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FOXP3, ICOS and ICOSL **polymorphisms in an Italian population affected by systemic sclerosis**

PhD thesis

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List of abbreviations and synonyms

APC, antigen presenting cell
ATG5, autophagy protein 5
B7RP-1, B7-related protein-1
BAFF, B cell activating factor
BAFFR, B-cell activating factor receptor
BCL-6, B cell lymphoma 6
BCR, B cell receptor
Breg, regulatory B cell
CCDC22, coiled-coil domain containing protein 22
CCR6, C-C chemokine receptor type 6
COMMD, copper metabolism gene MURR1 domain (protein)
CTLA-4, cytotoxic T lymphocyte-associated antigen 4
DNMT1, DNA (cytosine-5)-methyltransferase 1
EBI3, IL-27 subunit β
ENTPD1, ectonucleoside triphosphate diphospfohydrolase-1
FCRL3, Fc receptor-like protein 3
FOXP3, forkhead box P3 (transcription factor)
GC, germinal centre
GITR, glucocorticoid-induced TNFR family related gene
GWAS, Genome-wide association study
HRM, high resolution melting
ICAM3, intercellular adhesion molecule 3
ICOS, inducible T-cell costimulator; CD278
ICOSL, inducible T-cell costimulator ligand; B7-related protein 1; CD275
IFIT2, interferon-induced protein with tetratricopeptide repeats 2
ILD, interstitial lung disease
IRF5, interferon regulatory factor 5
iTreg,induced regulatory T cell
JNK, c-Jun N-terminal kinase
MAF, minor allele frequency
MAPK, mitogen activated proteinkinase
MBD4, methyl-CpG-binding domain
MHC, major histocompatibility complex
mTEC, medullary thymus epithelial cell
NFAT, nuclear factor of activated T cells
NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP1, NACHT, LRR and PYD domains-containing protein 1
nTreg, natural regulatory T cell
PAH, pulmonary arterial hypertension
PCR, polymerase chain reaction
PD-1, programmed cell death-1
PD-L, PD ligand
PLD4, phospholipase D family member 4
PTPN22, protein tyrosine phosphatase-non-receptor type 22
pTreg, peripherally derived regulatory T cell
RA*,* rheumatoid arthritis
RTKN2, Rhotekin 2
SNP, single nucleotide polymorphism

STAT1 (3) (4), signal transducer and activator of transcription 1 (3) (4)
SLE, systemic lupus erythematosus
SSc, systemic sclerosis
T1DM, diabetes mellitus type 1
TBK1,TANK-binding kinase 1
TCR, T cell receptor
TFH, T follicular helper
TIM-3, T cell immunoglobulin and mucin domain-3
TLR, Toll-like receptor
Treg, regulatory T cell
tTreg, thymus-derived regulatory T cell
USP18, ubiquitin specific peptidase 18
UTR, untranslated region

1. Introduction

1.1 FOXP3 and Tregs

Forkhead box P3 (FOXP3) is a member of the forkhead transcription factor family encoded by the *FOXP3* gene on the X chromosome. This protein suppresses the function of nuclear factor of activated T cells (NFAT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), thus leading to the underexpression of many genes including IL-2 and effector T-cell cytokines. FOXP3 is responsible for the transcriptional regulation of genes involved in the regulatory T cell (Treg) phenotypic and functional signature, including cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (Grant et al., 2015), CD2S, glucocorticoid-induced TNFR family related gene (GITR) and folate receptor 4 (Kim, 2009). FOXP3 is specifically expressed in $CD4^+CD25^+$ Treg cells, and is required for their development.

Treg cells have an immune suppressive capability, maintaining immune homeostasis and preventing autoimmunity induced by excessive, misdirected, or unnecessary immune activation (Fontenot et al., 2003). Genetic deficiency of FOXP3 induces dysfunction of Treg cells and immuno-dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome in humans (Nie et al., 2015). Interestingly, FOXP3-deficient Treg cells show decreased levels of Treg cell signature genes, such as CTLA-4, IL-27 subunit β (EBI3), IL-10, ectonucleoside triphosphate diphospfohydrolase-1 (ENTPD1). Conversely, this cell subset acquires the expression of T effector cytokine genes such as INF-γ, TNF-α, IL-4, and IL-17 (Gavin et al, 2007).

Treg cells are approximately 5–15% of the $CD4^+$ T cell compartment and can be divided into two subpopulations: thymus-derived Treg (tTreg) cells and peripherally derived Treg (pTreg) cells. tTreg cells are also known as natural Treg (nTreg) cells. In the thymus, $CD4^+CD8^+$ T cells undergo negative selection and become mature tTreg cells through IL-2, IL-15 and TGF-β signals. pTreg cells differentiate in the periphery after the contact between naïve $CD4^+$ T cells and the antigen and in the presence of TGF-β and IL-2. pTreg cells have similar suppressive role of tTreg cells *in vitro* and *in vivo* (Chen et al., 2003). Induced T regulatory T cells (iTreg) are another subpopulation of Treg cells, generated *in vitro* through TGF-β signals (Abbas et al., 2013). Furthermore, Treg cells can be classified basing on tissue specificity (central Tregs, effector Tregs, and tissue resident Tregs) and on homeostasis activity (highly dynamic and apoptotic) (Yang et al., 2015). Figure 1 summarizes the p- and tTreg development process. Another Tregs classification is based on ICOS expression (Vocanson et al., 2010).

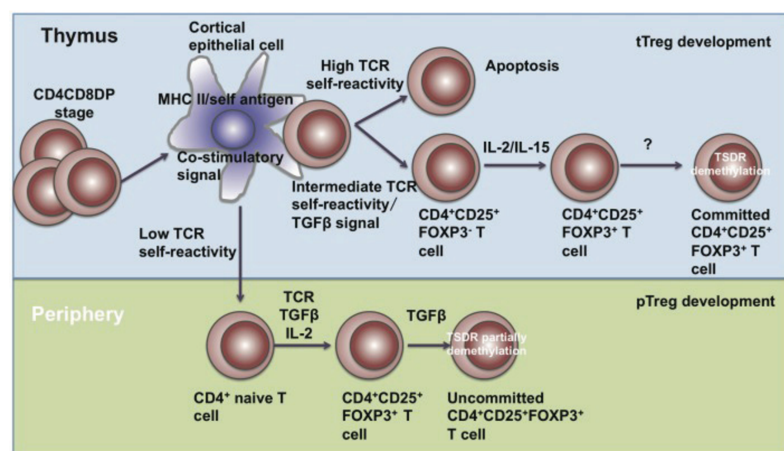

Figure 1. The development of Treg cells. Different subpopulation of Treg cells develop in different regions. In the thymus, CD4+CD8+ T cells undergo negative selection and become mature tTreg cells through IL-2, IL-15 and TGF-β signals. In the periphery, naïve CD4+ T cells encounter antigen and differentiate into pTreg cells in the presence of TGF-β and IL-2 (Abbas et al., 2013).

Tregs are able to suppress immune responses through different mechanisms (figure 2A):

- CTLA-4 on Tregs surface can down-regulate the co-stimulatory molecules CD80/CD86 limiting the initiation of an adaptive immune response (André et al., 2009).
- Tregs induce the apoptosis of effector T cell by the interaction between galectin-9 and the T cell immunoglobulin and mucin domain-3 (TIM-3), and by the release of granzymes, which enter T effectors via perforin pores (Zhang and Shan, 2014).
- Tregs can release TGF-β, IL-10 and IL-35 that are anti-inflammatory cytokines (Li et al., 2012).
- Tregs can enable the hydrolysis of pro-inflammatory adenosine triphosphate (ATP) into anti-inflammatory adenosine (ADO) through the expression of the ecto-enzymes CD39 and CD73 (Antonioli et al., 2013).

In physiological conditions, Tregs maintain tolerance by exerting suppression of effector T cells. In pathological conditions, such as in autoimmune diseases, Tregs fail to suppress autoreactive effector T cells, thus leading to target cell death. Treg cells failure may be due to several causes, including inadequate numbers of Tregs, impaired suppressive ability, Treg conversion into effector cells, and resistance of effector T cells to Treg-mediated suppression (Grant et al., 2015) (figure 2B).

