UNIVERSIDADE DE LISBOA

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Focusing on the epigenetic control of regulatory T cells

Bárbara Coelho da Silva Martins Saraiva

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Orientado por:

Dra. Ekaterina Minskaia

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ABSTRACT

FOXP3, encoded on X chromosome and mainly regulated epigenetically, plays a critical role in the development of regulatory T cells (Treg), essential for maintenance of immune tolerance and homeostasis, and is a widely used Treg-specific marker. Several regions on FOPX3 locus, such as enhancer, promoter and three Conserved Noncoding DNA Sequences (CNS 1-3) were identified as important for epigenetic control. Specifically, promoter and CNS 2, also known as Treg Specific Demethylated Region (TSDR), contribute to FOXP3 expression *via* demethylation of their CpG islands.

Differences in TSDR methylation levels can help distinguish Tregs from conventional T lymphocytes (Tcon). In addition, CAMTA1, encoded on chromosome 1, was shown to be the only other molecular marker that may help differentiate these T cell populations as its demethylation pattern is somewhat similar to that of TSDR. Currently, studies correlate autoimmune diseases (AID) and allergies with decreased function of Tregs and higher methylation levels of FOXP3 locus. Interestingly, females are more susceptible than males to AID which has been suggested to be due to X chromosome inactivation.

The aim of this study was to compare the methylation pattern of FOXP3 promoter, TSDR, and CAMTA1 between Tregs and Tcons of random healthy male and female donors.

$CD4^+$ T cells, isolated from peripheral blood, were sorted into $CD4^+CD25^{bright}CD127^{low}$ $FOXP3^+CD45RA^+CD15s^-$ naïve Treg cells and $CD4^+CD25^{low}CD127^+$ Tcon cells by FACS. Bisulphite converted genomic DNA was PCR-amplified, cloned into pGEM-T vector and sequenced. Sequences were analysed and levels of methylation defined.

We demonstrate that donors of both genders have higher demethylation levels in CAMTA1 in Tregs than in Tcons compared to previously undescribed methylation patterns of FOXP3 promoter and TSDR. CAMTA 1 showed two different patterns of demethylation in Tregs: demethylation of the first eight-ten CpGs or demethylation of CpGs 2 and 11.

In conclusion, CAMTA1 seems to be a more reliable marker differentiating between Tregs and Tcons. In future, methylation patterns of male and female donors with AID and allergies must be analysed to understand the impact of both X chromosome inactivation and clinical conditions in the methylation of FOXP3 locus and compared to that of CAMTA1 gene region.

Keywords: Epigenetics, Methylation; Treg; FOXP3; CAMTA1

RESUMO

O gene FOXP3, codificado no cromossoma X e principalmente regulado por vias epigenéticas, apresenta um papel fundamental no desenvolvimento de células T reguladoras (Treg), essenciais à manutenção da tolerância imunitária e homeostase, sendo amplamente utilizado como um marcador específico das células Treg. Existem diversas regiões no *locus* do FOXP3 reconhecidas pela sua importância no controlo epigenético: *enhancer*, promotor e três Sequências Conservadas de DNA Não Codificante (CNS), CNS 1-3. Especificamente, o promotor e o CNS2, também denominado de Região Desmetilada Específica de células Treg (TSDR), são responsáveis por este controlo através da desmetilação de ilhéus CpG das suas sequências.

Diferenças nos níveis de metilação da região TSDR contribuem para a distinção entre células Treg e linfócitos T convencionais (Tcon). A par da região TSDR, verificou-se que o gene CAMTA1, localizado no cromossoma 1, apresentava variações dos padrões de desmetilação semelhantes às verificadas no TSDR, apresentando-se como o outro marcador molecular que poderia contribuir para a distinção entre as duas populações.

Disfunções das células Treg e um padrão de elevada metilação do *locus* do FOXP3, têm sido correlacionados com doenças autoimunes e alergias. Notar ainda que as mulheres apresentam uma maior suscetibilidade para doenças autoimunes comparativamente aos homens, o que poderá ser explicado pela inativação do cromossoma X.

Propôs-se comparar os padrões de desmetilação do promotor do FOXP3, TSDR e do gene CAMTA1 entre células Treg e Tcon de homens e mulheres saudáveis.

Foram isoladas células T CD4+ a partir de sangue periférico, tendo sido sujeitas a citometria de fluxo (FACS) e separadas células Treg naïve $CD4^{+}CD25^{bright}CD127^{low}$ $FOXP^{3+}CD45RA^{+}CD15s^{-}$ e células Tcon $CD4^{+}D25^{low}CD127^{+}$. O DNA genómico, após tratamento com bissulfito, foi amplificado por PCR, clonado em vetores pGEM-T e sequenciado. As sequências foram analisadas e os níveis de metilação estabelecidos.

Foi demonstrado que, quer em dadores do sexo feminino quer do sexo masculino, a região do gene CAMTA1, nas células Treg, apresentou um padrão consistente de maior desmetilação comparativamente ao das células Tcon. Por outro lado, resultados inconsistentes e não descritos até à data foram apresentados para o promotor do FOXP3 e TSDR. O gene CAMTA1 apresentou ainda dois padrões diferentes de desmetilação nas células Treg: desmetilação das primeiras oito/dez posições CpG e desmetilação do segundo e décimo primeiro CpG.

Deste modo, a desmetilação do gene CAMTA1 poderá ser um marcador molecular adicional mais fidedigno para a distinção entre células Treg e Tcon quando a expressão de FOXP3 possa estar afectada. No futuro, será fundamental estudar os padrões de metilação em dadores com doenças autoimunes ou alergias com o objetivo de compreender o impacto que quer estas condições clínicas quer a inativação do cromossoma X poderão ter no padrão de metilação do *locus* do FOXP3 comparativamente à possível independência do gene CAMTA1 para com as condições supramencionadas.

Palavras-chave: Epigenética, Metilação; Treg; FOXP3; CAMTA1

O Trabalho Final exprime a opinião do autor e não da FMUL.

CONTENTS

LIST OF ABBREVIATIONS

anti-h - anti-human antibody
AID - autoimmune diseases
AP-1 - activator protein 1
aTreg - activated regulatory T cell
CAMTA1 – calmodulin binding transcription factor 1
CD3 - cluster of differentiation 3 T cell co-receptor
CD4 - cluster of differentiation 4
CD15s – sialyl Lewis x
CD25 – interleukin – 2 receptor alpha chain
CD45RA - cluster of differentiation 45RA
CD127 - interleukin-7 receptor-α
CNS - conserved noncoding DNA sequence
CREB/ATF - cAMP response element binding protein / Activating transcription factors
DNA - deoxyribonucleic acid
eTreg - effector regulatory T cell
FACS - fluorescence-activated cell sorting
FOXP3 - forkhead box transcription factor 3
gDNA - genomic DNA
GVHD - graft-versus-host disease
HTLV-1 - human T lymphotropic virus – 1
IPEX - immune dysregulation, polyendocrinopathy, enteropathy X - linked syndrome
iTreg - induced regulatory T cell
LKB1 - serine-threonine kinase 11
NFAT - nuclear factor of activated T-cells
nTreg - natural regulatory T cell
PCR - polymerase chain reaction
RNA - ribonucleic acid
rTreg - resting regulatory T cell
STAT-5 - signal transducer and activator of transcription 5
Tcon - conventional $CD4^+$ T cell
TCR - T cell receptor
TeT family - ten-eleven-translocation family
Treg - regulatory T cell
TSDR - Treg specific demethylated region

INTRODUCTION

Regulatory T cells (Treg), a subtype of $CD4^+$ T cells, have a crucial role in response to autoimmune stimuli, self-tolerance and immune homeostasis [1, 2], and have also been considered as a negative prognostic marker for numerous solid tumours [3-6].

