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VARIATIONS OF THE PERFORIN GENE IN PATIENTS WITH AUTOIMMUNE DISEASES

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SUMMARY

Perforin is involved in cell-mediated cytotoxicity which plays a key role in the immune response against viral infections, but it is also involved in downmodulation of the immune response through fratricide of activated immune cells. Since both viral infections and downmodulation of the immune response may be involved in development of autoimmune diseases, this thesis investigated whether variations of the perforin gene (*PRF1*) may contribute to development of autoimmunity, focusing on Autoimmune Lymphoproliferative Syndrome (ALPS) and Multiple Sclerosis (MS).

ALPS is an inherited autoimmune diseases caused by mutations decreasing function of the Fas death receptor involved in downmodulation of the immune response. ALPS is characterized by autoimmune manifestations and accumulation of non malignant lymphocytes in the lymphoid organs with expansion of double negative (DN) T cells lacking CD4 and CD8; this expansion is absent in an ALPS incomplete variant named Dianzani Autoimmune Lymphoproliferative Disease (DALD). ALPS is generally due to deleterious mutations of the *Fas* gene, but mutations of other genes have been detected in rare patients.

MS is a demyelinating autoimmune disease of the central nervous system. Its clinical course varies; at onset, approximately 15% of patients display a primary progressive form (PP), whereas the remainder start out with a relapsing remitting form (RR), and most of them switch to a secondary progressive form (SP) within 10-30 years. Both environmental and genetic factors are involved in its development/progression and several studies point to a complex inheritance involving interactions between combinations of loci that may influence the immune response.

This thesis reports the following findings:

i) **ALPS**. *PRF1* has been sequenced in 14 ALPS, 28 DALD, and 816 controls, and two variations have been detected: a N252S amino acid substitution in 2 ALPS, and an A91V amino acid substitution in 6 DALD. Frequency of N252S was higher in ALPS than in controls (7.1% vs 0.1%, p=0.0016) and conferred an OR=62.7 (95% CI: 6-654.9); frequency of A91V was higher in DALD than controls (12.5 % vs 4.6%, p=0.016) and conferred an OR=3 (95% CI: 1.2-7.1). Co-presence of A91V and variations of the osteopontin gene previously associated with DALD conferred an OR=17 (p=0.0007) for DALD. In one N252S patient, NK activity was strikingly defective in early childhood, but became normal in the late childhood. A91V patients displayed lower NK activity than controls. The thesis also describes an atypical ALPS patient carrying A91V and affected with epidermodysplasia verruciformis, characterized by increased susceptibility to human papilloma virus (HPV) infection.

ii) **MS**. By sequencing the entire *PRF1* coding region in 190 MS patients and 268 controls, A91V and N252S variations have been detected in both groups and six novel mutations (C999T, G1065A, G1428A, A1620G, G719A, C1069T) in patients (n=7) only; C999T, G1065A, G1428A, and A1620G were synonymous variations, whereas G719A and C1069T caused an R240H and an R357W substitution respectively. All together, allelic frequency of these variations was higher in patients than in controls (10% vs 4.5%, p=0.0016); moreover, genotypic analysis showed that carriers of the variations were more frequent in patients than in controls (OR=2.06, 95% CI: 1.13-3.77). Since A91V was the

most frequent variation and displayed a trend of association with MS in the first population of patients and controls (7.6% vs 4.3%, p=0.044), its frequency has been assessed in a second population of 966 patients and 1520 controls. Results showed that A91V allelic frequency was significantly higher in patients than in controls also in the second population (7.5% vs 5.8%; p=0.019). Genotypic analysis of the combined cohorts of 1156 patients and 1788 controls showed that A91V carriers were significantly more frequent in patients than in controls (OR=1.38, 95% CI=1.10-1.74).

These data suggest that *PRF1* variations are a susceptibility factor for ALPS and MS development, possibly because they affect either the anti-viral response or the immune response switching off. Other data show that these variations may also be involved in development of type 1 diabetes mellitus and systemic lupus erythematosus.

LIST OF PUBBLICATIONS

1: Zavattaro E, Azzimonti B, Mondini M, De Andrea M, Borgogna C, Dell'Oste V, Ferretti M, Nicola S, **Cappellano G**, Carando A, Leigheb G, Landolfo S, Dianzani U, Gariglio M. Identification of defective Fas function and variation of the perforin gene in an epidermodysplasia verruciformis patient lacking EVER1 and EVER2 mutations. *J Invest Dermatol,* 2008; 28:732-5.

2: Orilieri E, **Cappellano G**, Clementi R, Cometa A, Ferretti M, Cerutti E, Cadario F, Martinetti M, Larizza D, Calcaterra V, D'Annunzio G, Lorini R, Cerutti F, Bruno G, Chiocchetti A, Dianzani U. Variations of the perforin gene in patients with type 1 diabetes. *Diabetes,* 2008; 57:1078-83.

3: Cappellano G, Orilieri E, Comi C, Chiocchetti A, Bocca S, Boggio E, Bernardone IS, Cometa A, Clementi R, Barizzone N, D'Alfonso S, Corrado L, Galimberti D, Scarpini E, Guerini FR, Caputo D, Paolicelli D, Trojano M, Figà-Talamanca L, Salvetti M, Perla F, Leone M, Monaco F, Dianzani U. Variations of the perforin gene in patients with multiple sclerosis. *Genes Immun,* 2008; 9:438-44.

4: Clementi R, Chiocchetti A, **Cappellano G**, Cerutti E, Ferretti M, Orilieri E,Dianzani I, Ferrarini M, Bregni M, Danesino C, Bozzi V, Putti MC, Cerutti F, Cometa A, Locatelli F, Maccario R, Ramenghi U, Dianzani U. Variations of the perforin gene in patients with autoimmunity/lymphoproliferation and defective Fas function. *Blood,* 2006; 108:3079-84.

INTRODUCTION

1. AUTOIMMUNITY

The specific immune response involves T and B lymphocytes activated by *non self* antigens. B cells recognize every type of soluble macromolecules in their naive form, whereas T cells recognize peptides processed and presented on major histocompatibility complex (MHC) molecules expressed on the cell surface by antigen presenting cells (APC) (i.e. macrophages, dendritic cells and B lymphocytes). Lymphocyte activation is accompanied by modification of expression of several genes that code for surface molecules involved in lymphocyte proliferation and effector functions, which allow expansion of antigen-specific lymphocytes and their differentiation into effector cells, i.e. plasma cells secreting antibodies for B cells, activated T cells secreting cytokines for $CD4^+$ T helper (TH) cells, and cytotoxic T lymphocytes (CTL) for $CD8^+$ T cells. Activation also triggers expression of several genes coding for molecules involved in immune response switching-off, an active process that induces apoptosis of most effector lymphocytes several days later. A small subset of activated cells survives to form an expanded pool of memory lymphocytes and accounts for the more efficient response upon re-encounter of the same antigen (secondary response).

In the early ‘900 Paul Ehrlich realized that some diseases can be caused by an erroneous activation of the immune system which focuses its attack agaist *self*-antigens, instead of foreign antigens, and termed this condition “horror autotoxicus”. This early intuitions resulted to be true and it is now well known that the immune system use several mechanisms to ensure *self*-tolerance protecting the body from *self*-reactive lymphocytes,

but failure of these mechanisms can result in inappropriate responses against *self*-components and development of autoimmune diseases. In *organ-specific* autoimmune diseases, the immune response is directed to a target antigen unique to a single organ, so that clinical manifestations are largely limited to that organ. In *systemic* autoimmune diseases, the response is directed toward target antigens expressed by a broad range of organs and tissues, which are all damaged. Examples of organ-specific autoimmune diseases are Hashimoto's thyroiditis and Graves' disease, affecting the thyroid gland, and type I insulin-dependent diabetes mellitus (IDDM), which affects the pancreatic islets. Examples of systemic autoimmune disease are systemic lupus erythematosus (SLE) and primary Sjögren's syndrome, in which tissues as diverse as the skin, kidneys, and brain may all be affected.

Moreover, some autoimmune diseases are predominantly mediated by autoantibodies, whose productions is supported by T helper type 2 (Th2) lymphocytes secreting cytokines supporting the humoral response (mainly IL-4, IL-5 and IL-6), whereas other are predominantly mediated by T helper type 1 (Th1) lymphocytes, producing proinflammatory cytokines (IL-2, IFNγ and Lymphotoxin), and by T cytotoxic (Tc) cells. Examples of the former are autoimmune hempocytopenias, pemphigous vulgaris, systemic lupus erytematosus, miastenia gravis, Graves disease; examples of the latter are insulin-dependent diabetes mellitus (IDDM) and multiple sclerosis (MS).

1.1 The aetiology of autoimmune diseases

In the ‘60s, it was believed that all *self*-reactive lymphocytes were eliminated during their development in the bone marrow and thymus which generated a *central*

tolerance for *self*-antigens and that failures to eliminate these lymphocytes led to autoimmunity.

Since late ‘70s, a broad body of experimental evidence has countered that belief, revealing that not all *self*-reactive lymphocytes are deleted by central tolerance. Instead, normal healthy individuals normally possess mature, recirculating, *self*-reactive lymphocytes which do not cause disease because they are controlled by several mechanisms generating a *peripheral tolerance* and acting by inducing deletion, anergy, or suppression of *self*-reactive lymphocyte clones. A breakdown in this regulation can lead to activation of *self*-reactive clones of T or B cells, generating humoral or cell-mediated responses against *self* antigens. These reactions can cause serious damage to cells and organs, sometimes with fatal consequences.

The role of environmental factors in the aetiology of autoimmune diseases is clear when considering the disease concordance rate between monozygotic twins. More than 50% and sometimes 70% or 80% of monozygotic twins are discordant for major autoimmune diseases even if they generally share the same environment, at least during childhood.

A large bulk of data suggest that a key role is played by infectious agents, in particular viruses. Infections may trigger autoimmunity by 3 mechanisms:

i) The first mechanism is *antigen mimicry* and suggests that the immune response against infectious agents may trigger autoimmune diseases against *self* antigens sharing epitopes with the *non self* antigens. This hypothesis is supported by abundant epidemiological, clinical, and experimental evidences of the associations of infectious diseases with autoimmune disease and by the observations that a

number of bacterial or viral proteins display the amino acid stretches with high homology with sequences of *self* proteins.

ii) The second mechanism is the *release of sequestered antigens* and suggests that infections may damage tissues causing release of *self* antigens which had not been previously accessible to the immune system and are therefore recognized as *non self*.

iii) The third mechanism is the *adjuvant effect* and suggests that infections trigger tissue inflammation that support the autoimmune response by inducing secretion of high amounts of cytokines and expression of costimulatory molecules by antigen presenting cells (APC), which is the effect of adjuvant in vaccine formulations.

These mechanisms are not non mutually exclusive and may act together in chronic autoimmune disease to cause the “epitope spreading”, i.e. the progressive expansion of the autoimmune response against multiple epitopes (intramolecular epitope spreading) and antigens (intermolecular epitope spreading) during the disease progression which is a phenomenon.

1.2 Genetics of autoimmune diseases

Studies of recurrence risk in families and twins suggest a complex mode of inheritance involving interactions of different combinations of loci influencing the immune response. Despite several whole genome surveys, potent locus have not been detected for most autoimmune diseases. It is likely that most susceptibility genes have a small effect, with the possible exception of the MHC locus, with a high degree of genetic heterogeneity in different individuals. Susceptibility genes may include genes coding for molecules involved in immune response control and immune effector functions. Further genes may be those involved in the immune response switching off, which leads to homeostatic control of the

size of the peripheral lymphocyte pool and reduce the risk of autoimmunity due to cross-reactions between *non-self* and *self* antigens. Intriguingly, some of these genes are also involved in clearance of viral infections. However, even if many studies detected a statistical association of autoimmune diseases with particular alleles of specific genes, often it is not clear whether these alleles are directly involved in the disease development or the statistical association is due to *linkage disequilibrium*, i.e the physical association of that allele with the allele of a nearby gene which is the real predisposing factor. Moreover, many alleles have been found to be associated in some populations but not in other, which may be explain with the genetic heterogeneity of the populations.