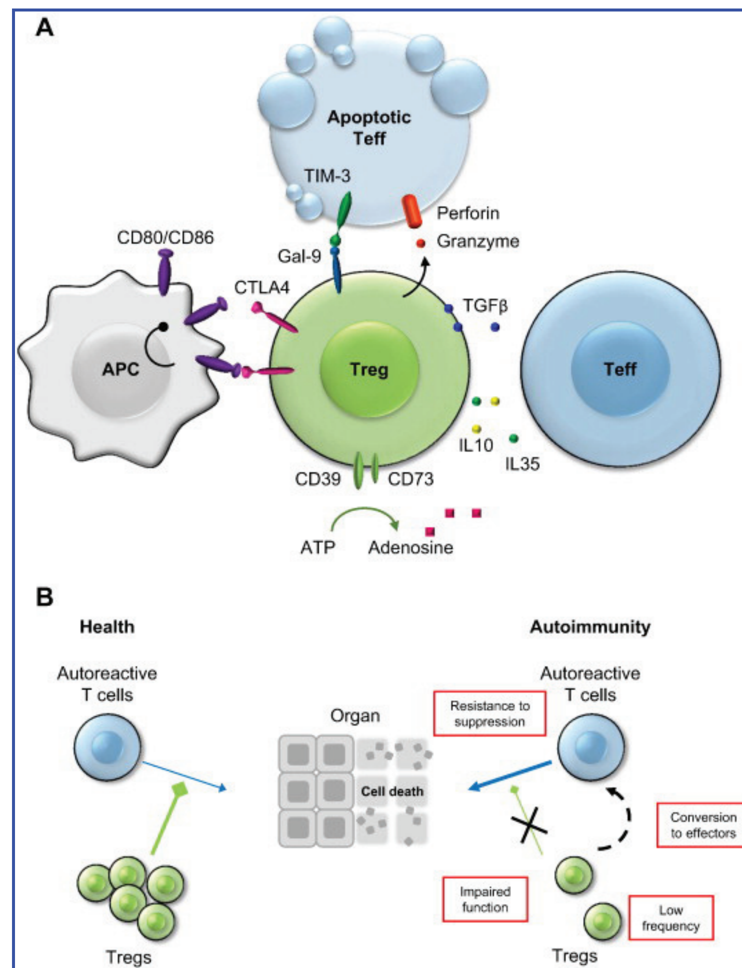

Figure 2. Main Treg functions. For explanation, refer to text (Grant et al., 2015).

Recently, Authors have found that Treg cells are able to transfer microRNAs to various immune cells, including T_H1 cells, suppressing their proliferation and cytokine secretion, playing also an epigenetic role in the modulation of the immune response (Okoye et al., 2014; Rothstein and Camirand, 2016).

1.2 ICOS and ICOSL

The beginning of conventional T cell activation requires the interaction between the T cell receptor with MHC class I or class II-peptide complexes. Moreover, this activation requires also secondary co-stimulatory signals that, synergistically with TCR stimulation, are able to facilitate T cell activation (Sharpe and Freeman, 2002). Many studies have shown that CD28, a T cell-specific cell-surface receptor (Hutloff et al, 1999) plays a crucial role in the secondary co-stimulatory signal, promoting cellular proliferation and survival after T cell activation (Wikenheiser and Stumhofer, 2016). CD28 can bind to B7.1 (CD80) and B7.2 (CD86) placed on APC (Sansom, 2000) (figure 3).

CTLA-4, the CD28 homologous, compensates the CD28-mediated signals, preventing the overstimulation of the immune system (Hutloff et al., 1999). A third member of CD28 family, ICOS, has significant homology to the co-stimulatory molecule CD28 and the immune-attenuator CTLA-4 (Rudd and Schneider H, 2003).

ICOS is a type-I transmembrane molecule that consists of a single immunoglobulin (Ig)V-like domain, a transmembrane region of 23 amino acids, and a cytoplasmic tail of 35 amino acids, and it shows a close structural resemblance to CD28 and CTLA-4 (Hutloff et al., 1999). Location of both ICOS and CD28 on chromosome 2, adjacent to each other, suggests the occurrence of evolutionary gene duplication (Beier et al., 2000).

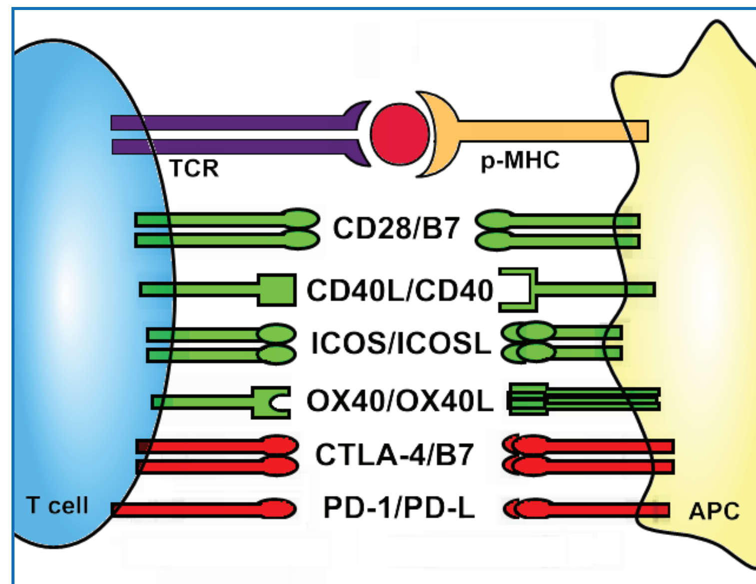

Figure 3. There are several costimulatory pathways including both activating (depicted in green) and inhibitory (depicted in red) pathways. The activating pathways are critical for T and B cell activation. Inhibitory pathways downregulate T cell activation.

ICOS is not constitutively expressed on resting T cells. Indeed, it is rapidly induced after TCR cross-linking and/or CD28 co-stimulation (Wikenheiser and Stumhofer, 2016). Together with CD28 and CTLA-4, ICOS is expressed on activated $CD4^+$ and $CD8^+$ T cells, underlining its involvement in the adaptive T cell response to a foreign antigen (Watanabe et al., 2005). ICOS binds to its ligand (ICOSL; B7RP-1) expressed on B cells and dendritic cells (Yoshinaga et al., 1999).

ICOSL expression is regulated by B-cell activating factor receptor (BAFFR) and non-canonical NF-kB signalling (Hu et al., 2011). ICOS stimulation induces, through the recruitment of class IA phosphatidylinositol 3-kinase (PI3K), the production of phosphatidylinositol 3,4,5-trisphosphate (PIP3), which is known to activate Akt pathway. This pathway promotes cell proliferation and survival (Coyle et al., 2000; Arimura et al., 2002; Fos et al.,

2008). ICOS stimulation induces the mitogen activated proteinkinase (MAPK) pathway activation by phosphorylation of p46 c-Jun N-terminal kinase (Arimura et al., 2002). Furthermore, ICOS cytoplasmic tail has a characteristic motif, termed Iprox, which is able to recruit the serine/threonine-protein kinase, named TANK-binding kinase 1 (TBK1), a member of the inhibitor of NF-κB kinase (IKK) family (Pedros et al., 2016). The recruitment of TKB1 plays a crucial role in promoting the transition from early T follicular helper (Tfh) cell to germinal centre (GC)-resident Tfh cell (Pedros et al., 2016). Figure 4 summarizes the molecular events underlying PI3K and MAPK pathways, as well as TBK1 recruitment by ICOS/ICOSL.

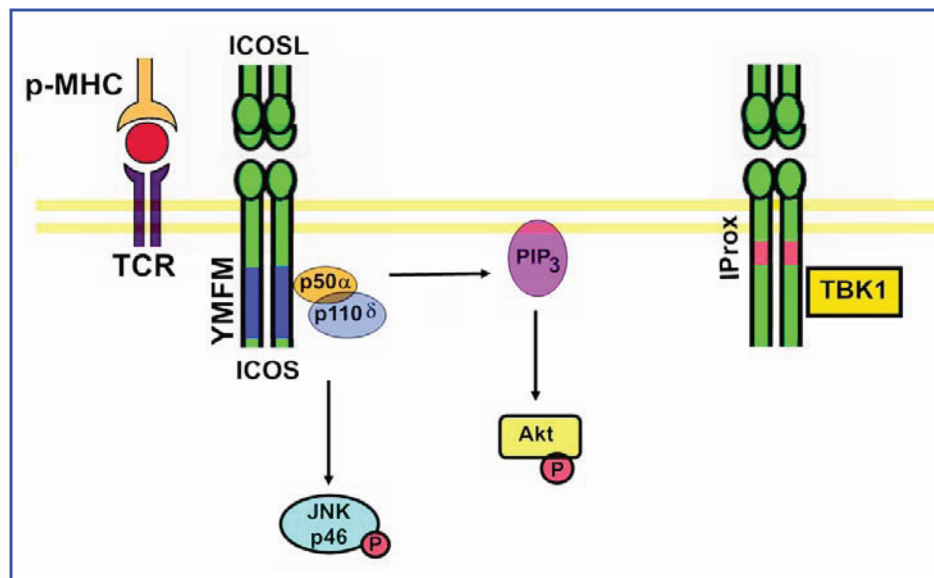

Figure 4. ICOS/ICOSL interaction induces, through its YMFM signalling motif, the recruitment of p50α regulatory subunit of the PI3K pathway, resulting in PIP3 production and Akt phosphorylation. Moreover, ICOS/ICOSL interaction may induce c-Jun N-terminal kinase (JNK) p46 phosphorylation. Intracellular IProx domain of ICOS induces the recruitment of TBK1, an inhibitor of NF-κB kinase.

ICOS, first considered a key player only in T-cell responses to a foreign antigen, as well as in secretion of lymphokines and upregulation of molecules that mediate cell–cell interaction (Hutloff et al., 1999), has recently been discovered to have other important functions. In particular, it has been found that the interaction of ICOS with ICOSL triggers key activities of T cells, including cytokine production and differentiation into Tfh cells of secondary lymphoid tissues (Leconte et al., 2016). Tfh cells are crucial for the formation and maintenance of GCs. Inside GCs, these cells provide help for the generation of high-affinity memory B cells and long-lived plasma cells (Crotty, 2011). This process guarantees that, following antigen encounter, only B cells with the highest affinity for that antigen differentiate into plasma cells and memory B cells (Victora and Nussenzweig, 2012).