Treg cells constitutively express interleukin – 2 receptor alpha chain (CD25) and forkhead box transcription factor 3 (FOXP3). FOXP3, located on the Xp11.23 chromosome, plays a critical part in Treg cell development, differentiation and function, and is widely used as a Treg-specific marker [7,8]. However, it is now known that it is not exclusively expressed in Treg cells but also in other human T cells, such as conventional $CD4^+$ T cells (Tcon) after their activation [9-12].
FOXP3 expression is mainly regulated by epigenetic processes [1], such as DNA methylation, histone modifications, nucleosome positioning and microRNAs [13]. These processes are reversible and alter chromatin structure but not the nucleotide sequence [13-15]. The focus of this project is on DNA methylation, where methylation of cytosines within CpG islands is usually responsible for gene silencing while demethylation is associated with opening of the locus [8], active transcription and expression [1,8].

Baron et al. identified several regions within FOXP3 locus with variable DNA methylation patterns [16]. Subsequent studies demonstrated that several regions on the FOPX3 locus were important for its epigenetic control: enhancer [17], promoter [18] and three evolutionary Conserved Noncoding DNA Sequences (CNS elements), CNS 1-3 [8, 16-20]. Enhancer [17], promoter [18] and CNS 2, also known as Treg Specific Demethylated Region (TSDR), are involved in this process *via* DNA methylation [8, 16-20].
More specifically, methylation levels of TSDR can help to distinguish Treg cells from Tcon cells, as it is usually more demethylated in Treg cells and methylated in Tcon cells [8,16]. Also, it can help to differentiate several maturation stages of lymphocytes:

I. thymocites showed incomplete demethylation of TSDR;
II. natural Treg (nTreg) cells, which proliferate from the thymus, showed complete demethylation of TSDR;

III. Treg cells induced in periphery from Tcon cells (iTreg cells), through a TGF-β dependent process associated with a suboptimal TCR triggering, showed incomplete demethylation despite high FOXP3 expression [8,19];

IV. Although FOXP3 can be transiently expressed in Tcon cells, TSDR remains methylated [19].

The fact that demethylation of CpGs in TSDR seems to maintain stability of FOXP3 expression [8, 19, 21-23] means that TSDR is used as a molecular biomarker that helps to measure Treg lineage commitment and quantify Treg cells [21].

It was also demonstrated that Treg cell development can be achieved by two independent processes, both induced by TCR stimulation: FOXP3 expression and TSDR hypomethylation. Demethylation of this region begins in the thymus and continues in periphery. It can be established without FOXP3 expression and, at the same time, demethylation of TSDR is not required for initiation of FOXP3 expression [24].

While FOXP3 expression can be used as a specific marker for Treg cells, demethylation of TSDR is an epigenetic marker better correlated with and required for lineage stability that results in a full suppressive phenotype [24,25].

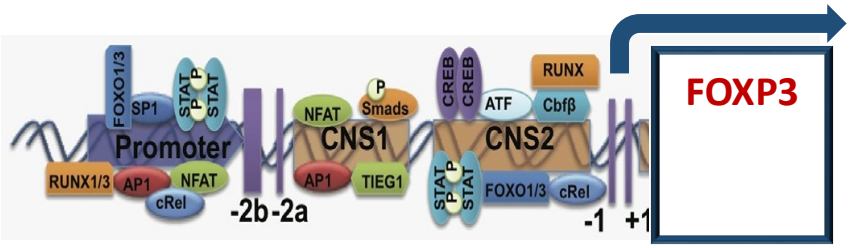

Figure1 - Transcriptional factors binding to FOXP3 locus
(adapted from Nie, J. et al., 2015) [26].

Transcriptional activity (see Figure1) of this gene is dependent both on TCR stimulation and TSDR demethylation. After triggering of TCR, several NFAT and AP-1 transcriptional factors bind to FOXP3 promoter, and in turn are positively regulated by FOXP3 expression [27]. During nTreg development, TSDR demethylation and FOXP3 stability involve the upregulation of Ten-eleven-translocation (TeT) family [28-30], Lkb1[31], and hydroxylation of methylated cytosines [28]. When TSDR is demethylated, CREB/ATF and STAT-5 bind to this region [18] to promote its transcription.

Mutations in FOXP3 result in severe autoimmune diseases (AID) as can be observed in IPEX syndrome (Immune dysregulation, Polyendocrinopathy, Enteropathy), a rare X -

linked syndrome [32]. Decreased number and functional deficiency of Treg cells have been also associated with myasthenia gravis, ulcerative colitis and multiple sclerosis [33-36]. Nowadays, studies correlate AID, allergies and even some viral infections not only with a decreased number and function of Treg cells but also with a higher methylation level of the FOXP3 locus. This was shown for tropical spastic paraparesis associated with HTLV-1 retrovirus infection [37] and ankylosing spondylitis [38]. Moreover, FOXP3 demethylation was significantly lower in children with cow's milk allergy [39] and may help predict atopic dermatitis [40,41]. As part of the physiopathology of certain diseases, iTreg cells, with a methylated TSDR, were increased in Diabetes type I [42].

Focusing on the predictive value of Treg cells, the increased number of cells and TSDR demethylation were correlated with a worse survival in colon cancer [43] and adult T-cell leukemia [44]. On the other hand, demethylated TSDR contributes to a positive outcome in sepsis early-phases [45].

Hypermethylation of FOXP3 locus was also linked to air pollution and a higher carbon exposure, increasing asthma morbidity [46, 47] meanwhile hypomethylation of the locus was associated with more physical activity [47].

Females are more susceptible than males to AID and it is hypothesized that these differences may be contributed to the influence of X-linked genes, including hormonal differences [21]. It is known that X chromosome inactivation affects approximately 85% of X-linked genes [48].

The majority of published studies which focused on TSDR methylation pattern used samples obtained from male human donors or mice in order to avoid bias related to X chromosome inactivation. However, Baron et al. observed hemi-methylation of female FOXP3 locus [16] and Stockis et al., while looking for Treg clones with a demethylated TSDR, observed that 80% were derived from male patients whereas only 23-45% from females [26].

In addition to TSDR, Baron et al. investigated other human genes that could be used as potential Treg markers. Calcium-dependent calmodulin-binding transcription factor 1, CAMTA1, encoded on chromosome 1, was shown to be the only other molecular marker that may help to distinguish Treg cells from Tcon cells as its demethylation

pattern is similar to that of FOXP3 TSDR [16]. The role of CAMTA1 in T cell development and function has not been studied until now [1].

It is known that calcium plays a crucial role in gene transcription. Calcium-dependent transcription mechanisms are numerous and include various signal transducers, such as calmodulin. They contribute to the activity of a number of transcriptional factors. Therefore, the interaction of CAMTAs with co-activators and co-repressors is important for induction of gene expression and regulation [49].

Moreover, calcium also has a critical role in $CD4^+$ T cells especially in TCR signalling, essential for their effector function: TCR stimulation promotes calcium intracellular depletion and activates calcium entry to raise the intracellular calcium concentration [50].

$FOXP3^+CD4^+$T cells are a heterogeneous population in terms of phenotype and function [51]. Miyara et al. divided this population in three phenotypically and functionally distinct subpopulations: $CD45RA^+FOXP3^{low}$ resting Treg cells (rTregs); $CD45RA^-FOXP3^{hi}$ activated Treg cells (aTregs); and cytokine secreting $CD45RA^-FOXP3^{low}$ nonsuppressive T cells. Most of aTreg cells derive from the thymus from recently activated rTreg cells, although a minority of them can arise from nonTreg cells for an undefined time in periphery [52]. In addition to CD45RA, they also discovered that CD15s was expressed in $CD15s^+CD45RA^-FOXP3^{hi}$ effector Tregs (eTregs), among aTreg cell population. eTreg cells are the most terminally differentiated and the most suppressive $FOXP3^+$ Treg population [53]. Finally, Myiara et al. showed, once more, that CD127 remained the most discriminative down regulated marker in Treg cells [53], as previously described [54].