1.3 Gender as a risk factor for autoimmune diseases

Many autoimmune diseases have a different frequency in female and the male and most of them (such as systemic lupus erythematosus, myasthenia gravis, scleroderma, multiple sclerosis, and Sjögren's syndrome) are much more frequent in females than in males. Conversely, ankylosing spondylitis is more common in males. The reasons for this different susceptibility are not known with certainty, but one possibility is that a role is played by sex hormones. It is unclear whether the sex steroid hormones have a direct impact on the lymphocytes, but this possibility is suggested by the fact that other steroids such as cortisone, have a very powerful effect. However, also other hormones are differentially expressed in male and female and may play a role, for instance, prolactin, whose receptor is expressed by both T and lymphocytes and that can modulate these cell activation *in vitro*. Sex hormones can modulate the immune response during pregnancy, mainly directing the immune response to Th2 type responses, in order to support IgG responses which are protective to the fetus and reduce cell-mediated responses that might

attack the placenta. Indeed, pregnancy exacerbates some autoimmune diseases, which are mainly antibody-mediated (and therefore favoured by the Th2); instead, it reduces other autoimmune diseases which are mainly mediated by Th1 and inflammatory cells. Therefore, the different immune responsiveness influenced by the different levels of sex hormones may be a factor influencing the initiation and evolution of the autoimmune response in males and females. However, this is not the only explanation of female predisposition to develop autoimmunity since recent experiment on mice showed that an independent effect may be directly mediated by genetic factors located in the sex chromosomes.

1.4 Association with HLA haplotype

The best-known genetic factor predisposing to autoimmunity is the HLA haplotype, since most autoimmune diseases have been found to be associated with specific HLA alleles. Most frequently association has been found with class II HLA alleles, although in some cases class I alleles are involved. For instance, the risk of developing IDDM is about 20 times higher in subjects expressing HLA-DR3 and DR4 molecules, whereas the probability of developing multiple sclerosis is 5 fold higher in DR2 carriers and that of myasthenia gravis is 5 fold higher in DR3 carriers. In several instances the association has been ascribed to the capacity of the predisposing molecule to "present" the *self* peptides responsible for the autoimmune disease to the *self* reactive lymphocytes.

2. PERFORIN

2.1 Functional features of perforin

Perforin is produced by cytolytic lymphocytes and is stored within cytoplasmic granules of these cells. This cytolytic mediator owes its name to the fact that it can 'perforate' target membranes by forming transmembrane pores, such that target cells lose membrane integrity and appear to die of colloid osmotic lysis, at least in vitro.

Perforin has been purified from cytotoxic lymphocytes of mice, rats and humans. Mature mouse perforin protein comprises 534 amino acid residues (after the cleavage of a signal peptide of 20 residues in length), with approximately 70% and 85% identity, respectively, to human and rat perforin. Its molecular mass of 66kDa or 70kDa **[1-2]**, determined by SDS-PAGE under reducing conditions, suggests that perforin molecules are post-translationally modified at potential N-glycosylation sites (three in mouse and rat; two in human). Indeed, N-glycanase, but not endoglycosidase-H, has been shown to remove the carbohydrate moieties from the polypeptide backbone of perforin. Following synthesis in the rough endoplasmic reticulum, perforin molecules travel through the Golgi apparatus, where post-translational modification continues, and are finally packaged into lysosome-like cytoplasmic granules. Although another granule protein, granzyme A, has been shown to be targeted to the granules through the mannose 6-phosphate-dependent pathway, perforin molecules do not seem to use the same targeting signal during their intracellular journey **[3]**. However, it is clear that the release of perforin molecules is predominantly controlled by regulated secretion. Upon conjugation with the appropriate target cell, the cytoplasmic granules within the killer cell reorient towards the cell-cell contact site, as demonstrated by electron microscopic (EM) studies **[4]**. The contents of the granules, including perforin, are then released into the intercellular space **[5]**. The release of perforin,

and the execution of its lytic function, are strictly dependent on presence of calcium ions (Ca2+). It is generally accepted that perforin monomers can bind and insert into the target membrane, and polymerize to form aggregates comprising various numbers of monomers **[6]**. These aggregates form pores allowing entry of granzymes, which are also released from the cytotoxic granules, in the target cell cytoplasm. Granzymes are serine proteases cleaving several substrates including several pro-caspases, which are in turn activated to initiate the apoptotic death of the target cell.

Perforin is an essential lymphocyte effector molecule; in fact, in mice, absence of functional perforin cause severe immune deficiency and/or affects lymphoid and myeloid homeostasis **[7-8]**. For instance, gene-engineered mice with two disrupted *PRF1* alleles are killed by 10.000-100.000 fold fewer infectious ectromelia (mouse poxvirus, corresponding to human smallpox or bovine cowpox) particles than wild-type mice **[9]**. However, perforin-deficient mice are not impaired for clearance of other viruses, such as vaccinia virus, vesicular stomatitis virus, murine cytomegalovirus, Semliki Forest virus **[10]**, cowpox virus **[9]**, rotavirus **[11]**, coxsackie B virus **[12]**, and murine herpesvirus-68 **[13]**, a mouse homologue of Epstein–Barr virus (EBV). Perforin-deficient mice are also highly prone to fatal, disseminated B cell lymphoma that develops in >60% of mice, generally above one year of age **[14]**.

2.2 Features of the human perforin

The human perforin gene, located on chromosome10q22, consists of three exons. The coding sequence is located in exons 2 and 3. Perforin is a 555 amino acid protein that has a 21 amino terminal signal sequence, an approximately 300 amino region that shares homology to the C9 complement protein, a 36 amino acid epidermal growth factor-like

domain, and a 132 amino acid domain homologous to the C2 domain of protein kinase C. The protein contains two N-linked glycosylation sites. **[15-16]**. Perforin is synthesized as an inactive precursor, which must be cleaved at its carboxy terminus, releasing the approximately 20 carboxy terminal amino acids, to yield the active form of the protein **[16]**. The C2 domain of perforin is important for its binding to the phospholipid bilayer of cytoplasmic membranes and conserved aspartate residues at positions 429, 435, 483, and 485 are essential for calcium-dependent plasma membrane binding and cell lysis. The C2 domain in the uncleaved form cannot bind to the membrane, because of presence of a bulky N-linked glycan on the carboxy terminal domain of the protein. Proteolytic cleavage of the carboxy terminal domain allows the C2 domain to bind to the membrane **[17]**.

Perforin is harmful to cell membranes; however, the granule membrane encompassing the protein is not destroyed by perforin. The pH inside granules is very acidic (pH <5) and this probably keeps perforin in an inactive state. When exocytosis occurs and perforin is released, pH becomes neutral and perforin becomes active. In addition, proteoglycans usually cover and inactivate perforin in the granule **[18-19]**.

When the granule content is released from the cell, perforin separates from proteoglycans and binds to cell membranes of target cells. Cathepsin B, a lysosomal protease, moves to the surface of CTLs during granule exocytosis **[20]**; since cathepsin B can cleave perforin, the membrane-bound form of cathepsin B is thought to destroy perforin on the surface of CTLs protecting their membrane from perforin aggression **[20]**.

2.3 Deficit of perforin in the Familial Hemophagocytic Lymphohistiocytosis (FHL)

In humans, genetic defects impairing perforin synthesis, function, or release can result in Familial Haemophagocytic Lymphoistocytosis (FHL).

FHL is an autosomal recessive disease affecting infants and young children who are usually healthy at birth. Most patients display symptoms during the first year of life **[21]**, but some as late at the third decade of life **[22]**. Signs and symptoms include fever, splenomegaly, hepatomegaly, anaemia, thrombocytopenia, neutropenia, hypertriglyceridaemia, hypofibrinogenaemia, and cerebrospinal fluid pleocytosis **[23]**. Lymphadenopathy, rash, neurological abnormalities, leucopenia, elevated liver function tests, elevated ferritin, and hyponatraemia may also be present. Neurological abnormalities range from irritability and hypotonia to seizures, cranial nerve deficits and ataxia. The inciting event is thought to be initiated by infection. In late disease, bone marrow biopsy usually shows hypoplasia or aplasia. Patients who are not treated, or who do not respond to therapy, usually die within a few months from onset of symptoms. Most deaths are the result of infections, disseminated intravascular coagulopathy with uncontrolled bleeding, or central nervous system disease. Haemophagocytosis is a prominent feature of the disease. Haemophagocytosis occurs when activated macrophages (histiocytes) ingest erythrocytes and sometimes platelets and leucocytes. The bone marrow is the most common site of haemophagocytosis, although it can also be detected in the spleen, liver and lymph nodes. In the early stage of the disease, histiocytes are present in focal areas of the bone marrow; in later stages, they are distributed diffusely throughout the bone marrow. When haemophagocytosis is prominent in the bone marrow, it can be accompanied by decreased numbers of hemopoietic precursors and pancytopenia. In the lymph nodes, histiocytes are increased in the sinuses and T-cell areas, and lymphocyte depletion is often observed. In the spleen, histiocytes are distributed diffusely and erythrophagocytosis is frequently present. NK cell and CTL cytotoxicity are severely impaired. Uncontrolled immune responses result in infiltration and destruction of tissues by activated macrophages (CD68+) and CD8+ T

cells. Activated macrophages and T cells are often present in the bone marrow, spleen, lymph nodes, liver and central nervous system; other organs including the lungs, heart, intestine, thymus, kidney and pancreas may also be infiltrated. Uncontrolled activated macrophages and T cells release pro-inflammatory cytokines, which result in elevated serum levels of IFN-γ, TNF-α, IL-1 receptor antagonist, soluble IL-2 receptor, IL-6, IL-10, IL-12, IL-18, IP-10, and M-CSF. High levels of these cytokines contribute to haemophagocytosis, cellular infiltration, and organ damage. Lipoprotein lipase deficiency is associated with the elevated levels of serum triglycerides **[24-31]**. Linkage analysis studies showed that FHL is linked to three loci.

About 10% of FHL are linked to chromosome 9q21-22 (FHL1), 20-40% to chromosome 10q21 (FHL2), while most cases are to other mutations **[32,33]**. While the gene responsible for FHL1 is unknown, perforin mutations are responsible for FHL2 **[34].** A further gene is Munc 13-4, whose mutations result in FHL3 **[35]**; Munc 13-4 is located on chromosome 17q25 and is important for fusion of the granule membrane with the cell membrane. Finally, mutations in the coding region of the syntaxin gene cause FHL4; these mutation impair degranulation of the cytotoxic granules **[36]**.

Stepp et al **[34]** first showed that the perforin gene (*PRF1*) is responsible for FHL in several patients. These patients were homozygotes or double heterozygtes for mutations of *PRF1* that dramatically decreased expression of perforin and impaired cell-mediated cytotoxicity in vitro. In contrast, levels of granzyme B, were normal. The heterozygous parents were asymptomatic but expressed reduced levels of perforin in NK, CD8+, or CD56+ T cell and some of them also displayed decreased NK function in vitro, but they are asymptomatic. **[37,38]**.

A variety of mutations, including stop codons, single amino acid mutations or deletions, have been reported in the *PRF1* coding sequence in patients with FHL2 **[22,33,34,37,39-40]**. Most of them cause decreased CTL- and NK cell-mediated cytotoxicity. Certain mutations are typical of patients from specific ethnic backgrounds. For instance, the most frequent mutations in Japanese patients are nucleotide changes resulting in frameshift mutations at amino acid 69 (reported in 38% of Japanese patients) or amino acid 364 (reported in 63%) **[41]**. A stop codon at amino acid 374 is frequent among Turkish patients **[22,33,34,42]**. A nucleotide deletion resulting in a frameshift mutation at amino acid 17 is frequent among Afro–American patients **[40]**.