Tfh cells differ from T_H1 and T_H2 cells for the simultaneous expression of the chemokine receptor CXCR5, programmed cell death-1 (PD-1), and B cell lymphoma 6 (BCL-6), which is regulated by ICOS signalling (So and Fruman, 2012). After Tfh activation, BCL6 induces the production of IL-6 and IL-21 that are necessary for the differentiation of B cells into Ab-producing cells (Diehl et al., 2012). Interruption of ICOS-ICOSL interaction, ICOS deficiency (ICOS−/− mice) or alteration of ICOS expression lead to impaired GC responses (Crotty, 2011). In particular, ICOS−/− mice develop an autoimmune lupus-like phenotype that resembles the clinical condition of patients with lupus. According to studies on mice models, phenotypic characterisations of patients with an ICOS null genotype (ICOS−/−) showed significant reductions in circulating memory Tfh, class-switched memory B cells and serum IgG (Grimbacher et al., 2003). The overexpression of ICOS pathway through a point mutation in RC3H1

(roquin 1), a repressor of ICOS, leads to the development of a lupus-like systemic autoimmune disease in mice, suggesting a direct link between ICOS-ICOSL and autoimmunity (Mittereder et al., 2016).

As mentioned above, ICOS expression on Treg surface allows the classification of an important Treg cells subset (Vocanson et al., 2010). $CD25^+FOXP3^+ICOS^-$ cells produce TGF-β, while the $CD25^+FOXP3^+ICOS^+$ subpopulation is able to secrete both IL-10 to suppress dendritic cell function and, TGF-β to suppress T cell function (Ito et al., 2008). The survival and proliferation of the two subsets of Treg cells were differentially regulated by CD28 or ICOS, respectively. The $CD25^+FOXP3^+ICOS^+$ subset has two mechanisms for immunosuppression: the IL10-mediated suppression of APC function and TGF-β-mediated T cell-T cell-contact-dependent suppression. In contrast, the $CD25^+FOXP3^+ICOS^-$ subset shows only a TGF-β-mediated T cell-T cell contact-dependent suppression. In addition, ICOS and CD28 costimulation has opposing effects on $ICOS^+$ Treg cells; although ICOSL is able to costimulate their proliferation, experimental anti-CD28 signalling inhibits their proliferation. Conversely, anti-CD28 signalling strongly promotes $ICOS^-$ Treg cells proliferation (Ito et al., 2008).

1.3 Autoimmune diseases

Affecting approximately 4% of the population in industrialized countries, autoimmune diseases are the result of an interplay between predisposing genes and environmental factors. This interplay leads to loss of self-tolerance and an immune-mediated destruction of autologous cells and/or tissues. (Lie and Thorsby, 2005). Immune system in healthy subjects is characterized by

tolerance to molecules recognized as ‘‘self,’’ and thus does not respond to elements (carbohydrate, nucleic acid, or protein) that are expressed in endogenous tissues. When self-tolerance is lost, the immune system becomes reactive against one or more self-molecules (Janeway et al., 2001). Self-tolerance is maintained through different mechanisms. The ‘‘central tolerance’’ takes place mainly in the thymus.

In the thymic cortex, naïve T_H0 lymphocytes, that ignore self-elements, are positively selected, while T cells precursors, that exhibit any affinity for self-antigens, undergo apoptosis. T_H0 lymphocytes then migrate to the thymic medulla where they are exposed to self-antigens in the presence of MHC type I receptors. T cells which bind self-antigens with high affinity in the absence of MHC type II and a co-stimulatory signal are negatively selected and redirected into a TCR-mediated apoptosis (Bolon, 2011). Apoptosis pathway, responsible for this central tolerance, is controlled by interactions between Fas and Fas ligand. The lack or mutation of these molecules reduces autoreactive T cells selection, promoting systemic autoimmunity (Nagata and Suda, 1995). The “peripheral tolerance” takes place in secondary lymphoid organs and/or sites of inflammation and includes suppression of autoreactive cells by Treg cells and anergization of lymphocytes which encounter antigen in the absence of the co-stimulatory signals, or in the presence of co-inhibitory signals. Breakdown of one or more of these central or peripheral T cell–based tolerance mechanisms is responsible for the onset of autoimmune diseases (Murphy and Kenneth, 2012). The induction of autoimmunity is often considered to be an acquired response, but innate immune cells play important roles in moderating self-tolerance (Waldner, 2009).

In the innate immune system, three endosomal TLRs have been identified as major participants in some autoimmune diseases (Trivedi and Greidinger, 2009). These molecules are receptors able to recognize microbial or viral nucleic acid: TLR-3 for double-stranded RNA, TLR-7 for single-stranded RNA and TLR-9 for double-stranded DNA. Once bound with the appropriate nucleotides, TLRs induce a pro-inflammatory signal. Unfortunately, these TLRs are thought to have also a central role in autoimmune diseases by directing the cells that express them to attack self-molecules and enhance their expression of pro-inflammatory cytokines (Waldner, 2009).

Cytokine imbalances in the acquired immune system are well known to have a central role in the induction and progression of autoimmune diseases. In normal tissues cellular subsets, especially T lymphocytes and macrophages, express both anti-inflammatory cytokines and soluble cytokine inhibitors to down-regulate the immune response. Conversely, B lymphocytes and many non-immune but activated cells (i.e. endothelium, fibroblasts, synoviocytes) in inflamed tissues produce pro-inflammatory cytokines. The opposite behaviours of these cell subsets underline that autoimmune diseases may develop when the normal balance between pro-inflammatory and anti-inflammatory signals within a target organ is disrupted, leading to a chronic relative excess of local pro-inflammatory stimuli (Bolon, 2012).

Enhanced activity of $CD4^+$ T_H cells is responsible for the common pro-inflammatory phenotypes in autoimmune diseases. Among them, three distinct phenotypes are crucial: T_H1, T_H2, and T_H17. Organ-specific autoimmune diseases are T_H1-driven, mainly due to IL-2 and INF-γ release. In contrast, systemic autoimmune diseases are due to T_H2-dependent cytokines (such as

IL-4, IL-5, and IL-10), massive autoantibody production and immune complex deposition. T_H17 subset contributes to the pathogenesis of autoimmune diseases by producing IL-23, acting as a neutrophil chemotactic and activating factor (Zambrano-Zaragoza et al., 2014). T_H1 and T_H17 subsets generally differentiate in parallel because of the overlap, even if only partially, in their functions (Martin-Orozco et al., 2009).

Many autoimmune diseases could be correlated to a dysregulated intracellular signal transduction. Indeed, reduced CTLA-4 expression in Treg cells leads to an inappropriate activation of naïve T cells and promotes retention of autoreactive T cells in organs (Jain et al., 2010). Furthermore, autoimmunity can be due to altered levels of signal transducer and activator of transcription 3 (STAT3). This protein controls the phenotype of naïve $CD4^+$T cells, thus playing an important role in the selection between T_H17 and Treg subset. Moreover, STAT3 is involved both in T cells growth and survival, and in the transcription of pro-inflammatory genes (Zhernakova et al., 2013).

Dysregulated NF-κB signalling, a master regulator of gene transcription in cells of the innate and acquired immune systems, has been shown to play an important role in several autoimmune diseases (Dozmorov et al., 2013).

Moreover, many studies suggest the involvement of oxidative stress in the pathogenesis of chemically-induced autoimmune diseases. Indeed, the imbalance between oxidants and antioxidants, with a prevalence of the former, leads to interruption of redox signalling and damage of cell membranes, lipids, proteins and nucleic acids (Mateen et al., 2016).

Genetic risk factors in the development of autoimmune diseases have been discovered through genome-wide association studies (GWAS), a standard

approach to the identification of susceptibility genes for complex traits (Gutierrez-Arcelus et al., 2016). A GWAS study usually screens up to 1,000,000 SNPs by comparing the allele/genotype frequency between subjects affected by the disease and healthy controls (Kochi, 2016). GWAS can only identify risk loci associated with the disease. To determine the responsible genes or disease-causing variants in the loci, further analyses are required. Coding and regulatory variants are known to be enriched in the GWAS loci of complex diseases; these variants can qualitatively and/or quantitatively affect the function of genes. Even before GWAS, MHC genes, in particular HLA ones, were subject of intensive investigation since they play an essential role in antigen presentation in adaptive immune responses. HLA complex, located on the short arm of chromosome 6 (6p21.3), is known to harbour major genetic determinants for autoimmune diseases, underlining its role as strong predisposing genetic factors (Thorsby and Lie, 2005).

Studies based on SNP array data have identified relevant alleles of all classical HLA genes. In rheumatoid arthritis (RA), the shared epitope of HLA-DRB1 (amino acid positions 70–74) has previously believed to be responsible for this disease (Gregersen, 1987), but the most recent amino-acid-based association analysis have revealed that the amino acid at residue 11 is also crucial (Raychaudhuri, 2012). Single amino-acid nucleotide in HLA, at position 9, and HLA-DPB1, at position 11, have been also associated with RA independently of HLA-DRB1 (Viatte et al., 2012). Additional association in HLA-A, at position 77, was also found in patients with autoantibodies for anti-citrullinated proteins (Stahl et al, 2010).