In our research, we were interested in comparing the demethylation patterns of FOXP3 promoter, TSDR, and CAMTA1 between Treg and Tcon cells of random male and female human donors as we hypothesized that differences seen in the methylation pattern of the FOXP3 locus might be related to imbalances between genders while in CAMTA1 should not. In order to distinguish Treg from Tcon cells, in our project, we used $CD4^+CD25^{bright}CD127^{low}FOXP3^+CD45RA^+CD15s^-$ naïve Treg cells and $CD4^+CD25^{low}CD127^+$ Tcon cells.

One of the main research areas in João Forjaz Lacerda Lab is post-transplant complications, such as graft-versus-host disease (GVHD), and therapeutic strategies that

improve patient survival. In GVHD, a therapy with adoptive transfer of donor Treg cells is currently being investigated. Therefore, the aim of this project is not only to get insight into gender differences in the epigenetic control of Treg cell differentiation but also, in a long-term, to allow the optimization of therapies and contribute to a better patients' quality of life.

METHODS

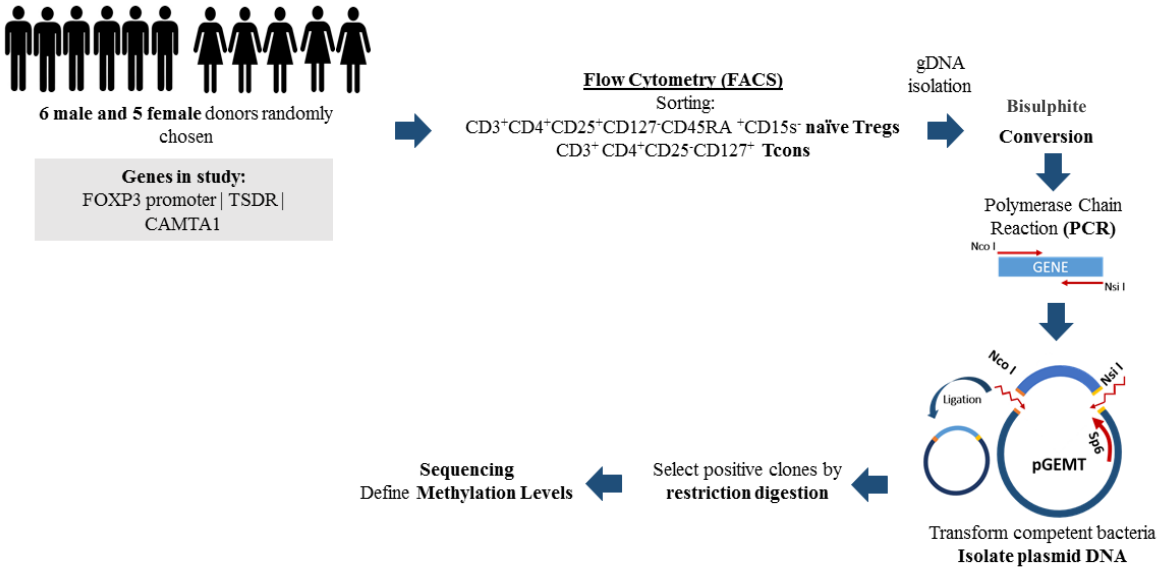

Figure2 - Methods: A schematic presentation of the workflow

1. Patient and sample collection

Blood samples were obtained from six male (M_1 – M_6) and five female (F_1 – F_5) young healthy volunteers with an unknown personal and family clinical history of AID and allergies.

2. Isolation of cells and fluorescent activated cell sorting (FACS)

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient.

Tcon and Treg cells were then isolated from a negative fraction of $CD34^+$ cell selection using EasySep™ Human CD34 Positive Selection kit (STEMCELL Technologies). For 25 minutes, cells were incubated at room temperature in PBS (2% human serum) with pre-titrated antibodies:

- anti-hCD3 (-PerCP, clone OKT3, eBiosciences);
- anti-hCD4 (-APC, clone RPA-T4, eBiosciences);
- anti-hCD45RA (-FITC, Myltenyi Biotec);
- anti-hCD25 (-Pe-Cy7, BD Biosciences);
- anti-hCD127 (-APC-Cy7, clone eBioRDR5, eBiosciences);
- CD15s (-PE, BD Biosciences).

Cells were then washed in PBS and sorted on a BD FACSARIAIIu. For intracellular staining for FOXP3, in addition to the antibody combination described above, cells

were stained with anti-hFOXP3 (PE 470 from eBioscience) using FOXP3 Staining Buffer Set (e-Bioscience).
Data was analysed with FlowJo ® LLC.

3. Genomic DNA isolation

Genomic DNA (gDNA) from the two populations of CD4+ T cells was isolated using Quick-gDNA MiniPrep kit (Zymo Research). Cells were resuspended in 100 µL of PBS and lysed in 400 µL of Genomic Lysis Buffer for 10 minutes. Zymo-Spin columns were loaded with samples and centrifuged at 12000 rpm for 1 minute. Columns were then washed with 200 µL of DNA Pre-Wash Buffer and 500 µL of gDNA Wash Buffer.
gDNA was eluted from the columns in 45 µL of DNA Elution Buffer.

4. Bisulphite conversion treatment

Bisulphite conversion treatment modifies all nonmethylated cytosines into uracils while methylated cytosines remain unchanged. To avoid potential biased results, gDNA from Treg and Tcon cell populations was isolated and bisulphite treated at the same time followed by polymerase chain reaction (PCR) and cloning of the three gene regions (CAMTA1, FOXP3 promoter and TSDR).
gDNA from the two cell populations was converted using EZ DNA Methylation Lightning Kit (Zymo Research), shown to convert 99.5% of nonmethylated cytosines to uracils while 99.5% of methylated cytosines remained unmodified. 20 µL of gDNA were mixed with 130 µL of Lightning Conversion Reagent and the samples were incubated at 95°C for 8 minutes followed by 54°C for 60 minutes. The samples were then loaded into Zymo-Spin IC Columns containing 600 µL of M-Binding Buffer, mixed well and centrifuged at 12000 rpm for 30 seconds. Columns were then washed with 100 µL of M-Wash Buffer and incubated with 200 µL of M-Desulphonation Buffer at room temperature for 15 minutes. Then, columns were washed twice with 200 µL of M-Wash Buffer. Bisulphite-treated gDNA was eluted in 12 µL of M-Elution buffer and used as a template.

5. Polymerase chain reaction (PCR)

FOXP3 promoter, TSDR and CAMTA1 were PCR amplified with gene-specific primers (Table1) using the following optimized conditions [Initial denaturation: 98º -

30seconds »» 45cycles at: 98º (10seconds) – 60/58º (20seconds) – 72º (45seconds) »» 72º (8minutes) »» 4º (∞)].

PCR products were then run on 1% agarose gel, bands of appropriate size excised and DNA purified using NZY GelPure Kit (NZYTech).

Table 1 – Primers sequences used in this research

Sequence	Primer Sequences, 5' → 3' **Foward (Fw)** and **Reverse (Rev)** Primers *Restriction sites are in bold*	Amplicon size (bp)	Annealing Temperature (ºC)
FOXP3 promoter	**Fw:** GCGC**CATGGT**TGTTTGTTTCGGGTTGGTTTTGTGATTTATTTTAG **Rev:** GCG**ATGCAT**ACAAAAAAAAATCAACCTAACTT	451	60
FOXP3 TSDR	**Fw:** GCG**CCATGG**TTAGGGATATTGGTTTATATATATTGAGATTTTGG **Rev:** GCG**ATGCAT**CCCCAAACACATATAAAATAACCTAACTCAACAAAAC	700	58
CAMTA1	**Fw:** GCG**CCATGG**AATAAGTAAAGAAAATTTAGTTGGTAATAAAATAAGGG **Rev:** GCG**ATGCAT**ATAACAATCTCATCTAAATCAACCTATAACAAACACC	470	58
SP6	**Rev:** GTGACACTATAGAATACTC	---	---

Table1 – Primers sequences used in this research. FOXP3 promoter, TSDR and CAMTA1 gene regions were PCR amplified with specific primer sets yielding amplicons of different sizes.