Mutations in the coding sequence of *PRF1* are responsible for approximately 20–40% of FHL patients among Japanese, European and Middle Eastern countries **[33,43]**, and 58% patients from North America **[40]**. The impaired activity of perforin in these patients can be the result of reduced expression, instability, or incorrect trafficking of the protein, failure of the protein to bind to target cells, or failure of the protein to lyse the cells. **[8,44]**.

2.4 Involvement of perforin in other human diseases

Clementi et al described a patients with FHL2, who developed B cell lymphoblastic lymphoma, suggesting that perforin-dependent cell-mediated cytotoxicity plays a protective role against development of lymphoproliferative disorders not only in mice but also in human **[45,46]**.

Solomou et al **[47]** identified mutations in *PRF1* in 5 unrelated patients with adult-onset aplastic anemia. Four of them showed hemophagocytosis in bone marrow biopsy, but none had clinical manifestations of FHL. Perforin protein levels in these patients were very

low or absent and NK cell activity was substantially decreased. *PRF1* mutations may explain the aberrant proliferation and activation of cytotoxic T cells in aplastic anemia. Moreover, germline mutations in the coding region of *PRF1* have been described in patients with anaplastic large cell lymphoma (ACCL), and they have been suggested to play a predisposing role **[48]**.

SECTION I

3.1 Autoimmune Lymphoproliferative Syndrome (ALPS)

Inherited factors that may play a role in development of autoimmunity are those causing functional defects of the Fas death receptor **[49-50]**. Fas belongs to the Tumor Necrosis Factor Receptor (TNFR) superfamily and induces cell death upon triggering by FasL **[51-52]**. It is expressed by activated lymphocytes and plays a role in depletion of effector cells and switching off the immune response.

The association between Fas defects and autoimmunity was first observed in mice homozygous for the *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) characters, due to mutations of the Fas and FasL genes respectively. These mice develop an autoimmune/lymphoproliferative picture with lymphadenopathy, splenomegaly and autoimmunity. Moreover, they show peripheral expansion of T cells expressing the TCRαβ but lacking both CD4 (marking Th) and CD8 (marking Tc) and therefore named "double negative" (DN) T cells. Causal relationship of this picture with defective Fas function was confirmed in Fas-/- *knock-out* mice **[53]**.

In humans, a similar picture has been described in patients carrying mutations of the *Fas* gene **[49,54-57]** and the disease has been named autoimmune lymphoproliferative syndrome (ALPS). ALPS is characterized by (1) defective function of Fas, (2) autoimmune manifestations that predominantly involve blood cells (i.e. thrombocytopenia, anemia, neutropenia), (3) polyclonal accumulation of lymphocytes in the spleen and lymph nodes with lymphadenomgaly and/or splenomegaly, and (4) expansion of DN T cells in the peripheral blood **[58-60]**. To date, several genetic lesions have been associated with ALPS (OMIM 601859). A classification names ALPS type 0 the disease due to homozygous mutation of the *Fas* gene, causing complete deficiency of Fas expression; ALPS type Ia is

due to heterozygous mutations of *Fas*; ALPS type Ib to mutations of the *FasL* gene; ALPS type II to mutations of the *caspase 10* gene **[61-65].** The term ALPS type III is used to define the disease due to unknown genetic defects causing defective Fas function. ALPS type IV has been provisionally used for a patient displaying normal Fas function but defective mitochondrial-induced apoptosis carrying a mutation of the N-Ras gene **[66]**.
An ALPS variant has been described in patients displaying the first 3 criteria but lacking expansion of DN T cells and mutations of the *Fas*, *FasL* o caspase 10 genes. This disease has been provisionally named DALD (Dianzani autoimmune Lymphoproliferative disease) by McKusick (OMIM reference no. 60523; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM).

ALPS-like disorders do not behave as classical monogenic diseases. This is true in *lpr* and *gld* mice and even more evident in ALPS and DALD. The *lpr* and *gld* mutations cause the disease in homozygosity, but its expression greatly depends on the genetic background, since it is much milder in BALB/c than in MRL mice **[53]**. Most ALPS type-Ia patients are heterozygous for the Fas mutation, but the parent carrying the mutation is generally healthy. Other complementary factors may thus be required in function of the severity of the mutation **[54]**. One possibility is that mild Fas mutations only induces ALPS when cooperate with mutations of other genes impairing function of the Fas system itself or other systems involved in similar functions. These genes may act as “genes modifiers” affecting the phenotypic expression of another gene. DALD, too, seems genetically heterogeneous: different genes may be involved in different families and multiple gene alterations may be required to develop the overt disease. This is suggested by the observation that, generally, both parents of such patients display defective Fas function, but are healthy.

A concurrent factor may be production of high levels of osteopontin (OPN), a cytokine involved in inflammation that inhibits activation-induced cell death (AICD) of lymphocytes. ALPS/DALD patients indeed display increased frequency of polymorphic variants of the OPN gene (OPN^{high} gene variants), that cause increased production of OPN by stabilizing its mRNA and increase the risk of ALPS/DALD by about 8-fold **[67]**. The increased osteopontin levels may favour ALPS/DALD because of the effect on AICD whose inhibition affects a mechanism to switch off the immune response that is partly alternative to Fas.

ALPS patients also display increased risk to develop lymphomas **[68]**. A study on 130 patients carrying Fas mutations showed that the risk of developing Hodgkin lymphomas is increased by about 50 fold, while that of non-Hodgkin lymphomas is increased by about 14 fold than the matched control population **[69-71]**. This might be ascribed to the altered control of lymphocyte homeostasis or to defective immune surveillance.

3.2 Epidermodysplasia verruciformis

Epidermodysplasia verruciformis (EV) is a rare disorder, characterized by persistent human papillomavirus (HPV) infection with an autosomal recessive inheritance **[72]**. It is associated with disseminated HPV infection and immunological abnormalities **[73-74]**. EV results from an abnormal susceptibility to specific human papillomavirus (HPV) genotypes and to the oncogenic potential of some of them, mainly HPV5 **[75]**. This disorder was first described by Lewandowski and Luiz in 1922 **[76]**.

Although the pattern of inheritance displays autosomal recessive inheritance in most cases, cross-linked inheritance and sporadic mutations have also been reported **[72,77]**.

Several abnormalities of cell-mediated or humoral immunity have been suggested to play a role in the pathogenesis of EV **[73,78]**. The first evidence of an impaired cell-mediated immunity in EV patients was obtained 30 years ago **[79]**. Decreased T cell counts and $CD4^+/CD8^+$ T cell ratio, as well as a reduced T cell responsiveness to mitogens have also been reported. By contrast, patients with EV were found to display normal or increased natural killer (NK) cell activity using the standard K562 cell killing assay **[80-81]**.

Although EV has been recently classified as a primary deficiency **[82]** of innate immunity to specific HPV genotypes with a central role assigned to keratinocytes, the molecular mechanisms underlying abnormal susceptibility to a single type of weakly pathogenic infectious agent are still unclear.

Ramoz et al mapped two susceptibility loci for EV, EV1 located at chromosome 17q25 (EV1) and EV2 at chromosome 2p21-p24 **[83]**. In 2002, two novel genes, EVER1 and EVER2, have been identified in the EV1 locus **[84]**; their function is unknown, but nonsense mutations in these genes have been associated with EV in some consanguineous families and sporadic cases **[85-86]**.

3.3 RATIONALE

A previous work from our group described a patient affected with ALPS type Ia who carried a heterozygous mutation of *PRF1* together to a heterozygous mutation of the *Fas* gene **[46]**. These mutations were inherited from distinct parents who were healthy, which suggested that their co-transmission was responsible for the son's ALPS. This observation was intriguing since both Fas and perforin are involved in cell-mediated cytotoxicity and control of lymphocyte homeostasis. Systematic evaluation of the role of *PRF1* in ALPS was not undertaken on that occasion. The study reported in the **paper 1** has thus been performed to extend the analysis of *PRF1* to a larger number of patients and evaluate its role in the development of ALPS and DALD**.** The **paper 2** describes a peculiar patient, not included in paper 1, displaying defective function of Fas together with a mutation of the *PRF1* gene, and displaying signs of ALPS together with a EV picture, which has never been previously associated with Fas or perforin defects.

These papers showed that *PRF1* variations may be a genetic concurrent factor favouring development of ALPS-like pictures in subjects carrying defects of Fas function. Moreover, paper 2 suggests that further genetic alterations affecting the anti-viral response, i.e. those involved in EV development, may be co-involved in some patients.

3.4 RESULTS

3.4.1 *Paper 1*

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Variations of the perforin gene in patients with autoimmunity/lymphoproliferation and defective Fas function

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Mutations decreasing function of the Fas death receptor cause the autoimmune lymphoproliferative syndrome (ALPS) with autoimmune manifestations, spleen/lymph node enlargement, and expansion of CD4/CD8-negative T cells. Dianzani Autoimmune Lymphoproliferative Disease (DALD) is a variant lacking this expansion. Perforin is involved in cell-mediated cytotoxicity and its biallelic mutations cause familial hemophagocytic lymphohistiocytosis (HLH). We previously described an ALPS patient carrying heterozygous mutations of the Fas and perforin genes and suggested that they concurred in ALPS. This work extends the analysis to 14 ALPS, 28 DALD, and 816 controls, and detects an N252S amino acid substitution in 2 ALPS, and an A91V amino acid substitution in 6 DALD. N252S conferred an OR = 62.7 (*P* = .0016) for ALPS and A91V conferred an OR = 3 (*P* = .016) for DALD. Copresence of A91V and variations of the osteopontin gene previously associated with DALD conferred an OR = 17 (*P* = .0007) for DALD. In one N252S patient, NK activity was strikingly defective in early childhood, but became normal in late childhood. A91V patients displayed lower NK activity than controls. These data suggest that perforin variations are a susceptibility factor for ALPS/DALD development in subjects with defective Fas function and may influence disease expression. (Blood. 2006;108:3079-3084)

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Introduction

Fas is a death receptor belonging to the tumor necrosis factor receptor (TNFR) superfamily and induces cell death upon triggering by FasL.[1-3] In the immune response, it is highly expressed by activated effector lymphocytes and is involved in switching off the immune response, limiting clonal expansion of lymphocytes, and favoring peripheral tolerance. Moreover, FasL is expressed by cytotoxic T cells and NK cells and is involved in killing of target cells expressing Fas. Fas induces cell apoptosis by triggering a cascade of caspases through 2 partly interconnected pathways: the extrinsic pathway involves caspase-8–mediated direct activation of the cascade, whereas the intrinsic pathway proceeds through mitochondrial release of cytochrome c and activation of caspase-9. Both pathways converge in the activation of effector caspases, such as caspase-3, -6, and -7.[1-3]

Defective Fas function leads to the unwanted accumulation of lymphocytes and favors autoimmunity possibly by impairing the switching off of autoreactive lymphocytes. This has been shown in the autoimmune lymphoproliferative syndrome (ALPS), an inherited disease characterized by (1) defective function of Fas, (2) autoimmune manifestations that predominantly involve blood cells, (3) polyclonal accumulation of lymphocytes in the spleen and lymph nodes with lymphoadenomegaly and/or splenomegaly, and (4) expansion of TCRαβ + CD4/CD8 double-negative (DN) T cells in the peripheral blood. Moreover, ALPS patients are predisposed to develop lymphomas in adulthood.[3-11] ALPS is generally due to deleterious mutations of the Fas gene *(TNFRSF6)* and is classified as ALPS type-Ia, but rare mutations of other genes have been detected, for instance, the FasL genes in ALPS-Ib, and the caspase-10 gene in ALPS type-IIa, whereas the mutated gene is not known in other patients. Mutations of the Fas and the FasL gene detected in MLR *lpr/lpr* and *gld/gld* mice, respectively, give rise to a disease that overlaps ALPS. We described an ALPS variant that fulfils the first 3 criteria but lacks expansion of DN T cells and mutations of the Fas, FasL, or caspase-10 genes.[12,13] Since the complete paradigm of ALPS could not be demonstrated, this disease has been provisionally named Dianzani Autoimmune Lymphoproliferative Disease (DALD) by McKusick (OMIM reference #605233; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = OMIM).

