Methylation Analysis in Thymomas of Patients with Myasthenia Gravis

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Table S1. Correlation among age, gender, thymic histology, Masaoka classification, MG onset, MG Osserman classification, and methylation levels of the studied genes in blood tumor tissue of all TAMG patients (n = 69). The effect of clinical and pathological features on mean methylation levels was assessed by means of analysis of variance (ANOVA), including age at sampling and gender as covariates. Linear regression analysis was performed to search for a correlation between age and methylation levels in the blood and tumor tissue.

	Age	Gender	Histology	Masaoka	MG Onset	Osserman
MTHFR blood	p = 0.65; r = −0.05	p = 0.93	p = 0.79	p = 0.59	p = 0.25	p = 0.35
MTHFR tumor	p = 0.98; r = 0.001	p = 0.95	p = 0.08	p = 0.68	p = 0.37	p = 0.58
DNMT3A blood	p = 0.17; r = −0.16	p = 0.96	p = 0.18	p = 0.29	p = 0.82	p = 0.84
DNMT3A tumor	p = 0.28; r = 0.13	p = 0.77	p = 0.32	p = 0.45	p = 0.72	p = 0.81
DNMT3B blood	p = 0.70; r = −0.05	p = 0.38	p = 0.52	p = 0.97	p = 0.75	p = 0.91
DNMT3B tumor	p = 0.11; r = 0.19	p = 0.68	p = 0.63	p = 0.83	p = 0.80	p = 0.56
DNMT1 blood	p = 0.72; r = −0.04	p = 0.85	p = 0.53	p = 0.24	p = 0.62	p = 0.48
DNMT1 tumor	p = 0.94; r = 0.008	p = 0.57	p = 0.56	p = 0.43	p = 0.14	p = 0.30