6. Bacterial Transformation and Plasmid DNA isolation

Both PCR products and pGEM®-T Easy Vector (Promega) were prepared for cloning by digestion with Nco I and Nsi I restriction enzymes (NEB) for 1.5 hours at 37ºC, according to manufacturer's requirements, and purified using NZY GelPure Kit. Ligation reactions were set up overnight at 4ºC with T4 DNA ligase (Promega). NZY Star Competent Cells were transformed by incubating on ice for half an hour, followed by 45 second heat shock at 42ºC and incubation with LB media in the shaker at 37ºC.

Bacterial cultures were plated on LB agar plates supplemented with ampicillin (100mg/1mL), IPTG (0.5mM) and X Gal (40mg/mL).

7. Selection of positive clones

Minimum 20 white colonies, expected to contain inserts, were picked from each plate. 4mL of LB media containing ampicillin (100mg/mL) were inoculated with one white colony and grown overnight in a shaker at 37ºC.

Isolation of plasmid DNA was performed using NZYMiniprep kit (NZYTech). Bacterial pellets were resuspended in 250µL of buffer A1 and transferred to an eppendorf tube. 250µL of lysis buffer A2 were added by mixing 2-3 times. Finally,

350µL of Buffer A3 were added to neutralize the reaction and the samples were centrifuged for 7 minutes at 13000 rpm. The supernatant was loaded into a spin column and centrifuged for 1 minute at 12 000 rpm. The columns were washed first with 500µL of Buffer AY and then with 600µL of buffer A4.

Plasmid DNA was eluted in 45 µL of elution buffer. 4µL of pasmid DNA were digested with restriction enzymes Nco I and Nsi I to confirm the identity of clones.

Restriction digestion reactions were run on 1% agarose gel and positive clones were identified by the presence of inserts based on the size of DNA products. Correct clones had two bands: vector (3.5kb) + insert (see table 1).

8. Sequencing and definition of methylation levels

10µL of DNA were sequenced with 3µL of reverse SP6 sequencing primer (see table 1) by StabVida.

Obtained sequences were aligned to the respective reference sequences of CAMTA1, FOXP3 promoter and TSDR using SeqMan software (DNA Star Lasergene 8).

In sequence alignments, methylated cytosines were presented as cytosines and demethylated cytosines as thymidines. The percentage of the methylation in each CpG position was determined by defining the proportion of methylated cytosines in the total of 20 sequences.

RESULTS

In order to compare the methylation patterns of FOXP3 promoter, TSDR, and CAMTA1 in Treg and Tcon cells of male and female donors, eleven random healthy donors were chosen: six male (M_1 – M_6) and five female (F_1 – F_5).

Using FACS, cells were sorted into $CD4^+CD25^{bright}CD127^{low}FOXP3^+CD45RA^+CD15s^-$ naïve Treg cells and $CD4^+CD25^{low}CD127^+$ Tcon cells. gDNA from these cell populations was then isolated and subjected to bisulphite conversion. Bisulphite-converted DNA was PCR-amplified and cloned into pGEM-T vector. Bacterial competent cells were transformed with ligation reactions and plated onto LB plates to allow for individual colonies to grow, as can be observed in Figure3. At least twenty white colonies were picked, expected to contain DNA inserts, and their plasmid DNA was isolated.

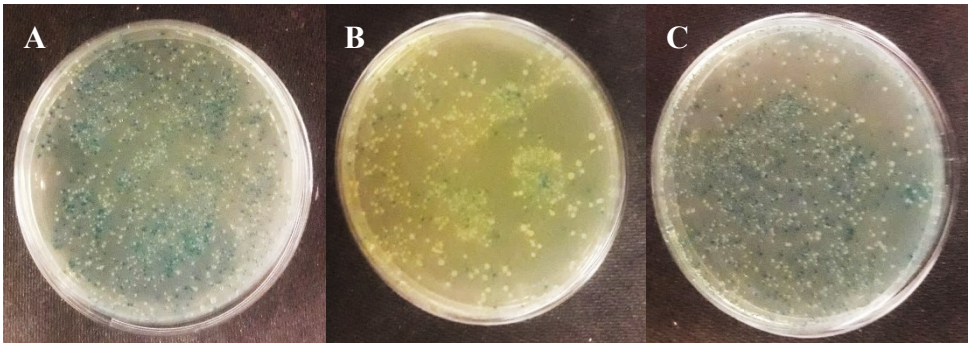

Figure3 – LB agar plates with grown colonies. White colonies were expected to contain vectors with inserts while blue colonies contain an empty vector. At least twenty white colonies were picked for each gene from each cell population of each donor. Plates with colonies with FOXP3 promoter (A), TSDR (B) and CAMTA1 gene region (C), from Treg cells of donor M_1 are shown as an example.

After, plasmid DNA was subjected to restriction digestion and positive clones were selected after the analysis of the reaction digestion patterns in agarose gel, as explained in Figure4.

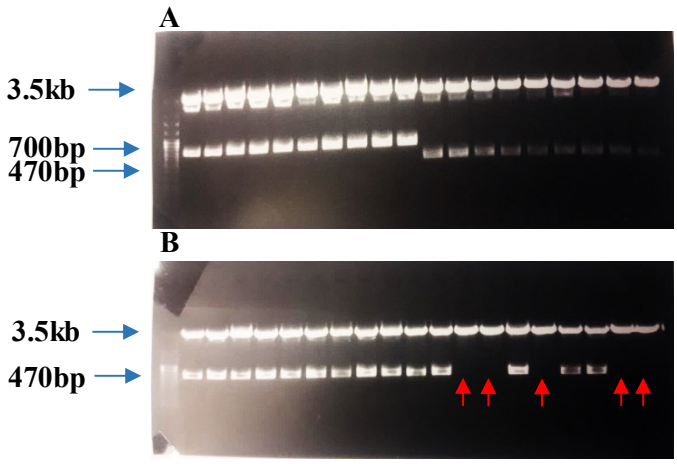

Figure4 – Selection of positive clones based on the electrophoresis pattern in 1% agarose gel. A. Positive clones were identified by the presence of two bands: the first, heavier band, corresponds to the vector with molecular weight of 3.5kb. The second band is the cloned insert: TSDR (700bp) or CAMTA 1 gene region (470bp). **B.** Clones that did not contain a DNA insert could be identified by the presence of only one band – an empty vector (3.5kb). Red arrows show clones without inserts during the screen for CAMTA1.

Finally, DNA from the positive clones was sequenced and aligned with the respective reference sequence, as shown in Figure5.

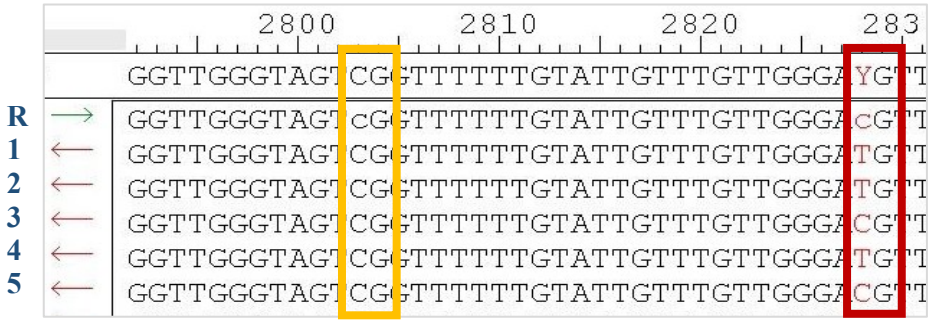

Figure5 – Sequence alignment (SeqMan software). Five DNA sequences (CAMTA1 gene region of Treg cells of donor M_1) were aligned to the respective reference sequence (**R**). The yellow square shows cytosines which remained unmodified after bisulphite conversion treatment meaning that all cytosines in the first CpG position of the original sequence are methylated. The red square shows that CpG position 2 in sequences 1, 2, and 4 contains a thymidine instead of a cytosine which means that a cytosine in the original sequence is not methylated.