ALPS-like disorders do not behave as classic monogenic diseases.[3-8] This is true in *lpr/lpr* and *gld/gld* mice and even more evident in ALPS and DALD. The lpr and gld mutations cause the disease in homozygosity, but its expression greatly depends on the genetic background, since it is much milder in BALB/c than in MLR mice. Most ALPS type-Ia patients are heterozygous for the Fas mutation, but the parent carrying the mutation is generally healthy. Other complementary factors may thus be required in

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function of the severity of the mutation.[14] The same observation is true in DALD patients, since both parents generally display defective Fas function, but are healthy.[13] We suggested that a concurrent factor may be production of high levels of osteopontin (OPN), a cytokine involved in inflammation that inhibits activation-induced cell death (AICD) of lymphocytes. We found that DALD patients display increased frequency of polymorphic variants of the OPN gene (OPN^{high} gene variants) that cause increased production of OPN by stabilizing its mRNA and increase the risk of DALD by about 8-fold.[15,16]

A second concurrent factor may be inherited alterations of the perforin gene *(PRF1)* that decrease the function of this protein, which is stored in the lytic granules of cytotoxic cells and plays a crucial role in cell-mediated cytotoxicity by forming pores in the target-cell membrane.[17] Biallelic mutations of *PRF1* cause about 30% of cases of familial hemophagocytic lymphohistiocytosis (HLH), a rare life-threatening immune deficiency ascribed to decreased capacity of CD8+ T cells and NK cells to kill virus-infected cells.[17-22] A further 25% of patients display mutations of the *MUNC 13-4* gene involved in perforin storage in the lytic granules and exocytosis.[23] HLH is a recessive disease and subjects carrying heterozygous PRF1 mutations are generally healthy.

We have identified a heterozygous mutation of *PRF1* in an ALPS patient who also carried a heterozygous mutation of the Fas gene. Since these mutations were inherited from distinct parents who were healthy, we suggested that their cotransmission was responsible for the son's ALPS.[24]

Systematic evaluation of the role of *PRF1* in ALPS was not undertaken on that occasion. The present study has thus been performed to extend the analysis of *PRF1* to a larger number of patients and evaluate its role in the development of ALPS and DALD.

Patients, materials, and methods

Patients

We analyzed 14 ALPS and 28 DALD Italian patients (some have already been presented in Dianzani et al,[12] Ramenghi et al,[13] and Campagnoli et al[25]). Diagnosis of ALPS was based on the presence of all the following criteria: (1) autoimmune manifestations; (2) chronic nonmalignant lymphadenopathy (2 or more lymph nodes enlarged over 2 cm in diameter) and/or splenomegaly; (3) defective Fas-induced apoptosis in vitro; and (4) mutations in the Fas, FasL, or caspase-10 genes and/or expansion of DN T cells in the peripheral blood. The Fas, FasL, caspase-10, and OPN genes were sequenced from genomic DNA, as previously reported.[12,13] Seven ALPS patients (patients 1-7) carried heterozygous mutations of the Fas gene.

Diagnosis of DALD was based on the presence of the first 3 criteria, but lack of the fourth one. Two DALD patients (DALD-2 and -24) carried a heterozygous variation of the caspase-10 gene, causing a V410I amino acid substitution, initially associated with ALPS in homozygosity,[10] but then recognized as a polymorphism.[25,26]

No patients displayed the diagnostic criteria for HLH. Controls (n = 816) were ethnically matched, healthy individuals. All patients and controls were unrelated, white and Italian. Peripheral-blood specimens and serum were obtained from patients and healthy controls with written informed consent, which was obtained in accordance with the Declaration of Helsinki. The study was performed according to the guidelines of the local ethics committee of the Ospedale Maggiore of Novara (Novara, Italy).

Flow cytometry

Analysis of lymphocyte subpopulations in peripheral-blood mononuclear cells (PBMCs) was performed by direct immunofluorescence and flow cytometry. Perforin expression was evaluated in fixed and permeabilized cells (Cytofix-Cytoperm; BD PharMingen, San Diego, CA) using a phycoerythrin (PE)–conjugated antiperforin antibody (BD PharMingen) and flow cytometry.

Fas function assay

Fas-induced cell death was evaluated as previously reported on T-cell lines obtained by activating PBMCs with phytohemagglutinin at days 0 (1 μg/mL) and 15 (0.1 μg/mL) and cultured in RPMI 1640 + 10% fetal calf serum (FCS) + rIL-2 (2 U/mL) (Biogen, Geneva, Switzerland). Fas function was assessed 6 days after the second stimulation (day 21).[12,13] Cells were incubated with control medium or anti-Fas MAb (CH11, IgM isotype) (1 μg/mL) (UBI, Lake Placid, NY) in the presence of rIL-2 (1 U/mL) to minimize spontaneous cell death. Cell survival was evaluated after 18 hours by counting live cells in each well by the trypan blue exclusion test and by flow cytometry of cells excluding propidium iodide and unstained by annexin V–FITC; the 2 methods gave overlapping results. Assays were performed in duplicate. Cells from 2 healthy donors were included in each experiment as positive controls. Results were expressed as specific cell-survival percent, calculated as follows: (total live-cell count in the assay well/total live-cell count in the control well) × 100.

Fas function was defined as defective when cell survival was less than 82% (the 95th percentile of data obtained from 200 healthy controls).

Amplification of *PRF1* and mutation detection

Genomic DNA was isolated from PBMCs, and exon 2 and 3 of the perforin coding region were amplified in standard polymerase chain reaction (PCR) conditions. The primers used for amplification have been previously described. PCR products were purified with the EXO/SAP kit.[24] Sequencing was performed with the ABI PRISMR BigDye Terminator kit (Applied Biosystems, Foster City, CA) on an automatic sequencer (Applied Biosystems 3100 Genetic Analyzer) according to the manufacturer's instructions with the amplification primers plus 2 internal primers (forward 5'-CAGGTCAACATAGGCATCCACG-3'; reverse 5'-GAACAGCAG-GTCGTTAATGGAG-3') for exon 3. OPN gene variants were typed as previously reported.[15]

Cytotoxicity assays

NK activity of PBMCs was assessed by a standard 4-hour ^{51}Cr release assay with K562 cells as the target. Results are expressed as specific lysis percent calculated as follows: (sample ^{51}Cr release-spontaneous release)/(maximal release-spontaneous release) × 100.

Statistical analysis

Comparisons of NK activity, perforin expression, and NK-cell distribution were performed with the nonparametric Mann-Whitney *U* test. Genotype distributions were analyzed with the χ^2 test or the Fisher exact test as reported. All *P* values are 2-tailed, and the significance cut-off was *P* below .05.

Results

The work involved 14 patients with ALPS and 28 with DALD. Both groups displayed autoimmune manifestations, lymphadenopathy and/or splenomegaly, and defective Fas function; ALPS patients alone also displayed mutations of the Fas gene and/or peripheral-blood expansion of DN T cells. Figure 1 shows Fas function of T cells from all patients and available parents. That of ALPS-4 and -7 was borderline, but they were included in the ALPS group because they also carried a Fas gene mutation and expansion of DN T cells. Most parents, too, displayed defective Fas function.

The coding region of *PRF1* was sequenced from genomic DNA in all patients and 816 random controls in the search for variations previously associated with HLH. Only 2 HLH-associated missense variations were detected, a C/T substitution in position 272 of the cDNA (numerations are referred to cDNA clone M28393,

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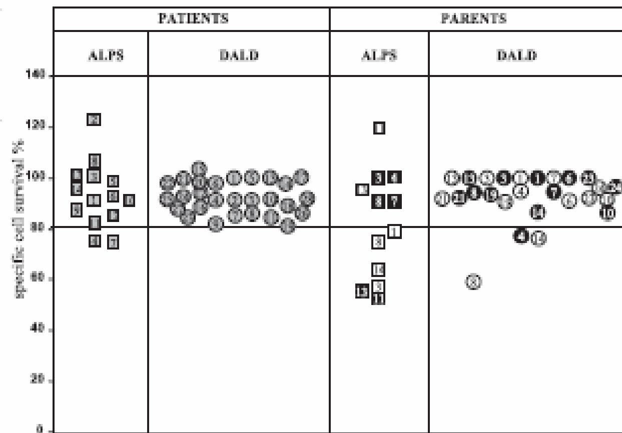

Figure 1. Fas-induced T-cell death in patients with ALPS or DALD and several of their parents. Data from ALPS patients' families are marked with squares, those from DALD patients' families with circles; gray symbols mark patients; black symbols, the fathers; and white symbols, the mothers. Numbers correspond to the code assigned to each patient. Long-term T-cell lines were treated with anti-Fas Mab, and survival was assessed after 18 hours. Results are expressed as relative cell-survival percent. The horizontal lines indicate the upper limit of the normal range, calculated as the 95th percentile of data obtained from 200 healthy donors (median cell survival of controls was 60%; the 5th-95th percentile range was 36%-82%). In the control wells (ie, in the absence of apoptotic stimuli), spontaneous cell loss was always less than 10% of the seeded cells and similar in cultures from the patients and healthy donors. ALPS patients 1 to 7 carried heterozygous mutations of the Fas gene.

ATG = +1) and an A/G substitution in position 755, which caused an A91V and an N252S amino acid substitution at the protein level, respectively. The genotypic distributions of these variations did not deviate significantly from the Hardy-Weinberg equilibrium in either group.

The N252S substitution was found in 2 ALPS patients (ALPS-5 and ALPS-11), 2 controls, and no DALD patients. The overall genotype distributions (Table 1) were significantly different in ALPS and controls ($P = .0016$). The N252S allelic frequency was significantly higher in the ALPS patients (7.1% vs 0.1%, $P = .0016$) and conferred an OR = 62.7 (95% CI: 6-654.9). This variation had been previously reported by some of us in ALPS-5.[24]

The A91V variation was carried by 6 DALD patients only: it was heterozygous in 5 (DALD-3, -6, -9, -14, -25) and homozygous in one (DALD-10). Moreover, it was detected in 72 controls: heterozygous in 69 and homozygous in 3. The overall genotype distributions (Table 1) were significantly different in DALD and controls ($P = .01$). The A91V allelic frequency was significantly higher in the DALD patients (12.5% vs 4.6%, $P = .016$) and conferred an OR = 3 (95% CI: 1.2-7.1).

Table 1. Frequency distribution of the A91V and N252S PRF1 variations and the *OPN*^{high} genotypes in 14 ALPS and 28 DALD patients

	ALPS*		DALD†		Controls	
Genotypes according to variation	N	%	N	%	N	%
PRF1 N252S						
NN	12	85.7	28	100	814	99.8
NS	2	14.3	0	0	2	0.2
SS	0	0	0	0	0	0
PRF1 A91V						
AA	14	100	22	78.5	744	91.2
AV	0	0	5	17.9	69	8.4
VV	0	0	1	3.6	3	0.4
PRF1 and *OPN* susceptibility genotypes‡						
None	4	28.6	6	21.4	51	38.1
PRF1 alone	2	14.3	0	0	10	7.5
OPN alone	8	57.1	16	57.2	70	52.2
PRF1 + *OPN*	0	0	6	21.4	3	2.2

*The overall genotypic distribution of N252S was significantly different from controls ($P = .0016$, Fisher exact test).