Involvement of alleles in multiple HLA genes has been also shown in other autoimmune diseases, such as type 1 diabetes (Hu et al, 2015), multiple sclerosis and Graves' disease (Moutsianas et al., 2015). Recent studies have identified additional susceptibility genes, shared by most autoimmune diseases and involved in common pathways, such as protein tyrosine phosphatase-non-receptor type 22 (PTPN22), interferon regulatory factor 5 (IRF5), signal transducer and activator of transcription (STAT4), B-cell scaffold protein with ankyrin repeats 1 (BANK1) and intercellular adhesion molecule 3 (ICAM3) (Zhernakova et al., 2013).

The analysis of epigenetic alterations in $CD4^+$ T cells from patients has revealed that DNA methylation pattern is profoundly changed compared to healthy controls. Many autoimmune diseases, including systemic lupus erythematosus (SLE), RA and systemic sclerosis (SSc) have been shown to be characterized by a hypomethylation pattern, which is associated with a decreased expression of methylation-related genes, such as DNA (cytosine-5)-methyltransferase 1 (DNMT1) and methyl-CpG-binding domain (MBD4) (Lei et al., 2009). In particular, $CD4^+$ T cell subset from these patients shows an important reduction in the levels of DNA methylation on the promoter of CD40LG (which encodes the B cell costimulatory molecule CD40L; also known as CD154). These data are thought to be important in autoimmune diseases pathogenesis since CD40–CD40L interaction has a key role in T_H17 cells differentiation and IL-17 production (Iezzi et al., 2009). Interestingly, it has been found that demethylation of CD40L regulatory elements on the female inactive X chromosome upregulates CD40L expression in $CD4^+$ T cells, giving a

possible explanation for the susceptibility of women to these autoimmune diseases (Lu et al., 2007).

Patients with SLE, SSc and primary Sjögren's syndrome show a hypomethylation of CD70 (also known as TNFSF7 or CD27L) gene that probably contributes to the overexpression of its protein on $CD4^+$ T cells (Jiang et al., 2012). CD4+ T cells from patients with SLE and primary Jorgen's syndrome show a hypomethylation pattern in INF-regulated genes, such as IRF5, interferon-induced protein with tetratricopeptide repeats 2 (IFIT2), STAT1 and ubiquitin specific peptidase 18 (USP18). This hypomethylation is present in T cells before their activation and differentiation, and it persists in the immune system throughout and even beyond active stages of the disease. These data may provide an explanation for the important role that type I interferon pathway plays in the pathogenesis of these autoimmune diseases (Absher et al., 2013; Altorok et al., 2014; Coit et al., 2013). It is noteworthy that not all overexpressed genes in autoimmune diseases are characterized by an epigenetic alteration. For this reason, further mechanisms leading to their overexpression need to be investigated.

Genes in lymphocytes could also be hypermethylated. Indeed, it has been reported that the hypermethylation of regulatory regions of FOXP3 in peripheral blood $CD4^+$ T cells from patients with SSc, RA and diabetes mellitus type 1 (T1DM) modifies FOXP3 expression, which is required for the generation of Tregs (Wang et al., 2014). These findings might indicate an epigenetic alteration responsible for the loss of immune homeostasis in development of SSc, T1DM and RA.

1.4 Systemic Sclerosis

Systemic sclerosis is an autoimmune disorder of unknown aetiology associated with substantial morbidity and mortality (Nikpour et al., 2010). SSc is characterized by vasculopathy, inflammation, progressive perivascular and interstitial fibrosis (Desbois and Cacoub, 2016). Clinical manifestations of SSc include skin thickening, Raynaud’s phenomenon, digital ulcers, gastro-oesophageal reflux disease, intestinal hypomotility and pseudo-obstruction, pulmonary arterial hypertension (PAH), interstitial lung disease (ILD) and renal diseases (van den Hoogen et al., 2013a).

SSc has different phenotypes and could be classified, basing on symptomatology and a physical exam, in:

- limited cutaneous systemic sclerosis (lcSSc): distal cutaneous portion of the neck, elbows and knees are affected
- diffuse cutaneous systemic sclerosis (dcSSc): cutaneous areas of the neck, elbows and knees are affected proximally
- systemic sclerosis sine scleroderma: vascular manifestations and serologic changes are compatible with diffuse cutaneous scleroderma without cutaneous sclerosis
- polyautoimmunity (van den Hoogen et al., 2013a)

Clinical diagnosis of SSc is based on serum levels of autoantibodies. Autoantibodies in SSc patients are heterogeneous, reflecting the complexity of the disease. Anti-topoisomerase-I (Scl70) antibodies have high specificity for SSc and are present in up to 45% of patients. Topoisomerase-I reactive cells are significantly increased in subjects with ILD and were associated with the

decline of pulmonary volumes (Fava et al., 2016). The quantification of these autoreactive T cells may be used to predict the presence and progression of ILD in SSc.

Up to date, SSc pathogenesis remains unknown, but there is growing evidence suggesting a close connection between environmental factors and SSc pathogenesis and many clinical trials have started (Clinical Trials Gov. Systemic sclerosis. 2016. Available at: http://bit.ly/2cQnXdi). In particular, most of available data suggest that environmental and genetic factors, together with regulatory epigenetic mechanism may play a central role in the pathogenesis of SSc, as seen for many other autoimmune diseases (Barsotti et al., 2016).

DNA methylation is one of the most widely studied epigenetic mechanisms in SSc (Altorok and Kahaleh, 2015). GWAS DNA methylation studies in dermal fibroblasts from SSc patients have identified a hypomethylation in the gene encoding for integrin-α9, a membrane glycoprotein that mediates cell-cell and cell-matrix adhesion. Consequently, this gene can produce an overexpression of integrins that contributes to myofibroblast differentiation (Carracedo et al., 2010) and activation of TGF-β. Hypomethylation was detected also in genes encoding for collagen that was overexpressed in SSc fibroblasts, and in some transcription factors (Altorok et al., 2015). Moreover, the demethylation of lysine 27 on histone H3 in $CD4^+$ T cells of patients with SSc leads to over-activation of several proteins, such as CD40L, CD70 and CD11a, which are strongly involved in the pathogenesis of SSc (Wang et al., 2015).

In the pathogenesis of SSc, regulatory proteins, implicated in several cellular processes such as transcription, apoptosis and cellular metabolism, can

be involved. Furthermore, both Sirtuin-1 (a regulatory protein involved in deacetylation of histones) and SMAD proteins have been discovered to be involved in the expression of antioxidants. In particular, Sirtuin-1 has been found to be a crucial regulator of TGF-β signalling, that plays a crucial role in the pathogenesis of SSc, particularly through the activation of collagen production that leads to fibrosis. The knockdown of Sirtuin-1 inhibits the release of collagen in fibroblasts by reducing TGF-β signalling. Interestingly, Sirtuin-1 is downregulated in SSc patient, but this decrease seems to be insufficient to counterbalance the extensive and persistent activation of TGF-β signalling in fibroblasts (Zerr et al., 2016). Moreover, TGF-β pathways are altered in SSc and it is possible to observe an increase of IL-13 synthesis in T cells from patients, involved in the collagen production, while in healthy subjects IL-13 synthesis is decreased by TGF-β (Baraut et al., 2015).

Recent studies have found that abnormal homeostasis and function of B cells are probably involved in the onset of SSc (Chizzolini and Boin, 2015). In particular, IL10-producing regulatory B cells (Bregs) are significantly lower in SSc patients compared to healthy controls. Lower Breg cell levels correlate with the presence of ILD (Barsotti et al., 2016). Interestingly, Breg cells are found to be increased in patients with SSc after treatment, and are inversely correlated with disease activity of SSc (Matsushita et al., 2016).

Expression of CD19 and CD21, of the costimulatory molecules CD80 and CD86, and B cell activating factor (BAFF) are upregulated in SSc patients (Sato et al., 2004; Matsushita et al., 2006). CD19 upregulation may induce the production of autoantibodies and skin fibrosis (Yoshizaki et al., 2008). B cells of

SSc patients may play a pathogenic role in BAFF and TGFβ1-dependent dermal fibrosis (Francois et al., 2013).

1.5 SNPs and systemic sclerosis

Up to now, more than 15,000 single nucleotide polymorphism (SNP) have been associated with complex clinical conditions and phenotypic traits (Hindorff et al. 2011; Welter et al., 2014).

GWAS is now a standard approach for the identification of susceptibility loci for complex traits. In the past decade, many genetic variants associated with the development of autoimmune diseases have been increasingly discovered (Kochi, 2016). In particular, several meta-analysis studies have identified over 100 loci for individual autoimmune diseases, such as RA (Okada et al., 2014), multiple sclerosis (IMSGC, 2014) and inflammatory bowel disease (Jostins et al., 2012). These genetic variants may be shared between different diseases (sometimes with opposite effects among different diseases). Combinations of these variants may determine the phenotype of individuals affected by a specific autoimmune disease.