In order to define the methylation levels of each CpG position, for each gene from each cell population (Treg and Tcon cells), the proportion of methylated cytosines was determined in the total of 20 sequences. This number was considered as representative of the entire cell population. Methylation patterns within three gene regions of one representative donor M_1 are shown in Figure6.

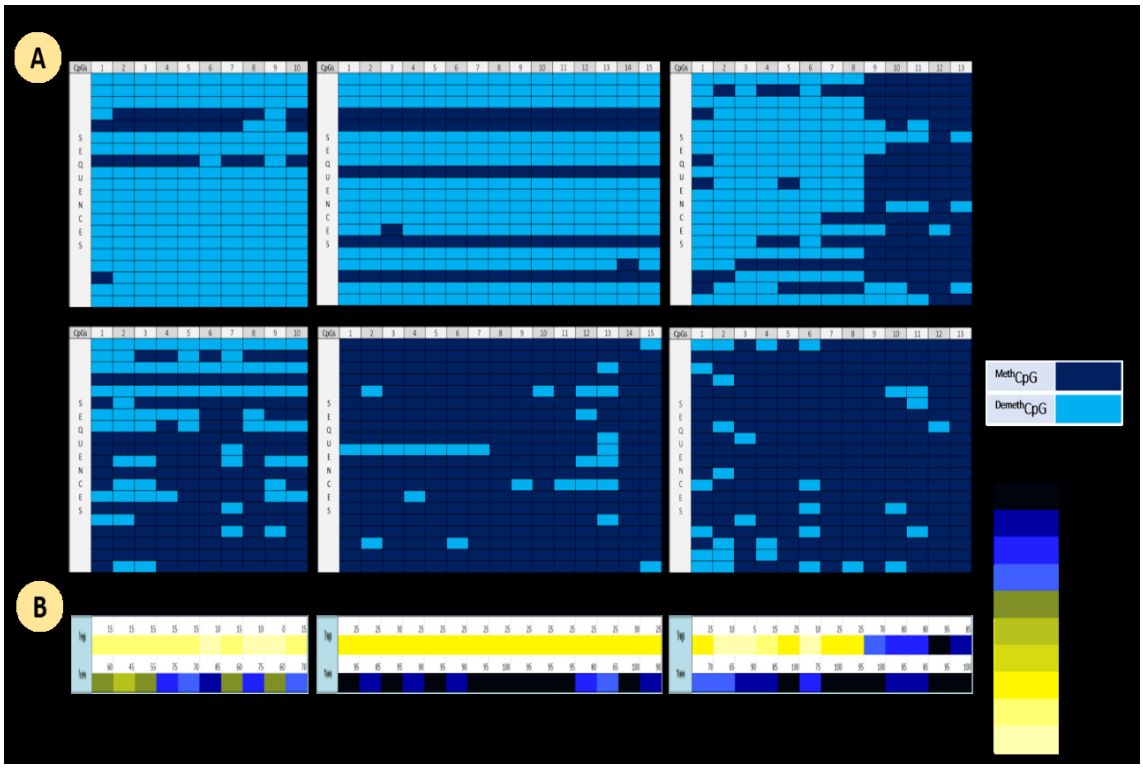

Figure6. Methylation patterns of FOXP3 promoter, TSDR, CAMTA1 in Treg cells and Tcon cells of male donor 1 (M_1). A. Analysed CpG positions from 20 DNA sequences of FOXP3 promoter, TSDR and CAMTA1 gene regions in Treg and Tcon cells. Each horizontal line represents one DNA and each column represents one CpG position. **B.** Merged methylation rates for each CpG position of each gene region of each donor. Darker colours represent higher methylation percentage while lighter colours represent higher demethylation percentage.

1. Methylation pattern of FOXP3 promoter region

FOXP3 promoter region is 450bp long and contains 10 CpG positions which were all studied in order to define their methylation status. As it can be observed in Figure7A, donors M_1, M_2 and F_1 presented a more methylated rate of FOXP3 promoter region in Tcon cells than in Treg cells. More specifically, the average methylation rate for Treg cells was 12,5%, 5,5%, and 0% for M_1, M_2 and F_1 donors respectively. On the other

hand, Tcon cells showed higher methylation rates of 65,5%, 50,5% and 38,5%, respectively.

This data supports previously published data on the methylation patterns of FOXP3 promoter in healthy donors.

However, promoter region remained completely demethylated in both Treg and Tcon cells in the majority of the remaining donors, showing no differences in the methylation pattern between these two cell populations (Figure7B).

Interestingly, no differences were found between male and female donors with average methylation levels of 0% in both cell populations.