†The overall genotypic distribution of A91V was significantly different from controls ($P = .01$, Chi square test); frequency of *PRF1*+*OPN* susceptibility genotypes was significantly higher than in controls ($P = .0009$, Fisher exact test).

‡Genotypes with the N252S (in ALPS) or A91V (in DALD) PRF1 variations (either homozygous or heterozygous) and/or the 282C-750T-1083A-1239C or 282C-750T-1083G-1239C *OPN*^{high} haplotypes.

Four other nucleotide variations were detected, but were not further evaluated since they did not change the amino acid nor influence the splicing sites. Two (C822T and T900C) had been previously reported as common polymorphisms not associated with HLH. Their frequency was similar in the patients and the controls. The other 2 (G435A and A462G) were in perfect linkage disequilibrium with the A/G substitution in position 755 (N252S) and were in fact only detected in the 4 subjects carrying this variation.

We had previously found that DALD development is favored by the 282C-750T-1083A-1239C and 282C-750T-1083G-1239C single nucleotide polymorphism haplotypes of the OPN gene (*OPN*^{high} gene variants).[15] To determine whether *PRF1* and *OPN* variations have a cooperative effect on ALPS/DALD development, we typed the OPN gene in all patients and 134 controls and evaluated the frequency of copresence of the *PRF1* and *OPN* genotypes conferring susceptibility to ALPS/DALD (Table 1). Copresence was displayed by 6 (21.4%) of 28 DALD patients, but only 3 (2.2%) of 134 controls, and increased the risk of DALD by 17-fold relatively to the absence of both factors (OR = 17; 95% CI: 2.7-122; $P = .0007$) and by 9-fold relatively to the presence of only one (OR = 8.8, 95% CI: 1.7-50.5; $P = .004$). By contrast, this cooperation was not detected in ALPS patients since none of them carried both factors.

We had previously shown that ALPS-5 carried a heterozygous mutation of the Fas gene.[24] His PRF1 N252S variation was inherited from the mother, whereas the Fas mutation also was carried by the father and a brother, and cosegregated with defective Fas function (Figure 2A). Since all 3 relatives were healthy, we suggested that co-inheritance of the Fas and perforin gene variations played a role in ALPS development in this patient. The mutated gene was not known in ALPS-11, and his inheritance pattern was determined by analyzing Fas function and sequencing *PRF1* in his parents. N252S was carried by his father only, whereas Fas function was defective in his mother only (Figure 2A). Once again, therefore, Fas and perforin alterations were inherited from different parents, who were both healthy. These data indicate that association of defective Fas function with the N252S variation strongly favors ALPS development.

A family analysis also was conducted for 3 DALD patients carrying the A91V variation (DALD-3, -6, -10, Figure 2B). In

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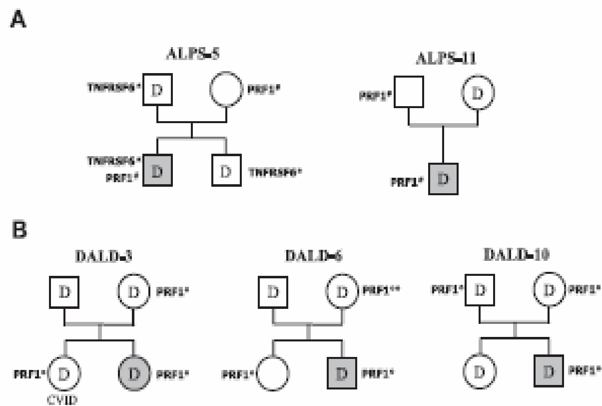

Figure 2. Pedigrees of patients ALPS-5 and -11 and DALD-3,-6, and -10. (A) Inheritance of the N252S PRF1 mutation (PRF1#) and defective Fas function (D) in ALPS-5 and -11; inheritance of the Fas mutation (TNFRSF6*) is also shown for ALPS-5. (B) Inheritance of the A91V PRF1 variation (PRF1°) and defective Fas function (D) in the 3 DALD patients. Subjects with ALPS/DALD are marked in gray; the sister of DALD-3 displayed CVID. PRF1°° marks A91V homozygotes. Fas function was evaluated as reported in Figure 1.

DALD-3's family, Fas function was defective in both parents and the sister, whereas A91V was carried by the mother and the sister: both parents were healthy, whereas the sister presented common variable immune deficiency (CVID). Intriguingly, DALD-3 also developed hypogammaglobulinemia some years after disease onset. In DALD-6's family, Fas function was defective in both parents, whereas A91V was carried by the mother (homozygous) and the sister; all these relatives were healthy. In DALD-10's family, Fas function was defective in both parents and the sister, whereas A91V was carried by both parents; all these relatives were healthy. Analysis of the OPN gene showed that all subjects in these 3 families also carried the OPN^{high} gene variants (data not shown). Association of defective Fas function and the A91V variation is thus not sufficient to induce DALD, even in the presence of the *OPN* susceptibility alleles, since 4 of 8 subjects with this association were healthy.

To assess whether N252S and A91V correlated with altered function and/or expression of perforin, NK activity was evaluated by a standard ^{51}Cr-release assay and perforin expression by flow cytometry in 5 patients: ALPS-5 and -11 with N252S, and DALD-3, -6, and -10 with A91V. Perforin expression was slightly decreased in ALPS-5, DALD-6, and DALD-10, but normal in the other subjects, whereas the proportion of NK cells, detected as CD3−CD56+ or CD3−CD16+ cells, was in the normal range in all subjects. NK activity was significantly lower in the patients with A91V than in the controls ($P = .015$), but not decreased in those with N252S (Table 2). However, in ALPS-11, previous analyses showed that NK activity was almost undetectable at the age of 3 (ie, at diagnosis), extremely low but detectable at the age of 5, and normal at the age of 12 (Table 2). In ALPS-5, NK activity was assessed at the age of 30, and no previous analyses were available.

Discussion

This paper follows a description of an ALPS patient (ALPS-5) with variations of both the Fas gene and *PRF1*.[24] It shows that his N252S variation is significantly more frequent in ALPS patients than in healthy controls. Moreover, the frequency of a second HLH-associated PRF1 variation, A91V, is significantly increased in DALD patients, who display an incomplete ALPS pattern. N252S was detected in 2 of 14 ALPS patients (ALPS-5 and -11). It increased susceptibility to ALPS by about 63-fold and was found only in 2 of 816 ethnically matched controls, as in other studies.[27]

Table 2. NK activity, perforin expression, and proportion of NK cells in PBMCs of ALPS/DALD patients carrying the N252S and A91V perforin variations

Subjects according to PRF1 variation*	Effector-target ratios for NK activity†			Perforin expression‡		Peripheral-blood NK cells, %	
	100:1	30:1	10:1	%	MFI	$CD3^-CD16^+$	$CD3^-CD56^+$
N252S							
ALPS-5	58	49	38	6§	9§	11	16
ALPS-11 (12 years)	35	28	18	24	50	13	15
(5 years)	7§	4§	2§	nd	nd	nd	nd
(3 years)	3§	1§	0§	nd	nd	nd	nd
A91V							
DALD-3	13	12	3	45	62	8	8
DALD-6	28	12	3	15§	61	13	13
DALD-10	18	11	4	38	17§	9	13
Median	18	12	3	38	61	9	13
Controls‖	31 (13-56)	23 (8-35)	12 (2-31)	23 (17-26)	47 (28-74)	11 (5-31)	17 (4-27)

nd indicates not determined.

*All patients were heterozygous for the indicated variation except for DALD-10, who was homozygous.

†NK activity is expressed as specific cell-lysis percent, and it is the mean of triplicate assays, whose standard deviation was always < 10% of the mean. Spontaneous cell lysis was always < 10% of maximal cell lysis.

‡Perforin expression is shown as percentage of positive cells and mean fluorescence intensity (MFI) in arbitrary units.

§< 5th percentile of controls.

‖Median (5th-95th percentile range) from 10 controls. The NK activity of these DALD patients was significantly lower than that of the controls ($P = .015$, Mann-Whitney test).

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N252S occurs within the membrane-attack-complex, a region critically involved in the pore-forming activity of perforin, but its functional significance has been debated since it has been associated with normal NK activity[17,28,29] as in both ALPS-5 and -11 at the time of this study. The finding that ALPS-11 displayed a striking defect of NK activity when he was 3 and 5 years old suggests that N252S is here associated with other factors decreasing NK function in early childhood, followed by normalization on the part of unknown compensatory mechanisms.

A91V was detected in 6 of 28 DALD patients. It increased susceptibility to DALD by 3-fold and was relatively frequent (4.6%) in the controls, as in other studies.[27,30,31] This variation decreases perforin function by altering its conformation, decreasing its cleavage to the active form and increasing its degradation.[28-30] In line with this view, our patients displayed lower NK activity than the controls, especially at low effector-to-target ratios.

These data suggest that some PRF1 variations that cause HLH when combined with a second PRF1 variation may favor ALPS/DALD development if inherited defects hitting Fas function also are present. Fas function is normal in HLH and hence is not a contributory of this disease.[21]

The family analyses showed that combination of A91V with the Fas defect was not sufficient to induce DALD since several healthy family members carried both alterations. This risk was significantly increased by copresence of the OPN^{high} gene variants, but even their combination with A91V and the Fas defect was not sufficient to induce DALD in 4 of 8 DALD family members. Combination of N252S with the Fas defect seems to have a stronger effect than A91V, since we found it in patients only. However, even this combination may not be sufficient for ALPS development, since Rieux-Laucat et al[32] have described an ALPS patient and his healthy father carrying both N252S and a Fas gene mutation. The observation that N252S is in perfect linkage disequilibrium with G435A and A462G raises the possibility that these variations or others included in the ancestral haplotype play a role in ALPS development. A second possibility is that concurrent roles are played by other factors. The fact that *OPN* did not cooperate with N252S in ALPS development may be related to the stronger effect displayed by N252S in ALPS than by A91V in DALD, and to the possibility that the genetic hit of Fas function is more severe in ALPS than in DALD.