Recently, *in silico* studies have shown that coding variants may explain < 10% of heritability in autoimmune diseases. The remaining proportion, about 80-90%, of heritability may be due to the presence of DNAse I hypersensitivity sites (indicating transcriptional activity of loci) (Gusev et al., 2014). These results suggest that genetic variants associated with autoimmune diseases may be regulatory which influence gene expression. By using a fine-mapping algorithm and integrating epigenome and transcriptome data, Farh et al. (2015)

have found that 60% of SNPs in autoimmune diseases were located in immune cell enhancers.

In autoimmune diseases, many SNPs may be located in genes mainly involved in the MHC-antigen presentation, in TCR and BCR signalling, and in regulatory T cells (Kochi, 2016). It is known that Tregs ($CD4^{+}CD25^{+}FOXP3^{+}$ T cells), play an important role in autoimmunity. Many genetic factors that influence Treg activity, through transcriptional regulation, have been identified. In RA, SNPs in the *H3K4me3* histone gene are particularly enriched in Treg cells (Trynka et al., 2013). In celiac disease, primary biliary cirrhosis and SLE, other variants may lie in acetylated *cis*-regulatory elements of Treg cells (Farh et al., 2015). A regulatory variant of C-C chemokine receptor type 6 (*CCR6)* gene has been associated with many autoimmune diseases. However, the interpretation of these association studies is complicated because this gene is expressed in both Th17 and Treg cells (Kochi et al., 2010). Additional variants in several genes, including those coding for *CTLA-4*, *IL2RA*, *IL2RB*, Fc receptor-like protein 3 (*FCRL3)* and Rhotekin 2 (*RTKN2),* associated with several autoimmune diseases, are up-regulated in Tregs (Kochi et al., 2005; Myouzen et al., 2012; Ferraro et al., 2014).

There exist several studies, which have shown association between SSc and genes implicated in the immune system (Jin et al., 2014; Barsotti et al., 2016).

Several polymorphisms of the major histocompatibility complex (MHC) class II (Arnett et al., 2010), and genes coding for proteins involved in the control of innate immunity, macrophage activation and T-cell functions, have been associated with SSc (Ramos et al., 2014).

Risk variants in genes of the innate immune system and associated with SSc include NACHT, LRR and PYD domains-containing protein 1 (*NLRP1*) (Dieudé et al., 2011a), Toll-like receptor 2 (*TLR2*) (Broen et al., 2012), autophagy protein 5 (*ATG5*) (Mayes et al., 2014), phospholipase D family member 4 (*PLD4*) (Terao et al., 2014). Several polymorphisms of genes involved in T-cell differentiation, proliferation, and activation have been associated with SSc. Such genes include those coding for protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*) (Rieck et al, 2007), signal transducer and activator of transcription 4 (*STAT4*) (Gourh et al., 2009), T-box transcription factor 21 (TBX21) (Gourh et al., 2009), tumor necrosis factor superfamily member 4 (TNFSF4) (Gourh et al., 2010), CD247 (Radstake et al., 2010), CD226 (Dieudè et al., 2011b), IL-21 (Diaz-Gallo et al., 2013). Other risk variants located in IRF5 (Dieudè et al., 2010) and IRF8 (Gorlova et al., 2011) have been identified in SSc.

2. Aims

Dysregulation of co-stimulatory and/or co-inhibitory signals, including ICOS signalling, can cause a breakdown of self-tolerance, thus leading to autoimmunity (Zhang e Vignali, 2016). Furthermore, ICOS has been linked to the function of Tregs. As a result of ICOS/ICOSL interaction, naïve $CD4^+$ T cells can differentiate into IL-10–producing Treg (Ito et al., 2007), which are known to play a pivotal role in autoimmune diseases, including SSc (Liu et al., 2016). Medullary thymus epithelial cells (mTECs), through ICOSL signalling, can induce the production of IL-2 by $CD25^-$ T cells, thus leading to the expansion of tTregs (Nazzal et al., 2014).

Although considerable progress has been made in the past few decades in elucidating how co-stimulatory and co-inhibitory pathways affect autoimmune diseases, many questions and gaps still remain.

The aim of the present study was to investigate the association between the FOXP3 rs2294020*, ICOS* rs6726035 *and ICOSL* rs378299 SNPs and the susceptibility to SSc in a North Italian Caucasian population. Furthermore, we have extended our association analysis of the FOXP3 rs2294020 SNP also in 14 GWAS datasets in order to reveal association between this SNP and susceptibility to other autoimmune diseases in individuals of European ancestry. Autoimmune diseases studied included psoriasis, celiac disease, Crohn's disease (CD), ulcerative colitis (UC), multiple sclerosis (MS), vitiligo, type-1 diabetes (T1D), rheumatoid arthritis (RA), and ankylosing spondylitis (AS).

3. Materials and methods

3.1 Patients and control subjects

The study, designed as a case-control study, was composed of 350 Italian unrelated subjects (280 females and 70 males). 166 (147 females and 19 males) patients with SSc were enrolled. 184 healthy subjects (133 females and 51 males) were used as control individuals. All patients and healthy subjects were recruited from the Department of Internal Medicine (University of Milan, Italy). Patients fulfilled the classification of the *American College of Rheumatology for SSc* (Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee, 1980). Patients were classified as having “early” or “definite” form of disease, as defined by LeRoy and Medsger (2001) and van den Hoogen et al. (2013b). In brief, scleroderma skin changes proximal to the metacarpophalangeal or metatarsophalangeal joints were sensitive and highly specific criteria for the classification of definite scleroderma. If these criteria were not present, seven additive items (with varying weights) were applied. Such additional criteria included skin thickening of the fingers, fingertip lesions, telangiectasia, abnormal nailfold capillaries, interstitial lung disease or PAH, Raynaud’s phenomenon, and SSc-related autoantibodies. The total score was determined by adding the maximum weight in each item. Patients with a total score of ≥ 9 were classified as having definite SSc.

At the time of enrolment, serum samples from SSc patients were analyzed for anticentromere (ACA), anti-topo I (Scl70) and antinuclear (ANA) antibodies, using indirect immunofluorescence staining and ELISA technique.

All subjects gave informed consent for the study, which was approved by the local ethics committee. The clinical characteristics of the patients are summarized in table 1.

Variable	Value
Age (years) of diagnosis of early SSc, mean ±, ESM	48.09 (±1.09)
Males	5.16%
"Definite" disease form	69.06%
Disease evolution time (months) (from early to definite form)	32.92 ((±3.17)
ANA nonspecific antibody	9.03%
ANA nucleolar antibody	10.32%
ACA antibody	61.93%
Scl70 antibody	18.06%

Table 1. Baseline demographic and clinical characteristics of SSc patients (abbreviations: SEM, standard error of mean; Scl70, anti-topoisomerase; ACA, anti-centromere antibody).

3.2 GWAS datasets

As already described in Chang et al. (2014), GWAS datasets were obtained from dbGaP (https://www.ncbi.nlm.nih.gov/gap) and Wellcome Trust Case Control Consortium 1 or 2 (WT1, WT2, respectively). GWAS datasets included different autoimmune diseases, as follow: celiac disease (dbGaP: phs000274; Ahn et al., 2012), multiple sclerosis (dbGaP:phs000171; Baranzini et al., 2009, and WT2; Sawcer et al., 2011), vitiligo (dbGaP:phs 000224; Jin et al., 2010, and (Jin et al., 2012), Chron's disease (dbGaP: phs000130; Duerr et al., 2006, and WT1, 2007), psoriasis (dbGaP (phs000019; Nair et al., 2009), type 1 diabetes (WT1; 2007), rheumatoid arthritis (WT1; 2007), ankylosing spondylitis (WT2; Evans et al., 2011), ulcerative colitis (WT2; UK IBD Genetics Consortium, 2009). Additional datasets used as healthy control subjects were obtained from dbGaP: phs000206 (Amundadottir et al., 2009; Petersen et al., 2010), phs000168 (Lee et al., 2008), phs000138 (Estrada et al., 2012),

phs000125 (Bierut et al., 2002), phs000092 [Bierut et al., 2002; 2008; Bierut, 2007).

3.3 SNP selection

On the basis of Ensembl linkage disequilibrium data (www.ensemble.org), the variant rs2294029 (c.747A>G) was identified as a tag polymorphism for FOXP3 gene with a minor allele frequency (MAF) >0.01. The SNP is located on chromosome X (49246763, forward strand). This variant overlaps 9 transcripts: 8 downstream gene variants for *FOXP3* and 1 synonymous variant for *CCDC22* (coiled-coil domain containing 22). rs2294020 variant is positioned in the putative 3'-UTR region of *FOXP3* gene and in close proximity to the *CCDC22* gene in the complementary strand (exon 7 of the coding CCDC22, http://www.ensemble.org)

Similarly to *FOXP3* SNP, ICOS rs6726035 and ICOSL rs378299 were selected on the basis of extensive database searching (http://www.ensemble.org, http://ncbi.nlm.nih.gov/SNP) and with MAF >0.01. The rs6726035 (12:g.204251279T>C) is a 3-near gene variant of *ICOS,* positioned in chromosome 2. The rs378299 (9:g.44241460C>T) is an upstream gene variant of ICOSL, located in chromosome 21.

3.4 DNA extraction and genotyping

Genomic DNA was extracted from whole peripheral blood with a commercial DNA isolation kit (Nuclear Laser Medicine, Italy), with a salting out method. Genotype analysis was performed by high-resolution melting (HRM) analysis.