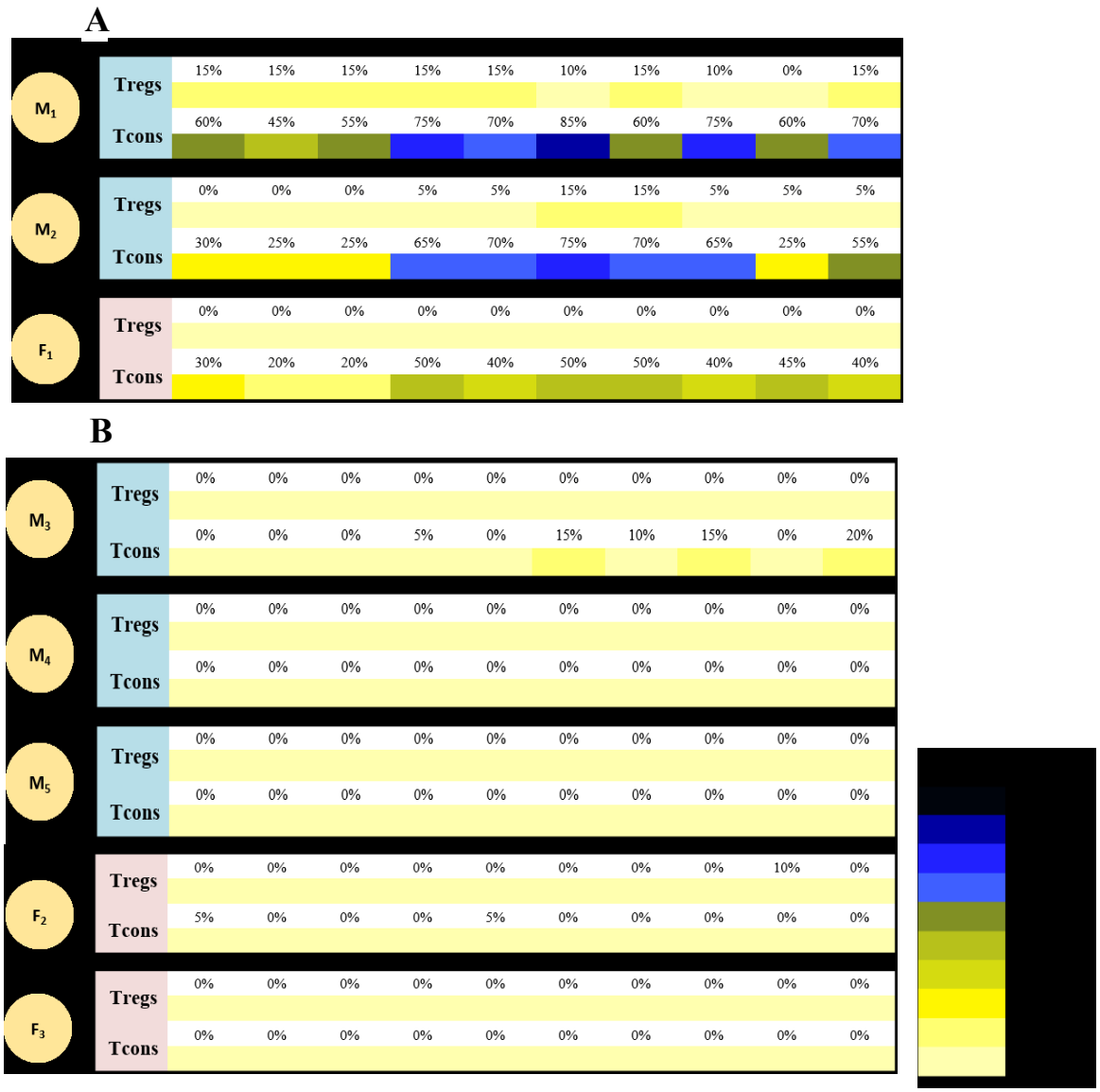

Figure7. Methylation pattern of FOXP3 promoter. A. Merged CpG methylation rates of FOXP3 promoter region of male donors 1 and 2 (M_1, M_2) and female donor 1 (F_1). **B.** Merged CpG methylation rates of FOXP3 promoter region of male donors 3-6 (M_3 – M_6) and female donors 2 and 3 (F_2, F_3).

2. Methylation pattern of FOXP3 TSDR region

The longest region that was studied was FOXP3 TSDR which is 700bp long and contains 15 CpG positions. As can be seen in Figure8A, M_1 and M_4 donors showed a pattern similar to that described in literature for healthy donors. FOXP3 TSDR in Tcon cells was more methylated compared to Treg cells: for M_1 the average methylation rate for Treg cells was 25,7% as compared to 91% for Tcon cells. Moreover, M_4 presented a complete methylation pattern for Tcon cells in contrast to an average methylation rate of 65,7% for Treg cells.

However, unexpected methylation patterns were found within TSDR region of other donors (Figure8B). High methylation levels in FOXP3 TSDR region were observed in both cell populations and in both genders, showing no significant differences.

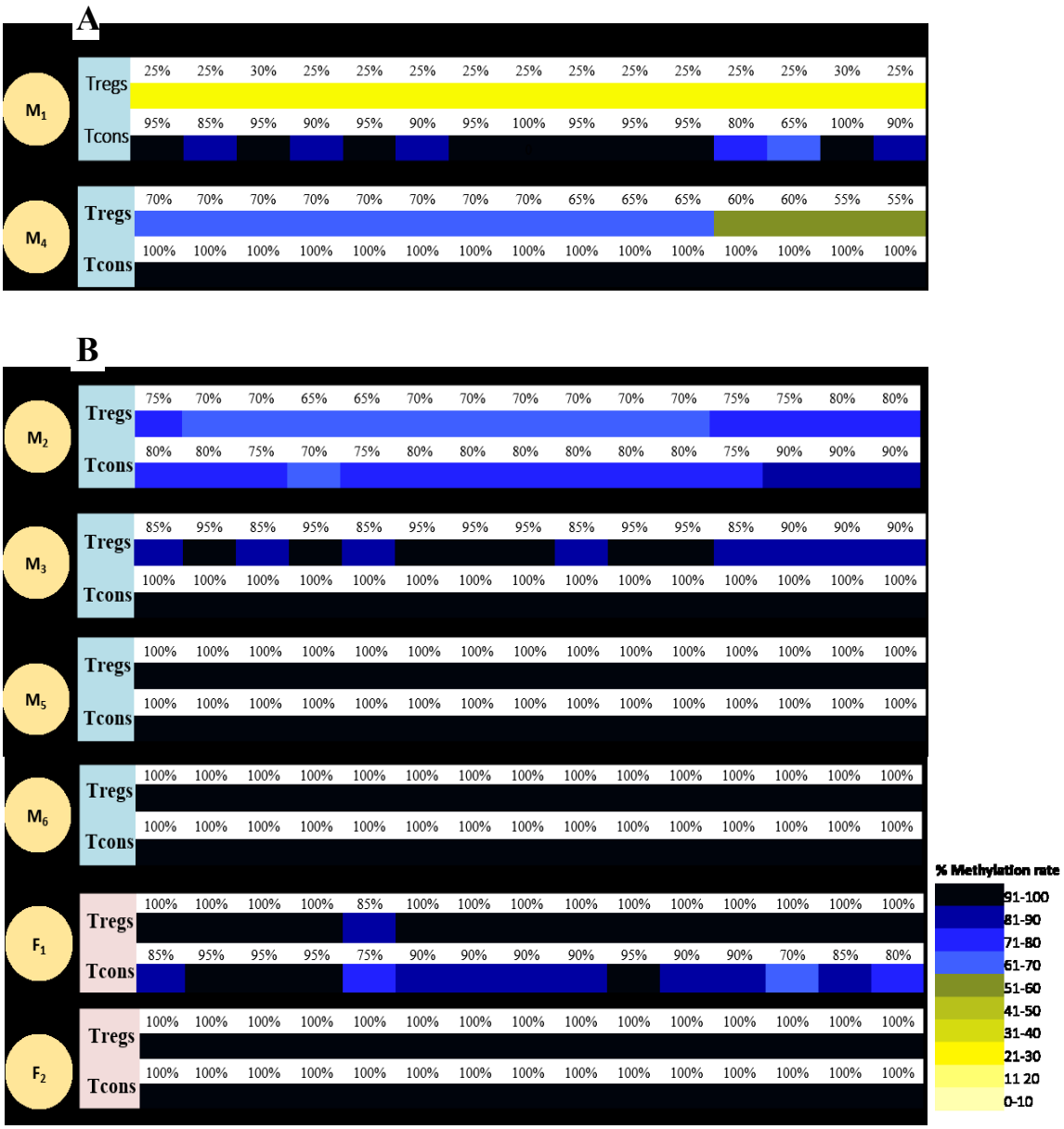

Figure8. Methylation pattern of FOXP3 TSDR. **A.** Merged CpG methylation rates of FOXP3 TSDR region of male donors 1 and 4 (M_1, M_4). **B.** Merged CpG methylation rates of FOXP3 TSDR region of male donors 2, 3, 5 and 6 (M_2, M_3, M_5, M_6) and female donors 1 to 5 ($F_1 – F_5$).

3. Methylation pattern of CAMTA1 gene region

Thirteen CpG positions within the 470bp region of CAMTA1 gene, located on chromosome 1, were analysed in this study.

As can be observed in Figure9, two patterns of methylation were present. The first 8 to 10 CpGs positions were more demethylated in Treg than in Tcon cells of the majority of donors (Figure9A). At the same time, interestingly, CpG positions 2 and 11 remained significantly more demethylated in Treg than in Tcon cells in donors that showed overall higher methylation rates in other CpG positions (Figure9B).

The average methylation rates for all CpG positions within the entire CAMTA1 region are presented in Figure9. In first group of donors (Figure9A), the average methylation rate for CpGs 1 to 10 was 19-53,5% in Treg cells and 37,5-90% in Tcon cells. In second group of donors (Figure9B), the average methylation percentage for CpGs 2 and 11 was 2.5-47,5% for Treg and 45,5- 87,5% for Tcon cells.

Overall, CAMTA1 gene region of Treg cells was more demethylated than that of Tcon cells. Once again, no significant differences were found between genders except the fact that male donors were more prone to display the first pattern (Figure9A).