Fas and perforin alterations may cooperate in affecting both the antiviral response and the switching off of the immune response. Both molecules are used by cytotoxic cells to kill virus-infected cells. Moreover, Fas is highly expressed by effector lymphocytes that are switched off by several FasL^{+} cell types, but a regulatory activity also has been ascribed to perforin-mediated killing of effector lymphocytes and antigen-presenting cells.[33-43] In this connection, it is noteworthy that OPN inhibits lymphocyte AICD, another mechanism involved in switching off the immune response. Lymphocyte accumulation and autoimmunity displayed by ALPS and DALD patients may be favored by both defective immune response switching off and decreased virus clearance that would prolong the immune response. This possibility opens the way to the view that ALPS/DALD may overlap both HLH and other inherited diseases characterized by lymphoproliferation and defective control of viral infections, such as the X-linked lymphoproliferative syndrome (XLP) due to mutations of the SAP gene altering function of the 2B4 NK coreceptor.[44] Intriguingly, both ALPS and XLP are associated with high susceptibility to lymphoid neoplasia, which also seems favored by inherited PRF1 variations.[45]

Acknowledgments

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References

1. Nagata S. Apoptosis by death factor. Cell. 1997; 88:355-365.
2. Krammer PH. CD95's deadly mission in the immune system. Nature. 2000;407:789-795.
3. Dianzani U, Chiocchetti A, Ramenghi U. Role of inherited defects decreasing Fas function in autoimmunity. Life Sciences. 2003;72:2803-2824.
4. Fisher GH, Rosenberg FJ, Straus SE, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell. 1995;81:935-946.
5. Rieux-Laucat F, Le Deist F, Hivroz C, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. Science. 1995;268:1347-1349.
6. Straus SE, Sneller M, Lenardo MJ, Puck JM, Strober W. An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. Ann Int Med. 1999;130:591-601.
7. Sneller MC, Dale JK, Straus SE. Autoimmune lymphoproliferative syndrome. Curr Opin Rheum. 2003;15:417-421.
8. Rieux-Laucat F, Fischer A, Le Deist F. Cell-death signaling and human disease. Curr Opin Immunol. 2003;15:325-331.
9. Straus SE, Jaffe ES, Puck JM, et al. The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis. Blood. 2001;98:194-200.
10. Wang J, Zheng L, Lobito A, et al. Inherited human caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. Cell. 1999; 98:47-58.
11. Rieux-Laucat F, Le Deist F, Fischer A. Autoimmune lymphoproliferative syndromes: genetic defects of apoptosis pathways. Cell Death Diff. 2003;10:124-133.
12. Dianzani U, Bragardo M, DiFranco D, et al. Deficiency of the Fas apoptosis pathway without Fas gene mutations in pediatric patients with autoimmunity/lymphoproliferation. Blood. 1997;89:2871-2879.
13. Ramenghi U, Bonissoni S, Migliaretti G, et al. Deficiency of the Fas apoptosis pathway without Fas gene mutations is a familial trait predisposing to development of autoimmune diseases and cancer. Blood. 2000;95:3176-3182.
14. Jackson CE, Fischer RE, Hsu Ap, et al. Autoimmune lymphoproliferative syndrome with defective Fas: genotype influences penetrance. Am J Hum Gen. 1999;64:1002-1014.
15. Chiocchetti A, Indelicato M, Bensi T, et al. High levels of osteopontin associated to polymorphisms in its gene are a risk factor for development of autoimmunity/lymphoproliferation. Blood. 2004;103:1376-1382.
16. Chiocchetti A, Comi C, Indelicato M, et al. Osteopontin gene haplotypes correlate with multiple sclerosis development and progression. J Neuroimmunol. 2005;63:172-178.
17. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science. 1999;286: 957-959.
18. Clementi R, zur Stadt U, Savoldi G, et al. Six novel mutations in the PRF1 gene in children with haemophagocytic lymphohistiocytosis. J Med Genet. 2001;38:643-646.
19. Ueda I, Morimoto A, Inaba T, et al. Characteristic perforin gene mutations of haemophagocytic lymphohistiocytosis patients in Japan. Br J Haematol. 2003;121:503-510.
20. Feldmann J, Le Deist F, Ouachee-Chardin M, et al. Functional consequences of perforin gene mutations in 22 patients with familial haemophagocytic lymphohistiocytosis. Br J Haematol. 2002; 117:965-972.
21. Fadeel B, Orrenius S, Henter JI. Familial hemophagocytic lymphohistiocytosis: too little cell death can seriously damage your health. Leuk Lymphoma. 2001;42:13-20.
22. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nature Rev Immunol. 2002;2:735-747.
23. Feldmann J, Callebaut I, Raposo G, et al. Munc13–4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). Cell. 2003; 115:461-473.

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24. Clementi R, Dagna L, Dianzani U, et al. Inherited perforin and Fas mutations in a patient with autoimmune lymphoproliferative syndrome and lymphoma. N Engl J Med. 2004;351:1419-1424.
25. Campagnoli MF, Garbarini L, Quarello P, et al. The broad spectrum of autoimmune lymphoproliferative disease: molecular bases, clinical features and long-term follow-up in 31 patients. Haematologica. 2006;91:538-541.
26. Zhu S, Hsu AP, Vacek MM, et al. Genetic alterations in caspase-10 may be causative or protective in autoimmune lymphoproliferative syndrome. Hum Genet. 2006;119:284-294.
27. Molleran Lee S, Villanueva J, Sumegi J, et al. Characterisation of diverse PRF1 mutations leading to decreased natural killer cell activity in North American families with haemophagocytic lymphohistiocytosis. J Med Genet. 2004;41:137-144.
28. Voskoboinik I, Thia MC, Trapani JA. A functional analysis of the putative polymorphisms A91V and N252S and 22 missense perforin mutations associated with familial hemophagocytic lymphohistiocytosis. Blood. 2005;105:4700-4706.
29. Risma KA, Frayer RW, Filipovich AH, Sumegi J. Aberrant maturation of mutant perforin underlies the clinical diversity of hemophagocytic lymphohistiocytosis. J Clin Invest. 2006;116:182-192.
30. Trambas C, Gallo F, Pende D, et al. A single amino acid change, A91V, leads to conformational changes that can impair processing to the active form of perforin. Blood. 2005;106:932-937.
31. Busiello R, Fimiani G, Miano MG, et al. A91V perforin variation in healthy subjects and FHLH patients. Int J Immunogen. 2006;33:123-125.
32. Rieux-Laucat F, Le Deist F, De Saint Basile G. Autoimmune lymphoproliferative syndrome and perforin. N Engl J Med. 2005;352:306-307.
33. Su MW, Pyarajan S, Chang JH, et al. Fratricide of CD8+ cytotoxic T lymphocytes is dependent on cellular activation and perforin-mediated killing. Eur J Immunol. 2004;34:2459-2470.
34. Badovinac VP, Hamilton SE, Harty JT. Viral infection results in massive CD8+ T cell expansion and mortality in vaccinated perforin-deficient mice. Immunity. 2003;18:463-474.
35. Badovinac VP, Tvinnereim AR, Harty JT. Regulation of antigen-specific CD8+ T cell homeostasis by perforin and interferon-gamma. Science. 2000;290:1354-1358.
36. Marks L, Levy RB. The cytotoxic potential of regulatory T cells: what has been learned from gene knockout model systems? Transplantation. 2004;77:S19-S22.
37. de Saint Basile G, Fischer A. Defective cytotoxic granule-mediated cell death pathway impairs T lymphocyte homeostasis. Curr Opin Rheumatol. 2003;15:436-445.
38. Voskoboinik I, Thia MC, De Bono A, et al. The functional basis for hemophagocytic lymphohistiocytosis in a patient with co-inherited missense mutations in the perforin (PFN1) gene. J Exp Med. 2004;200:811-816.
39. Zhou S, Ou R, Huang L, Moskophidis D. Critical role for perforin-, Fas/FasL-, and TNFR1-mediated cytotoxic pathways in down-regulation of antigen-specific T cells during persistent viral infection. J Virol. 2002;76:829-840.
40. Beilke JN, Kuhl NR, Van Kaer L, Gill RG. NK cells promote islet allograft tolerance via a perforin-dependent mechanism. Nat Med. 2005;11:1059-1065.
41. Bose A, Inoue Y, Kokko KE, Lakkis FG. Cutting edge: perforin down-regulates CD4 and CD8 T cell–mediated immune responses to a transplanted organ. J Immunol. 2003;170:1611-1614.
42. Ferlazzo G, Munz C. NK cell compartments and their activation by dendritic cells. J Immunol. 2004;172:1333-1339.
43. Della Chiesa M, Vitale M, Carlomagno S, Ferlazzo G, Moretta L, Moretta A. The natural killer cell–mediated killing of autologous dendritic cells is confined to a cell subset expressing CD94/NKG2A, but lacking inhibitory killer Ig-like receptors. Eur J Immunol. 2003;33:1657-1666.
44. Nichols KE, Ma CS, Cannons JL, Schwartzberg PL, Tangye SG. Molecular and cellular pathogenesis of X-linked lymphoproliferative disease. Immunol Rev. 2005;203:180-199.
45. Clementi R, Locatelli F, Dupre L, et al. A proportion of patients with lymphoma may harbor mutations of the perforin gene. Blood. 2005;105:4424-4428.

3.4.2 *Paper 2*

LETTER TO THE EDITOR

Identification of Defective Fas Function and Variation of the Perforin Gene in an Epidermodysplasia Verruciformis Patient Lacking EVER1 and EVER2 Mutations

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TO THE EDITOR

Epidermodysplasia verruciformis (EV), an infrequently reported lifelong clinical entity, is characterized by abnormal susceptibility to human papillomaviruses (HPVs). The natural course of the disease may at times be punctuated by the transformation of EV into squamous cell carcinoma; the lesions are preferentially located on sun-exposed sites. Nonsense mutations in two adjacent novel genes, named *EVER1* and *EVER2*, have recently been associated with the disease in some consanguineous families and sporadic cases (Ramoz *et al*., 2002; Orth, 2006). Despite these findings, we have recently described an EV case with a lack of EVER gene mutations and a remarkable CD8^{+} T-cell lymphocytopenia (Azzimonti *et al*., 2005). Although EV has recently been classified as a primary deficiency in innate immunity to specific HPV genotypes (Notarangelo *et al*., 2004), with the central role assigned to keratinocytes, the molecular mechanisms underlying abnormal susceptibility to a single type of weakly pathogenic infectious agent are still unclear.

A 59-year-old woman was admitted to our hospital with a diagnosis of EV. Physical examination revealed a limited number of multiple, flat, whitish and reddish papular lesions on the hands and forearms (Figure 1a and b) and a few pityriasis versicolor-like lesions on the trunk (Figure 1c). Surprisingly, the patient had never developed either cutaneous premalignant or malignant lesions, even in sun-exposed areas; thus, her forehead did not present any erythematous lesions. As reported in Figure 1d and e, biopsy specimens from lesions of the forearm and hand showed hyperkeratosis, acanthosis, and numerous large cells with pale staining of the cytoplasm and perinuclear vacuolization in the spinous and granular layers, resembling the typical histological features of EV (de Oliveira *et al*., 2003).

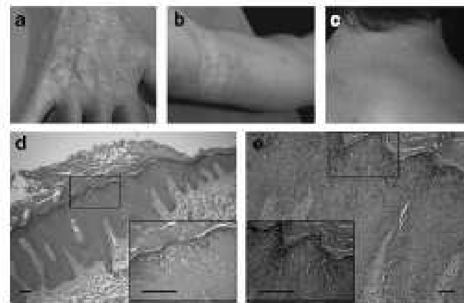

Figure 1. Clinical and histological findings from the study patient. Flat whitish and reddish papular lesions on the (a) hand and (b) forearm, (c) pityriasis versicolor-like lesions on the trunk. (d) A biopsy specimen from a papular lesion on the right forearm shows hyperkeratosis and acanthosis. The inset shows numerous large cells with pale staining of the cytoplasm and perinuclear vacuolization in the spinous and granular layers. (e) A biopsy specimen from a flat wartlike papule on the back of the hand, performed when she was 39 years old (1987), shows the same histopathological findings. Bar = 10 μm.

This study was approved by the Research Ethics Committee "Maggiore Hospital" Novara and conducted according to the Declaration of Helsinki Principles. Written informed consent was obtained from the patient.

HPV DNA analysis was performed on samples collected with prewetted cotton-tipped swabs from different sites of the skin and on a formalin-fixed, paraffin-embedded papular lesion from the forearm. The results obtained by PCR analysis are shown in Table 1.

Genetic analysis of *EVER1* (20 exons) or *EVER2* (16 exons) genes did not detect any mutation that would result in a loss of function. We only found a nonsynonymous heterozygous single nucleotide polymorphism in the *EVER2* gene (rs7208422) in position 1289 of the cDNA (numbering based on GenBank accession no. NM_152468), which caused the N306I amino-acid substitution at the protein level.