The primer sequences are summarized in table 2.

Gene	SNP	Forward (5'→3')	Reverse (5'→3')	Size (bp)
FOXP3	rs2294020	CTGCTTCCCCCGCCTTTTCT	GCCCTTAGGAGCACCAGTCTT	182
ICOS	rs6726035	CCACATGCCAGGATGCCATT	CCAGTTTCAAGCACCCACAG-3	186
ICOSL	rs378299	TTCCCTCCCTCCTTCCATTCA	CCAGGACTTAAGGCGAGTGAG	85

Table 2. Primer sequences of rs2294020 (FOXP3), rs6726035 (ICOS) and rs378899 (ICOSL) SNPs.

Polymerase chain reaction (PCR) was carried out in 25 µl reaction mixture: 12.5 µl 2×HRM PCR Master MIX, with EVAGreen® dye (Qiagen, Germany), 1 µl of genomic DNA (50 ng), 3.5 µl of primer mix (containing 0.7 µM of the forward and reverse primers), 8 µl water. Water was used as a negative control for PCR contamination. HRM analysis was performed on Rotor-Gene Q real time instrument (Qiagen). All the analyses were run according to the following conditions: 40 cycles of 95°C for 10 s, 55°C for 30 s, 72°C for 10 s, and a melt from 65°C to 95°C at intervals (ramps) of 0.02°C/s. To ensure genotyping quality, positive control and template negative controls were included for each genotype in each run. The analysis of the result was carried out using Rotor-Gene 6000 software (Corbett Life Science, Australia).

3.5 Direct sequencing

PCR-direct sequencing of the SNPs under study on 20% of randomly chosen samples was performed in order to verify the efficiency of real time-PCR HRM technique as well as the identification of each particular polymorphism.

The purified samples were analyzed with the ABI PRISM BigDyeTM Terminator kit (Applied Biosystems, USA) on the automatic sequencer 3100 Genetic Analyzer (Applied Biosystems). Sequences were assembled using the

ABI PrismDNA software 3.7 (Applied Biosystems). 100% concordance was obtained.

3.6 Statistical analysis

Allelic frequencies in case and controls were determined and tested by Fisher's exact test. For association analyses, estimates and tests were performed with the PLINK toolset v1.07 (Purcell et al., 2007). In SSc, FOXP3 analyses were stratified according to sex, since this gene is located on the X chromosome. Association analysis was performed assuming three models: codominant, dominant, and recessive models.

For association analysis of rs2294020 in GWAS, XWAS, a specialized-software implemented on the basis of PLINK (Purcell et al., 2007) for analysis of the X chromosome, was utilized (Gao et al., 2015). GWAS datasets were analyzed as described in Gao et al. (2015). Briefly, four association tests were used:

a) FM_{01}: a test which assumes skewed X-inactivation;

b) FM_{02}: a test which assumes complete female X-inactivation;

c) $FM_{combFisher}$: a test in which male and female subjects are analyzed separately. Subsequently, a combined value of significance was obtained from Fisher method. X-inactivation status is non influential;

d) $FM_{CombStouffer}$, similar to $FM_{combFisher}$, a combined value of significance was obtained from Stouffer method. This test accounts for different sample sizes between males and females.

As only a single SNP has been considered in this studies, nominal p-values where considered, without correction for multiple hypothesis.

The association of clinicopathological characteristics with disease progression (from early to definite forms) was assessed using the Kaplan-Meier analysis with long-rank test Multivariate Cox proportional hazards regression analyses were used to assess the effect of each SNP on disease progression with adjusting for known factors, including age, presence of specific autoantibodies. Statistical significance was set as a 2-sided p value < 0.05.

4. Results

In this study, the SNP genotyping results were successfully obtained from a total of 350 subjects including 166 individuals with SSc and 184 healthy controls. HRM analysis enabled us to identify the rs2294020, rs6726035 and rs378299 polymorphisms in each participant. As shown in figure 5, the melt profiles of each sample were normalized and the different genotypes were used as the baseline for difference curve plotting. In figure 6 representative electropherograms of the candidate SNPs are shown.

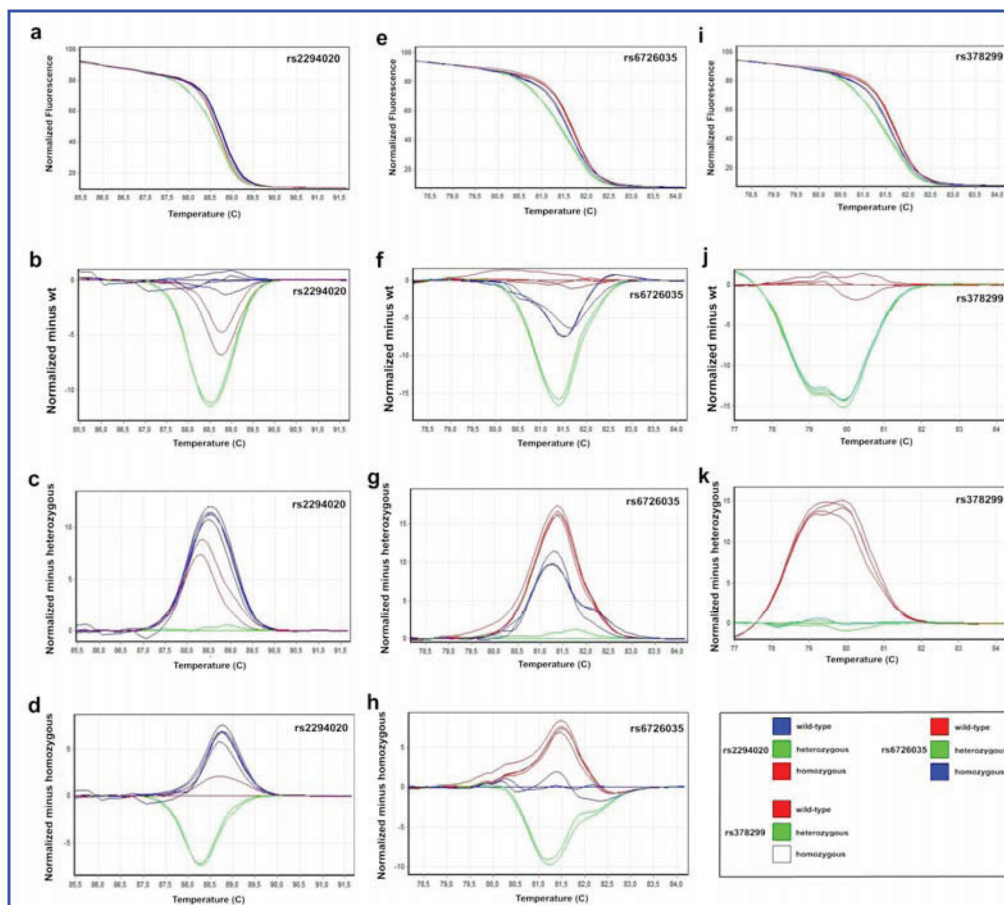

Figure 5. Representative plots of HRM analysis of rs2294020 (**a-d**), rs6726035 (**e-h**) and rs378299 (**i-k**) polymorphisms using *Rotor-Gene 6000* software. Melt profiles after normalization and difference curves for each polymorphism are shown.

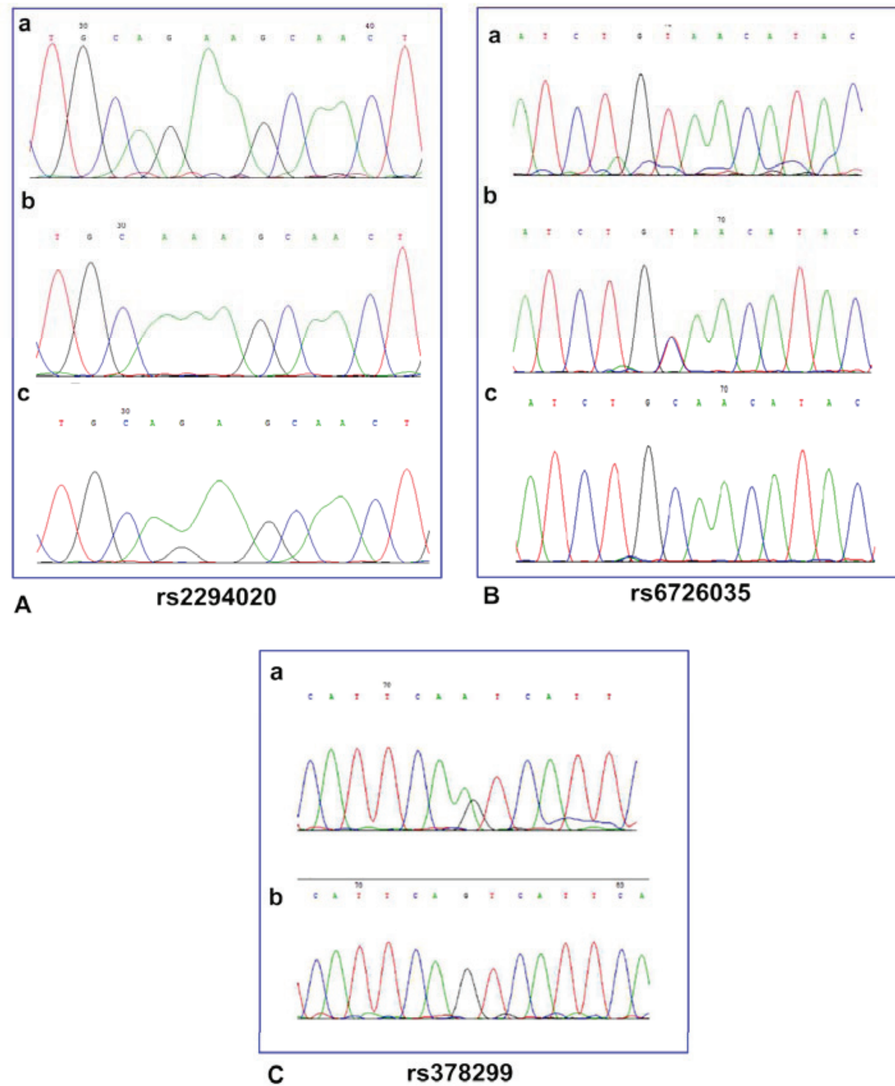

Figure 6. Representative pherograms of SNP detection from direct sequencing of amplified PCR products for rs2294020 (**A**), rs6726035 (**B**) and rs378299 (**C**). Homozygous dominant (**a**), heterozygous (**b**), and homozygous recessive genotypes are shown.