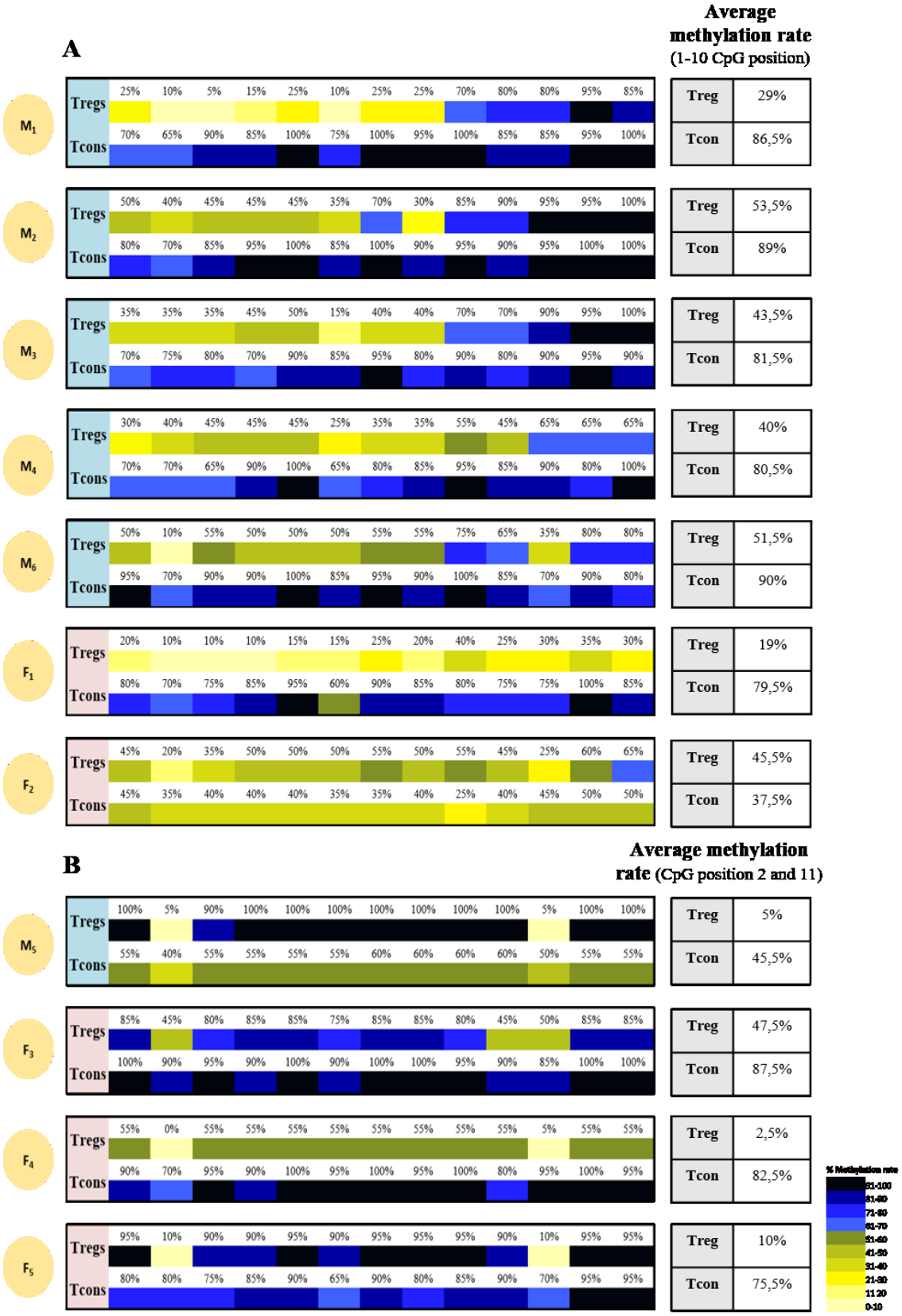

Figure9. Methylation pattern of CAMTA1 gene region. A. Merged CpG methylation rates of CAMTA1 gene region of male donors 1 to 4 and 6 (M_1 -M_4, M_6) and female donors 1 and 2 (F_1, F_2). **B.** Merged CpG methylation rates of CAMTA1 gene region of male donor 5 (M_5) and female donors 3 to 5 (F_3 – F_5).

DISCUSSION

Following the isolation of $CD4^+CD25^{bright}CD127^{low}$ $FOXP3^+CD45RA^+CD15s^-$ naïve Treg cells and $CD4^+CD25^{low}CD127^+$ Tcon cells and bisulphite treatment of gDNA from these cell populations, bisulphite converted DNA was amplified with gene-specific primers and cloned into pGEM-T vector. Twenty sequences from positive clones were then analysed to define the methylation status of individual CpG positions within each gene region. As shown in Figures 7, 8 and 9, methylation rates were defined for each CpG position of each gene region in study (FOXP3 promoter, FOXP3 TSDR and CAMTA1 gene region) from two cell populations of six male and five female random healthy donors.

M_1 and M_2 donors can be considered as standard donors based on the published literature: their Treg cells showed a more demethylated pattern not only in FOXP3 promoter region but also in TSDR region compared to Tcon cells, which would allow for the expression of FOXP3 protein in Treg cells. Similarly, CAMTA1 gene region, was more demethylated in Treg cells than in Tcon cells.

Interestingly, we observed two demethylation patterns in Treg cells of CAMTA 1 gene region: demethylation of the first eight-ten CpG positions (Figure9A), present in more donors, and demethylation of only two CpG positions: second and eleventh (Figure9B). Despite what was observed in the other two studied gene regions, and following the two patterns described above, CAMTA 1 remained significantly more demethylated in Treg cells than in Tcon cells in the majority of donors. It is, therefore, reasonable to state that compared to FOXP3 promoter and TSDR region, CAMTA1 seems to be a more reliable marker that differentiates Treg from Tcon cells.

In remaining male and female donors, FOXP3 promoter region showed complete demethylation in Treg and Tcon cells with no differences not only between the two subpopulations but also between genders. We hypothesize that promoter may have to be demethylated to allow the binding of transcription factors and the induction of transcription and expression. This could explain the existing phenomenon of FOXP3 expression in Tcon cells in transitory conditions while TSDR is completely methylated

or induction of a transient Treg phenotype (iTreg cells) with an incomplete demethylation of TSDR [8,19].

On the other hand, methylation of TSDR appears to be a more complicated process compared to what we initially gathered from literature. In fact, in most donors and without differences between the two genders, TSDR presented a complete methylation pattern not only in Treg cells but also in Tcon cells (Figure8B). These results were unexpected and did not confirm what has been published so far.
Remarkably, it came to light in preliminary work of our lab that followed this research that the demethylation of TSDR could be strand-dependent. In our recent findings (data not shown), the opposite strand of TSDR is significantly more demethylated in Treg cells than in Tcon cells and also shows differences between the two genders (unpublished data) that might be linked with X chromosome inactivation.

In order to have a more sustained conclusion for this study, it would be important to have a higher number of donors. Also, it would be interesting to know the exact family and personal clinical history of our donors as they were known so far as healthy and some clinical situations might have an impact on the epigenetic regulation and function of Treg cells [32-47].

In conclusion, the demethylation of CAMTA1 gene region in Treg cells is a gender independent process. Therefore, methylation pattern of CAMTA 1 gene region may be used to distinguish Treg cells from Tcon cells even when other specific molecular markers, such as TSDR, do not show any epigenetic differences.

In the future, the study of the epigenetic control of Treg cells will allow to understand exactly how and when the methylation mechanism occurs along the different maturations stages. Moreover, understanding the impact of the X chromosome inactivation on the methylation of FOXP3 locus and FOXP3 expression may contribute to understanding possible differences in clinical phenotypes between males and females in certain diseases, especially in AID.
Studying the methylation patterns of FOXP3 locus and CAMTA 1 gene region among patients with AID and allergies will be crucial not only in confirming recently published data on methylation of FOXP3 locus in Tregs of patients with these diseases but also in

understanding the role that CAMTA1 may have as an independent marker in these clinical situations.

In essence, these follow-up studies will allow us to better understand the epigenetic regulation mechanisms of Treg cells. This will open doors to finding new therapy targets focusing on Treg cells and also to optimizing cell therapies such as the ones already used in GVHD patients leading to a clinical outcome improvement in a wide range of diseases.