Analysis of serum Ig levels by ELISA showed that the patient displayed mildly decreased levels of IgA and normal amounts of IgM, IgG, and IgE (Table 2). Immunophenotype analysis of peripheral blood mononuclear cells by two-color immunofluorescence and flow cytometry (Table 2) showed a low reduction of helper T cells

Abbreviations: ALPS, autoimmune lymphoproliferative syndrome; EV, epidermodysplasia verruciformis; HPV, human papillomavirus; IL, interleukin; PRF1, perforin gene

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(CD3$^+$CD4$^+$) and a striking expansion (9%) of an atypical T-cell population expressing the TCRαβ, but double-negative for both CD4 and CD8 (double-negative T cells). The patient did not develop a contact allergy to dinitrochlorobenzene sensitization, and the purified protein derivative intradermal test was also negative. Analysis of cytokine secretion of peripheral blood T cells stimulated with anti-CD3 mAb showed a higher production of IL-10 in comparison to healthy controls (Figure 2a). Secretion of IL-4, IL-5, and tumor necrosis factor-α was also significantly increased. The proliferative response and natural killer activity were in the normal range (data not shown).

Expansion of double-negative T cells is a hallmark of the autoimmune lymphoproliferative syndrome (ALPS), an inherited disease mainly due to impaired apoptosis triggered by the Fas (CD95) death receptor (Straus *et al.*, 1999; Dianzani *et al.*, 2003; Worth *et al.*, 2006). Functional analysis of the Fas gene, performed by using *in vitro*-activated peripheral blood T cells stimulated with an anti-Fas mAb, revealed defective Fas function. By contrast, etoposide-induced cell loss, a topoisomerase II inhibitor chemotherapeutic agent that triggers a death pathway independent of Fas, was in the normal range (Figure 2b). Evaluation of Fas expression in these cells by immunofluorescence and flow cytometry detected normal levels (data not shown). Despite the double-negative T-cell expansion and defective Fas function, the patient did not show any clinical feature of ALPS.

ALPS is often caused by mutations of the Fas gene (*TNFRSF6*), but mutations of the FasL (*TNFSF6*), caspase-10 (*CASP10*), or caspase-8 (*CASP8*) genes have also been occasionally reported (Dianzani *et al.*, 1997). Moreover, we have recently shown that mutations of the perforin gene (*PRF1*) and polymorphisms of the osteopontin gene (*SPP1*) may cooperate with defective Fas function in development of ALPS-like diseases (Clementi *et al.*, 2004, 2006). No mutation was detected in *TNFRSF6*, *CASP10*, and *CASP8*, whereas a missense variation in *PRF1*, that is, a C/T substitution at position 272 of the cDNA (numerations are referred to cDNA clone M28393, ATG = +1), which caused an A91V amino-acid substitution, was found. She did not carry the ALPS-associated allele of *SPP1*, as she was homozygous for +1239A.

Table 1. HPV DNA presence in skin samples from different sites[1]

Sample	HPV type
Skin swabs	
Hand (papular lesion)	5, 14
Neck (pityriasis versicolor-like lesion)	14
Forehead (no lesion)	5, 14
Cheek bone (no lesion)	5, 14, 38
Surgical specimen	
Forearm (papular lesion)	14, 15, 24

[1]Skin samples collected with a saline-soaked cotton-tipped swab and from paraffin-embedded surgical specimen.

Table 2. *In vivo* immunological analysis of the EV patient

Parameter	Study patient	Normal range
Serum		
IgM	93[1]	40–230
IgG	1,050	700–1,600
IgA	48[2]	70–400
IgE	25	<150
PBMC immunophenotype		
T cells		
CD3$^+$	**465**[3] (40[4])	425–503
CD3$^+$CD4$^+$	**230**[2] (20)	280–332
CD3$^+$CD8$^+$	**226**[5] (19)	163–213
TCRα/β$^+$ CD4/CD8 DN	**105**[5] (9)	14–28
CD3$^+$/CD25$^+$	**161**[5] (14)	114–149
CD3$^+$/CD28$^+$	254 (22)	180–312
CD3$^+$/CD45$^+$RA	509 (44)	616–676
CD3$^+$/CD45$^+$RO	540 (46)	344–539
B cells		
CD19$^+$/CD20$^+$	217 (19)	110–243
NK cells		
CD16$^+$/CD56$^+$	29 (2)	15–47
Monocytes		
CD14$^+$	78 (7)	54–104
Skin DTH response		
DNCB	—	+++
PPD	—	+++

DTH, delayed-type hypersensitivity; DNCB, dinitrochlorobenzene; PBMC, peripheral blood mononuclear cells; PPD, purified protein derivative.
Numbers in bold indicate statistically significant difference compared to the normal range.
[1]Values are expressed as international units (IU) per ml.
[2]Absolute number lower than the normal range.
[3]Values are expressed as median number of cells per μl (percentage of peripheral blood mononuclear cells out of the total mononuclear cell population); similar results were obtained from blood sample analyses performed over a 1-year period.
[4]Median percentage of lymphocytes.
[5]Absolute number higher than the normal range.

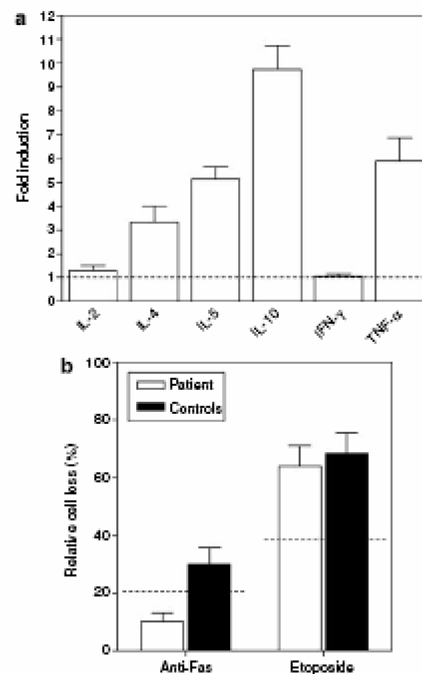

Figure 2. Evaluation of cytokine secretion and T cell survival in the PBMC of the study patient. (a) Cytokine secretion by peripheral blood mononuclear cells of the study patient versus healthy controls (for each cytokine, results are expressed as fold induction above the media of the controls, referred to 1 as represented by the dotted line). The peripheral blood mononuclear cells were stimulated with anti-CD3 mAb. Cytokines were measured on day-2-culture supernatants using a cytometric bead array kit. Results shown are averages of two experiments. IL, interleukin; IFN, interferon; TNF, tumor necrosis factor. (b) Fas- and etoposide-induced T-cell loss in the EV patient and three controls. T-cell lines derived from each subject were treated with the indicated reagent, and cell survival was assessed after 18 hours. Results are expressed as % relative cell loss and are the mean ±SD of two independent assays for the patient (white bars) and three controls run in parallel (black bars). The horizontal lines indicate the lower limit of the normal range calculated as the 5th percentile of data obtained from 200 normal donors. In the control wells (i.e., in the absence of apoptotic stimuli), spontaneous cell loss was always <10% of the seeded cells and was similar to that in cultures from the patients and normal donors.

In summary, this study describes an EV patient lacking mutations of the EVER genes, but carrying several immunological alterations typically found in patients with ALPS, such as peripheral blood expansion of double-negative T cells, decreased Fas function, and a strikingly increased secretion of IL-10. In the study patient, no mutations were detected in the Fas (ALPS type I) and caspase-10 or caspase-8 gene (ALPS type II). Therefore, her defect might be similar to that of ALPS type III, that is, ALPS lacking known mutations and presumed to carry unknown mutations of genes involved in the Fas signaling pathway. Moreover, the patient displayed the A91V variation of the perforin gene that has been associated with an incomplete variant of ALPS.

A number of reports on viral disease models, in which mice with deficiencies in one or more components of the two cytolytic pathways were used, demonstrated that perforin and Fas concur in virus clearance (Kagi *et al.*, 1994; Balkow *et al.*, 2001). In this context, it can be postulated that both Fas function and perforin defects may contribute to the abnormal susceptibility of the study patient to cutaneous HPV infection, possibly by decreasing viral clearance *in vivo*. Consistent with a role of defective cell-mediated cytotoxicity in some EV patients, we previously reported an EV patient displaying a dramatic CD8+ T-cell lymphocytopenia (Azzimonti *et al.*, 2005). Thus, a common pathogenetic mechanism can be found in the reduced number or decreased cytotoxic function of $CD8^+$ T cells that may impair the immune defenses against HPV and lead to lifelong infection. It remains to be explained why a cell-mediated cytotoxicity defect would selectively favor cutaneous HPV infection. One possibility is that the putative mild cytotoxicity defect, carried by the EV patient, may reveal itself only in the skin environment, which is a peculiar lymphoid tissue. A second, non-mutually exclusive possibility is that additional genetic or environmental factors may be present in such patients.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary text. Materials and Methods, Supplementary References.

REFERENCES

Azzimonti B, Mondini M, De Andrea M, Gioia D, Dianzani U, Mesturini R *et al.* (2005) CD8+ T-cell lymphocytopenia and lack of EVER mutations in a patient with clinically and virologically typical epidermodysplasia verruciformis. *Arch Dermatol* 141:1323–5

Balkow S, Kersten A, Tran TT, Stehle T, Grosse P, Museteanu C *et al.* (2001) Concerted action of the FasL/Fas and perforin/granzyme A and B pathways is mandatory for the development of early viral hepatitis but not for recovery from viral infection. *J Virol* 75:8781–91

Clementi R, Chiocchetti A, Cappellano G, Cerutti E, Ferretti M, Orilieri E *et al.* (2006) Variations of the perforin gene in patients with autoimmunity/lymphoproliferation and defective Fas function. *Blood* 108:3079–84

Clementi R, Dagna L, Dianzani U, Dupre L, Dianzani I, Ponzoni M *et al.* (2004) Inherited perforin and Fas mutations in a patient with autoimmune lymphoproliferative syndrome and lymphoma. *N Engl J Med* 351:1419–24

de Oliveira WR, Festa Neto C, Rady PL, Tyring SK (2003) Clinical aspects of epidermodysplasia verruciformis. *J Eur Acad Dermatol Venereol* 17:394-8

Dianzani U, Bragardo M, DiFranco D, Alliaudi C, Scagni P, Buonfiglio D *et al.* (1997) Deficiency of the Fas apoptosis pathway without Fas gene mutations in pediatric patients with autoimmunity/lymphoproliferation. *Blood* 89:2871–9

Dianzani U, Chiocchetti A, Ramenghi U (2003) Role of inherited defects decreasing Fas function in autoimmunity. *Life Sci* 72: 2803–24

Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ *et al.* (1994) Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* 369:31–7

Notarangelo L, Casanova JL, Fischer A, Puck J, Rosen F, Seger R *et al.* (2004) Primary immunodeficiency diseases: an update. *J Allergy Clin Immunol* 114:677–87

Orth G (2006) Genetics of epidermodysplasia verruciformis: insights into host defense against papillomaviruses. *Semin Immunol* 18:362–74

Ramoz N, Rueda LA, Bouadjar B, Montoya LS, Orth G, Favre M (2002) Mutations in two adjacent novel genes are associated with epidermodysplasia verruciformis. *Nat Genet* 32:579–81

Straus SE, Sneller M, Lenardo MJ, Puck JM, Strober W (1999) An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. *Ann Intern Med* 130:591-601

Worth A, Thrasher AJ, Gaspar HB (2006) Autoimmune lymphoproliferative syndrome: molecular basis of disease and clinical phenotype. *Br J Haematol* 133:124–40

SECTION II

4. MULTIPLE SCLEROSIS: an overview.

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) due to autoimmune aggression of myelin antigens. Its onset is usually between 20 and 40 years of age and displays a chronic development leading to substantial disability because of deficits of sensitive and motor functions. Its aetiology remains unclear and risk is greater in women than in men; the female-to-male ratios are between 1.5 and 2.5 in most populations, with a trend toward greater values in the most recent studies **[87]**. In high-risk populations, the lifetime risk for MS is about 1 in 200 for women and somewhat less for men.