We performed a basic association test, based on comparing allele frequencies between cases and controls. Table 3 shows the *p* values from basic association test of the *FOXP3* rs2294020*, ICOS* rs6726035 *and ICOSL* rs378299 SNPs in cases and control subjects. No comparison showed a significant *p* value.

Gene	SNP	all	males	females
FOXP3	rs2294020	-	0.1177	0.2610
ICOS	rs6726035	0.1585	0.7966	0.3408
ICOSL	rs378299	0.9492	0.8457	0.9621

Table 3. *p* values of basic association test of allele frequencies between SSc patients and controls.

Table 4 shows *p* values of chi squared test of frequencies between SSc patients and healthy controls according dominant and recessive genetic models. No association reached statistically significant *p* values. Similarly, logistic regression analysis showed no association between the occurrence of the SNPs under study and the susceptibility to the disease (table 5).

Gene	SNP	Model	all	males	females
FOXP3	rs2294020	-	-	0.1280	0.5293
ICOS	rs6726035	-	0.3681	0.5626	0.5155
ICOSL	rs378299	-	-	-	-
FOXP3	rs2294020	Dominant	-	0.1280	0.3677
		Recessive	-	0.1280	0.6830
ICOS	rs6726035	Dominant	0.2500	-	0.3063
		Recessive	0.3832	0.7218	0.9090
ICOSL	rs378299	Dominant	-	-	-
		Recessive	-	-	-

Table 4. *p* values of chi squared test of frequencies between SSc patients and controls according dominant and recessive genetic models.

Gene	SNP	Model	all	males	females
FOXP3	rs2294020	-	-	0.0692	0.2624
ICOS	rs6726035	-	0.3273	0.8125	0.3401
ICOSL	rs378299	-	0.9183	0.8420	0.9619
FOXP3	rs2294020	Dominant	-	0.0692	0.3061
		Recessive	-	0.0692	0.6830
ICOS	rs6726035	Dominant	0.3208	0.7167	0.2517
		Recessive	0.5823	0.4340	0.7765
ICOSL	rs378299	Dominant	0.9183	0.8420	0.9619
		Recessive	-	-	-

Table 5. *p* values of logistic regression of frequencies between SSc patients and controls according dominant and recessive genetic models.

Subsequently, we performed a logistic regression analysis of disease progression in patients affected by SSc. As shown in Materials and Methods section, patients affected by SSc were classified as having “early” or “definite” SSc. The “definite” SSc group included about 69% of patients. We found a significant association between the occurrence of the rs2294020 SNP of the *FOXP3* gene in female patients and the disease progression. In particular, the dominant genotypic model (GG vs. AA/AG) of rs2294020 showed an HR=1.5366 (*p*=0.0327, adjusted for age of patients) and an HR=1.5519 (*p*= 0.0359, adjusted for age of patients and presence of ACA, SCL70, and ANA autoantibodies) (tables 6 and 7, figure 7).

Gene	SNP	Model	all	males	females
FOXP3	rs2294020	-	-	0.6921	**0.0303**
ICOS	rs6726035	-	0.2566	0.0643	0.3035
ICOSL	rs378299	-	0.7796	0.6323	0.8962
FOXP3	rs2294020	Dominant	-	0.6921	**0.0327**
		Recessive		0.6921	0.3520
ICOS	rs6726035	Dominant	0.3972	0.0943	0.4983
		Recessive	0.2954	0.0574	0.2801
ICOSL	rs378299	Dominant	0.7796	0.6323	0.8962
		Recessive			

Table 6. *p* values of logistic regression of disease progression in SSc patients adjusted for age of patients. Significant *p* values are shown in bold.

Gene	SNP	Model	all	males	females
FOXP3	rs2294020	-	-	0.8568	**0.0295**
ICOS	rs6726035	-	0.3702	0.9960	0.4193
ICOSL	rs378299	-	0.5617	0.1859	0.6828
FOXP3	rs2294020	Dominant	-	0.8567	**0.0359**
		Recessive	-	0.8567	0.2832
ICOS	rs6726035	Dominant	0.4750	0.1960	0.5817
		Recessive	0.4489	0.1232	0.4115
ICOSL	rs378299	Dominant	0.5617	0.1859	0.6828
		Recessive	-	-	-

Table 7. *p* values of logistic regression of disease progression in SSc patients adjusted for age of patients and presence of specific sera autoantibodies. Significant *p* values are shown in bold.

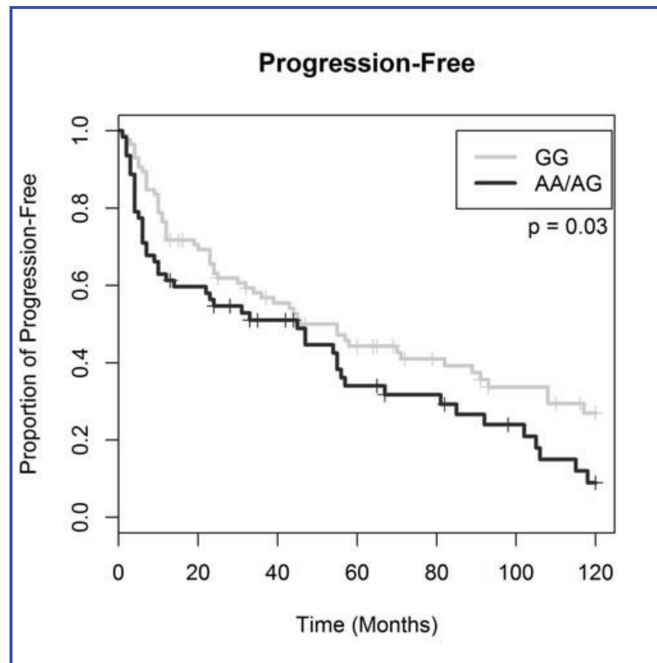

Figure 7. Impact of *FOXP3* rs2294020 on disease evolution in female patients affected by systemic sclerosis. Kaplan-Meier estimates of proportion of progression-fee patients by rs2294020 genotype.

Subsequently, we analyzed the association between rs2294020 SNP and 9 different autoimmune diseases, including celiac disease, multiple sclerosis, vitiligo, Crohn's disease, psoriasis, type-1 diabetes, rheumatoid arthritis, ankylosing spondylitis and ulcerative colitis. As shown in table 8, patients affected by multiple sclerosis (database WT2) showed a significant association (FM_{01} = 0.034; $FM_{combStouffer}$ = 0.030). However, these results were not replicated in the dbGaP dataset of multiple sclerosis patients. Both vitiligo datasets showed a significant association between the rs2294020 SNP and susceptibility to the disease (FM_{01} = 0.018 and 0.023, respectively). Furthermore, vitiligo GWAS1 showed a significant association (FM_{02} = 0.013; $FM_{combFisher}$ = 0.040; $FM_{comb\ Stouffer}$ = 0.016). The psoriasis GWAS dataset showed a significant

association between rs2294020 and the susceptibility to this disease (FM_{02} = 0.027; FM_0 = 0.039; $FM_{combStouffer}$ = 0.038).

Disease	MAF cases	MAF controls	FM_{02}	FM_{01}	FM comb Fisher	FM combStouffer
Celiac disease	0.2723	0.2902	0.793	0.580	0.598	0.559
Multiple Sclerosis	0.2491	0.2681	0.140	0.191	0.303	0.206
Multiple Sclerosis (WT2)	0.2812	0.2712	0.084	**0.034**	0.090	**0.030**
Vitiligo (GWAS1)	0.2593	0.3075	**0.013**	**0.018**	**0.040**	**0.016**
Vitiligo (GWAS2)	0.2362	0.2787	**0.044**	**0.023**	0.071	0.051
Chron's disease (NIDDK)	0.2929	0.2679	0.658	0.438	0.470	0.417
Chron's disease (WT)	0.2684	0.2546	0.439	0.485	0.729	0.498
Psoriasis	0.2536	0.2846	**0.027**	**0.039**	0.077	**0.038**
Type-1 Diabetes	0.2663	0.2674	0.255	0.390	0.466	0.445
Rheumatoid Arthritis	0.2581	0.2676	0.374	0.626	0.444	0.633
Ankylosing Spondylitis	0.2692	0.2706	0.211	0.685	0.024	0.625
Ulcerative Colitis	0.2751	0.2705	0.548	0.769	0.564	0.797

Table 8. GWAS datasets analysis for rs2294020 in patients affected by different autoimmune diseases Significant *p* values are shown in bold. (MAF, minor allele frequency; for other abbreviations refer to text).