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APPENDIX I – RESUMO EXTENSO EM PORTUGUÊS

O gene FOXP3, codificado no cromossoma X e principalmente regulado por vias epigenéticas, apresenta um papel fundamental no desenvolvimento de células T reguladoras (Treg), essenciais à resposta a estímulos autoimunes, à manutenção da tolerância imunitária e homeostase, sendo amplamente utilizado como um marcador específico das células Treg. Existem diversas regiões no *locus* do FOXP3 reconhecidas pela sua importância no controlo epigenético, o qual consiste em alterações reversíveis na estrutura da cromatina sem haver alterações na sequência de nucleótidos de DNA: *enhancer*, promotor e três Sequências Conservadas de DNA Não Codificante (CNS), CNS 1-3. Especificamente, o *enhancer,* o promotor e o CNS2, também denominado de Região Desmetilada Específica de células Treg (TSDR), são responsáveis por este controlo através da desmetilação de ilhéus CpG das suas sequências.

Diferenças nos níveis de metilação da região TSDR contribuem para a distinção entre células Treg e linfócitos T convencionais (Tcon). Além disso, os níveis de metilação desta região poderão ainda contribuir para a distinção entre diversos estadios de maturação dentro da população das células Treg, por exemplo, os timócitos apresentam uma incompleta metilação do TSDR enquanto as nTreg (células Treg que proliferam a partir do timo), têm a região do TSDR completamente desmetilada.

Foi ainda demonstrado que, enquanto o FOXP3 é um marcador específico das células Treg, a desmetilação do TSDR é responsável pela manutenção da expressão do FOXP3, pelo que pode ser considerada como um marcador da estabilidade da linhagem de células Treg e compromisso das células para com a mesma. Tal observação poderá ser complementada pelo facto de ser possível termos populações de células Tcon a expressarem transitoriamente FOXP3 com o TSDR completamente metilado.

A par da região TSDR, verificou-se que o gene Calcium-dependent calmodulin-binding transcription factor 1, também denominado, CAMTA1, localizado no cromossoma 1, apresentava variações dos padrões de desmetilação semelhantes às verificadas no TSDR, apresentando-se como o outro marcador molecular que poderia contribuir para a distinção entre as duas populações de células. Até à data, o papel do gene CAMTA1 não foi estudado nas células T, apesar de se saber que o cálcio apresenta um papel importante na transcrição, especialmente nos mecanismos de transdução.

Disfunções das células Treg e um padrão de elevada metilação do *locus* do FOXP3, têm sido correlacionados com doenças autoimunes e alergias e também com um pior

prognóstico e morbilidade. Notar ainda que as mulheres apresentam uma maior suscetibilidade para doenças autoimunes comparativamente aos homens, o que poderá ser explicado pela inativação do cromossoma X, um processo que afeta cerca de 85% dos genes ligados ao X.

Tendo como hipótese que possíveis diferenças nos padrões de desmetilação do gene do FOXP3 possam estar relacionadas com as desigualdades clínicas observadas entre géneros, propôs-se comparar os padrões de desmetilação do promotor do FOXP3, TSDR e do gene CAMTA1 entre células Treg e Tcon de seis homens e cinco mulheres saudáveis com uma história clínica e familiar, até à data, desconhecida, no que diz respeito a doenças autoimunes e alergias.

Foram isoladas células T $CD4^+$ a partir de sangue periférico, tendo sido sujeitas a citometria de fluxo (FACS) e separadas células Treg naïve $CD4^+CD25^{bright}CD127^{low}$ $FOXP^{3+}CD45RA^+CD15^{s-}$ e células Tcon $CD4^+D25^{low}CD127^+$. O DNA genómico, após tratamento com bissulfito, foi amplificado por PCR, clonado em vetores pGEM-T e sequenciado. As sequências foram analisadas e os níveis de metilação estabelecidos.

Foi demonstrado que, quer em dadores do sexo feminino quer do sexo masculino, a região do gene CAMTA1, nas células Treg, apresentou um padrão consistente de maior desmetilação comparativamente ao das células Tcon. Por outro lado, resultados inconsistentes e não descritos até à data foram apresentados para o promotor do FOXP3 e TSDR.

No caso do promotor do FOXP3, apesar da existência de dadores que apresentavam resultados comparáveis ao que está escrito na literatura, ou seja, onde as células Treg apresentavam esta região mais desmetilada em relação às células Tcon, a maior parte dos dadores, quer em células Treg quer em células Tcon tinha o promotor do FOXP3 desmetilado.

No que diz respeito à região do TSDR, também existem dadores que confirmam a informação já apresentada na literatura recente. No entanto, a maior parte dos dadores apresentou esta região completamente metilada.

O gene CAMTA1 apresentou dois padrões diferentes de desmetilação nas células Treg: um primeiro padrão, com desmetilação das primeiras oito/dez posições CpG, e um segundo padrão, com a desmetilação do segundo e décimo primeiro CpG mantendo-se as restantes posições CpG com altos níveis de metilação. No entanto, as células Treg apresentaram esta região significativamente mais desmetilada que as células Tcon.

Em qualquer um dos três genes não foram observadas diferenças entre géneros.

Assim sendo, podemos afirmar que existem dadores que podemos considerar "dadores standard" dado que apresentam resultados sobreponíveis aos que têm vindo a ser publicados acerca deste tópico.

Além disso, será interessante mencionar que, o facto de a região do promotor do FOXP3 se ter apresentado mais desmetilada na maioria dos dadores, sem quaisquer diferenças entre as populações de células Treg e Tcon, poderá ser justificada pela necessidade de esta região estar desmetilada para permitir a ligação de fatores de transcrição e consequente expressão. Assim, só desta forma se poderá compreender o porquê de, transitoriamente, termos células Tcon, que apresentam o TSDR metilado, a expressar FOXP3 ou a própria existência de células iTreg, células Treg induzidas de Tcon na periferia em resposta a estímulos autoimunes periféricos, que apresentam a metilação do TSDR incompleta.

Quanto à região do TSDR no gene do FOXP3, através dos inesperados resultados, é possível compreender que o processo de metilação do locus do FOXP3 mais complexo do que se pensava ao início. Dado a maioria dos dadores ter apresentado o TSDR completamente metilado em ambas as populações de células, impulsionou novas investigações no nosso laboratório onde foi analisado o padrão de desmetilação da fita de DNA oposta que demonstraram que o processo de metilação desta região poderá ser "dependente da fita de DNA" em questão. Neste recente estudo do nosso laboratório, a fita de DNA oposta encontrava-se mais desmetilada nas células Treg do que nas células Tcon e apresentou diferenças entre homens e mulheres, resultados esses que poderão vir a ser associados ao processo de inactivação do cromossoma X (resultados não apresentados).

Concluindo, a desmetilação do gene CAMTA1 é um processo independente de género e o padrão de metilação deste gene poderá ser considerado um marcador molecular adicional mais fidedigno para a distinção entre células Treg e Tcon quando a expressão de FOXP3 possa estar afetada e a região do TSDR não apresenta diferenças epigenéticas.

No futuro, é importante continuar a estudar o controlo epigenético das células Treg para que seja compreendido como exatamente ocorre e em que momentos da vida destas células acontece. Além disso, o impacto da inativação do cromossoma X no padrão de metilação do *locus* do FOXP3 poderá contribuir para justificar o desequilíbrio de prevalências de doenças autoimunes existente entre homens e mulheres. Por fím, o

estudo dos padrões de desmetilação em doentes com AID ou alergias poderá corroborar os dados já expressos na literatura recente em relação à região TSDR mas também perceber se o gene CAMTA1 pode ter algum papel como um marcador independente nestas situações clínicas. Tais descobertas abrirão portas para a pesquisa de novos alvos terapêuticos, otimização de terapias celulares já existentes, em buscar de melhores *outcomes* clínicos para múltiplas doenças.