5. Discussion

In this case-control study, we hypothesized that single nucleotide polymorphisms of *FOXP3* (rs2294020), *ICOS (rs726035)* and *ICOSL (rs378299)* genes may be involved in susceptibility to SSc in an Italian Caucasian population. In the present study, association analysis tests did not show significant association between the SNPs under study and SSc.

The results of this study show that, although rs2294020 of the *FOXP3* gene seems to be not associated to the susceptibility to SSc, its occurrence in female patients, may confer an increased risk to progression of SSc.

In order to examine in depth the possible role of the rs2294029 gene variant in the onset of autoimmune diseases, we explored its possible association with different autoimmune disorders in individuals of European ancestry. These diseases included psoriasis, celiac disease, Crohn's disease, ulcerative colitis, multiple sclerosis, vitiligo, type-1 diabetes, rheumatoid arthritis, and ankylosing spondylitis. The present study shows that rs2204020 may be associated with the susceptibility to vitiligo and psoriasis. Furthermore, we found a significant association between rs2294020 and multiple sclerosis. However, the significant association in multiple sclerosis was found only in a dataset. The reason of such discrepancy between the two datasets of multiple sclerosis patients may be due to different factors, such as population genetic heterogeneity, environmental factors, and different geographic regions, which may impact study replication (Liu et al., 2008).

Although the causes of SSc remain not well understood, there is a strong evidence that genetic predisposition contributes to SSc. In particular, genes

involved in immune regulation and inflammation, especially T-cell differentiation, proliferation, activation, B-cell signaling, and innate immunity, appeared to be the major factors contributing to the disease (Ma and Zhou, 2016).

The role of regulatory T cells ($CD4^+CD25^+FOXP3^+$ T cells) in murine autoimmunity is well established (Sakaguchi et al., 2008). In humans, however, the role of Tregs in complex autoimmune diseases remains still elusive, because of the existence of many genetic factors that influence the activity of Tregs. In fact, several autoimmune disease risk variants are involved in transcriptional regulation of genes that affect Treg cell function. Moreover, such genetic factors influence other $CD4^+$ T cell subsets, thus complicating the interpretation of the results (Kochi, 2016).

Several polymorphisms have been identified in various regions of the *FOXP3* gene. These variants may change the FOXP3 role functionally or quantitatively, thus leading to impaired function of $CD4^+CD25^+$ Tregs, which are known to contribute to the onset of autoimmune diseases (Oda et al., 2013).

It is known that gene variants associated with autoimmune diseases largely encompass functional sequences, including enhancer and regulatory elements, which can interact with specific transcription factors. Moreover, we know that about 90% of the gene variants involved in many autoimmune diseases, are non-coding. Finally, about 60% of these gene variants lie in immune cell enhancers (Farh et al., 2015). Functional polymorphisms are considered to be potential regulatory variants which may affect transcription (Benson et al., 2011).

rs2294020 SNP has been studied in juvenile idiopathic arthritis (Eastell et al, 2007), hay fever (Suttner et al., 2010) and alopecia areata (Conteduca et

al., 2014). In particular, Eastell et al. (2007), found no association between *FOXP3* rs2294020 polymorphism and juvenile idiopathic arthritis in a case-control association analysis of a UK population. However, these authors did not rule out FOXP3 as a candidate gene for this disease, due to the low statistical power obtained in male enrolled patients. rs2294020 gene variant has been found associated with hay fever in a German population, by using an additive effect-only logit model. The occurrence of the SNP rs2294020 has been also seen to slightly increase the risk for atopy (Suttner et al., 2010). Alopecia areata, an autoimmune disorder, has been shown to be associated with the rs2294020 polymorphism in an Italian population (Conteduca et al., 2014).

rs2294020 SNP is located in the 3' untranslated region (3'UTR). These gene elements are considered to contribute to mRNA stability and localization, and translational efficiency of the gene (Michalova et al., 2013). Moreover, alterations in the 3' UTR regions of *FOXP3* are known to result in a 90% decrease of protein expression, which in turn may induce impairment of Treg functions (Wan and Flavell, 2007).

It is worthwhile to note that the genetic variant rs2294020 is positioned on exon 7 of the coding coiled-coil domain containing 22 (*CCDC22)* gene, in close proximity to the *FOXP3* gene and in the complementary strand (www.ensembl.org). Since this SNP is a synonymous variant, its effect may be due to its position in 3’ UTR regions of *CCDC22* and/or *FOXP3* genes.

CCDC22 functions are still elusive. It has been shown that this protein may be involved in NF-kB activation through its interaction with copper metabolism gene MURR1 domain (COMMD) proteins (de Bie et al, 2006). Thus, the occurrence of rs2294029 variant could cause an impairment in

CCDC22 expression which, in turn, dysregulates the NF-kB signalling (Starokadomskyy et al., 2013). It is known that NF-kB is a master regulator of the immune response. Enhanced or inappropriate NF-kB activation has been shown to be involved in several autoimmune diseases (Herrington et al., 2016), including systemic sclerosis (Murdaca et al., 2016). In SSc, it has been proposed that an unknown trigger, together with a genetic predisposition, initiates an innate immune response through the activation of Toll-like receptors (TLRs), which induces a cascade of signal transduction that culminates in NF-kB activation, thus resulting in the production of acute phase cytokines, such as TNF-α and IFN-α (Johnson et al, 2015).

In conclusion, we cannot exclude the possibility that *FOXP3* and *CCDC22* genes could contribute independently to impaired Treg function. As shown above, an impaired Treg function could be achieved by defective expression of *FOXP3* gene, whose product is considered to be an essential key regulator for the induction and development of this cell subset. Alternatively, defective expression of *CCDC22* gene could be responsible for a dysregulated NF-kB signalling pathway, which is known to be a key regulator of FOXP3 expression (Long et al, 2009).

Interestingly, the data of this study show that the rs2294020 SNP display significant associations in autoimmune diseases involving the skin, such as psoriasis and vitiligo. Moreover, previous studies by Conteduca et al. (2014) showed that the rs2294020 gene variant is associated with susceptibility to alopecia areata, another autoimmune disease involving the skin. It would be intriguing to investigate in the near future the possible mechanisms involved in the pathogenesis of these skin-related autoimmune disorders. These studies

may be focused on the role of plasmacytoid dendritic cells (pDC), which represent one of the most powerful producers of type I IFN. It is known that chronic pDC activation contributes to the initiation of different autoimmune skin disorders, including psoriasis, vitiligo and alopecia areata. Moreover, a feedback regulatory loop is known to occur between DCs and $FOXP3^+$ T reg cells, which play a determinant role in maintaining self tolerance. In conclusion, an impaired FOXP3 function may induce a loss of Treg cells, which would increase proliferation and skin infiltration of DCs, thus inducing the initiation phase of autoimmune skin disease by type I IFN production (Darrasse-Jèze et al., 2009).

The limitation of our study is the absence of functional analysis and gene-targeted assays for this genetic variant. Furthermore, it should also be emphasized that our study is presumably underpowered given our sample size of patients affected by systemic sclerosis. Systemic sclerosis is a rare disease, with difficulties in diagnosis and classification, and with a highly variable timeline of symptomatic presentation.

6. Conclusion

This study provides evidence of a role of rs2294020 SNP in SSc progression, from the early to the definite form of the disease, in a Caucasian Italian population. Furthermore, rs2294020 gene variant seems also to be associated to vitiligo and psoriasis, that present themselves, among else, in the skin, thus suggesting that different autoimmune diseases may share common genetic factors.

Like many other gene variants associated with several autoimmune diseases (Gutierrez-Arcelus et al., 2016), the rs2294020 SNP shows to have a moderate effect size.

It is important to highlight that the lack of statistical significance in the association found in our study, does not exclude the possibility that the investigated gene variants are etiologically relevant polymorphisms. Indeed, when studied SNPs are located in non-coding regions, such as ICOS and ICOSL SNPs, the lack of association may not necessarily rule out a candidate gene (Martin et al, 2001). Owing to limited understanding of the mechanisms and physiological contexts of non-coding gene regions, interpretation of non-coding variants remains an important challenge. Unlikely mutations, which disrupt transcription factors motifs, alterations in non-canonical determinants may cause subtle but pivotal alterations to the immune response (Farh et al., 2015). Thus, much work remains to be done in order to characterize SNPs that may be involved in immune dysregulation. Understanding the regulatory mechanisms of the non-coding elements, most of which show a genetic links to immune regulators, could have broad implications for biology and treatment of

autoimmune diseases. The development of effective therapeutic approaches for SSc remains hampered by the poor knowledge of the mechanisms underlying the pathogenesis of this complex disease.

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