MS is rare in Asia and, in all continents, it is rare in the tropics and subtropics. Within regions of temperate climate, MS incidence and prevalence increase with latitude, both north and south of the equator **[87]**.

Italy shows a prevalence of 20/100.000, with a total number of patients estimated at about 50.000, with the highest prevalence in Sicily and Sardinia **[88-89]**. Genetic predisposition most likely contributes to geographic variations in MS incidence **[90]** with the highest risk in the Caucasian population. However, the remarkable differences in risk among people of common ancestry who migrate to areas of high or low MS prevalence suggest the role of environmental factors, which are confirmed by several epidemiological studies. These environmental factors seem to be predominantly effective in age childhood and first adolescence, since the immigrant populations tend to keep their disease risk similar to that detected in the area of origin if migration occurred after the fifteenth year of age, whereas they acquire the risk of the immigration area if migration occurred after that age **[91]**.

Three main clinical course of MS have been described: a) relapse remitting (RR), b) secondary progressive (SP), and c) primary progressive (PP) **[92]**. RR is the most common form and is the type most people are initially diagnosed with. It is characterized by relapses with a flare up of symptoms followed by remissions to symptom free phases. The SP form is an evolution of the RR form and it is characterized by incomplete recovery from relapses and chronic progressive evolution of disability; this evolution may happens within 5-20 years from onset. Around 65% of people diagnosed with RR MS go on to develop secondary progressive MS. About 15% of people with MS is affected by PP MS that is characterized by a lack of the RR phase; symptoms creep up slowly and steadily get worse. People with this type of multiple sclerosis tend to be diagnosed when they are older. About 85% of sufferers with this form of the disease develop walking difficulties because, unlike RR MS in which nerve damage is found in both the brain and spinal cord, the disease primarily attacks the spinal cord. They are unlikely to have the cognitive problems associated with damage to the brain **[92]**.

Two other types of MS have been described: (1) benign MS, in which one or two mild attacks, mainly affecting the sensory functions, are followed by complete recovery, and (2) progressive relapsing MS that is progressive from the outset with clear acute attacks and symptom flare ups, but without complete remissions since symptoms continue to get worse in between relapses.

4.1Aetiology

4.1.1 Genetic Factors

The earliest evidence supporting a genetic influence on MS susceptibility was derived from observations of familial aggregation and differences in MS risk among ethnic groups

residing in the same geographical regions. In Northern Europe, recurrence risk in a sibling is around 3% and the point prevalence is approximately 1 per 1.000 **[93-96]**. Second- and third-degree relatives of MS patients are also at an increased risk for MS, supporting the possibility that genetic factors, which are distinct from a common environmental exposure, influence familial susceptibility **[97]**. Studies carried out in Canada of adoptees **[98]**, half-siblings **[99]** and spouses **[100]** seem to confirm that genetics is primarily responsible for the co-incidence of MS within families. However, an intriguing association with month of birth was observed in the Canadian study, suggesting an interaction between genes and an environmental factors operating during gestation or shortly after birth **[101]**. Parent-of-origin genetic effects might influence both disease susceptibility and outcome **[102-104]**, and concordance in families for early and late clinical features indicates that, in addition to susceptibility, genes influence disease course and other aspects of the clinical phenotype **[105-108]**. Finally, twin studies from several populations consistently indicate that a monozygotic twin of an MS patient is at higher risk (~30% concordance) for MS than is a dizygotic twin (~5% concordance) **[109-112]**, providing additional evidence for a significant, but complex, genetic aetiology.

Two main issues have confounded efforts to identify susceptibility genes in complex diseases such as MS. First, the effects attributable to individual genes are modest. Second, the significant amount of genetic variations in the human population means that relevant signals have to be sorted from an overwhelming amount of noise **[113-114]**. The number of MS susceptibility loci, their effect size, and mode of inheritance can not be judged with certainty by studying the pattern of inheritance in multi-case families. However, the nonlinear relationship between familial recurrence risk and the degree of relatedness, as well as the available genetic data, suggest that between 20 and 100 common variants, each

increasing risk by only a modest factor of 1.2–1.5, would be sufficient to account for the prevalence and heritability of MS. Alternatively, many hundreds, if not thousands, of rare variants would be required to explain MS susceptibility, even if they individually increase the risk by as much as 10–20 fold **[115]**.

Despite many linkage and association studies performed over the years and in many populations, until very recently, the only replicated genetic risk factor remained the one mapping in the human Major Histocompatibility complex (HLA) region. The strongest association in this region is with the Class II allele HLA-DRB1*1501 (DR15) that in most tested Caucasoid populations increased the risk by about 3. Recent data point to the presence of a second MS risk modulating factor in the HLA-Class I region. Three associated alleles were recently reported by three different groups, namely HLA-A*2, HLA-C*05 and MOG-L142, all conferring a protective effect decreasing the risk by values between 0.49 and 0.7 independent of linkage disequilibrium with DR alleles **[116-118]**. While these data demonstrate that genes in the HLA class I region indeed exert an additional influence on the risk of MS, the identification of the primarily associated class I gene requires further studies.

The identification of non-HLA susceptibility loci has remained elusive, with no reliably replicated association, likely because most non-HLA susceptibility genes have a small phenotypic effect conferring marginal risk values that only extremely large patient and control datasets have the statistical power to detect. At last, thanks to the implementation of high throughput techniques and the employment of large data sets, some apparently reliable results are starting to be reported. A whole genome association study performed with about 300000 single nucleotide polymorphisms (SNPs) identified a strong association with two intronic SNPs within the interleukin-2 receptor alpha gene (IL2RA) ($p=2.9x10^{-8}$) and a

nonsynonymous SNP (T244I, rs6897932) in the interleukin-7 receptor alpha gene (IL7RA) ($p=2.9x10^{-7}$) **[119]**. The MS association of IL7R T244I SNP was replicated in independent data sets analysing a total of about 7.000 MS patients and 7.000 controls from Northern Europe/US. The associated allele conferred a MS risk ranging from 1.2-1.3 and induced a twofold increased skipping of exon 6 which produces a soluble IL7RA isoform **[120-121]**.

4.1.2 The role of infections

The relatively low concordance rate of identical twins indicates a contribution of nongenetic factors to MS aetiology. Viral and bacterial infections are candidates as environmental triggers of MS **[122]**. Evidences stem from experimental autoimmune encephalomyelitis (EAE), since almost 100% of transgenic mice expressing a TCR (T Cell Receptor) that is specific for an encephalitogenic peptide of Myelin Basic Protein (MBP) develop EAE when the transgenic mice are housed under nonpathogen-free conditions, whereas the same animals housed in a pathogenic-free facilities remain disease free.

Important clues may also come from the investigation of MS epidemics. Of the several reported, the most convincing, albeit not uncontroversial, occurred in the Faroe Islands **[123]**. The Faroes are a group of islands in the North Atlantic Ocean that have been a semi-independent unit of the Kingdom of Denmark since 1948. According to investigations by Kurtzke and others, MS was virtually absent among indigenous Faroese until the islands were occupied by the British troops in 1940. After the occupation, 25 cases were identified with onset between 1943 and 1960, most among residents of parishes where the troops were located **[123]**. A detailed analysis of the time course of the epidemic lead Kurtzke to postulate that there is a widespread transmissible agent that causes an asymptomatic persistent infection or “primary MS affection”; rarely, and years after the

primary infection, this agent would cause neurological symptoms (MS). Overall, the data support the possibility that an environmental factor introduced by the British troops caused an increase in MS incidence .

The viral aetiology of a number of human demyelinating disease [progressive multifocal leukoencephalopaty caused by papovirus JC; postinfectious encephalitis and subacute sclerosing panencephalitis (SSPE), both caused by measles virus; herpes simplex virus (HSV); HIV encephalopathy] explains the continued interest in virus as triggers for MS **[124-127]**. Moreover, animal models of virus-induced demyelinating diseases, such as encephalomyelitis by Theiler's murine encephalomyelitis (TMEV), neurotropic strains of mouse hepatitis virus, and rat- adapted measles virus also support the possible involvement of a virus in MS. Among virues that are pathogenic in humans, herpesviruses are of particular interest owing their neurotropism, ubiquitous nature, and tendency to produce latent, recurrent infections. Human herpesvirus (HHV-6) and Epstein-barr virus (EBV) are the leading candidates **[126-128]**.

HHV-6 can lead to meningo-encephalitis, and several observations suggest a role in MS, including its detection in oligodendrocytes in MS plaque tissue (but also in normal brains), the infection of astrocytes, and the presence of HHV-6 DNA and anti-HHV-6 IgG and IgM in serum and cerebro-spinal fluid (CSF) of MS patients **[126-127]**.

EBV is an ubiquitous herpesvirus that infects over 90% of the population. Primary infection generally occurs in early childhood and is usually subclinical; however, in older patients, it may manifest as infectious mononucleosis. After primary infection, EBV establishes latency in B cells and this persistent infection is etiologically linked to several lymphoid and epithelial tumors, including Burkitt lymphoma, nasopharyngeal carcinoma, and Hodgkin lymphoma. A meta-analysis estimated that the odds of MS are more than 10

times higher among EBV-positive versus EBV-negative subjects. Longitudinal studies showed that the risk for MS in apparently healthy adults was directly correlated with levels of anti-EBNA-1 IgG (EBV nuclear antigen 1) **[129]**. Interestingly, these anti-EBV antibodies are significantly increased up to 20 years before the onset of MS and then remain constant. A recent work found evidence of EBV infection in a substantial proportion of brain-infiltrating B cells and plasma cells in nearly all MS patients (investigated post-mortem). Expression of viral latent proteins was regularly observed in MS brains, whereas viral reactivation appeared restricted to ectopic B cell follicles and acute lesions. These findings provide evidence that EBV persistence and reactivation in the CNS play a role in MS immunopathology **[128-129]** .

Human herpesvirus 1 (HSV-1) and varicella zoster virus (VZV or HSV-3) have also been considered as MS-triggering agents on the basis either of CSF antibody studies or finding that VZV encephalitis is characterizied by demyelination. Among bacteria, Chlamydia pneumoniae has been implicated in MS **[130]**.

Two main mechanisms have been proposoed to explain how infections could induce MS: (a) molecular mimicry, i.e. the activation of autoreactive T cells by cross-reactivity between *self*-antigens and foreign antigens, can lead to the migration across the blood-brain barrier (BBB), CNS infiltration and finally to tissue damage by anti-viral lymphocytes cross-reacting against myelin antigens; and (b) bystander activation, which assumes that autoreactive cells are activated because of non-specific inflammatory events that occur during infection and work as adjuvant for the autoimmune response. A third proposal is that infections induce MS through a combination of these two mechanisms.

4.2 Pathogenesis of MS

Most studies on MS pathology and pathogenesis have been focused on focal demyelinated lesions in the white matter, named sclerotic plaque. Its characteristic pathological feature is demyelination. Both the myelin sheath and the oligodendrocyte itself are destroyed within lesions, following attack by lymphocytes that react with myelin-related epitopes, such as MBP. Immune attack involves both cellular immunity, with T cells directed at myelin and oligodendrocytes and inciting phagocytosis by macrophages, and humoral immunity with secretion of anti-myelin antibodies from B cells and subsequent fixation of complement and opsonization of the myelin sheath and oligodendrocytes.

The CNS has long been considered to be an immunoprivileged site with few, if any, lymphocytes present in the absence of active infections. However, accumulating evidence has demonstrated that a small number of T cells traffic through the CNS surveying for infection and injury, and that T cells activated in the periphery can penetrate the blood–brain barrier (BBB) and enter the CNS **[131-133]**. EAE can be induced using CNS homogenates, myelin proteins, or their encephalitogenic peptides in adjuvant. Myelin-specific CD4+ T cells are considered to be the initiators of disease in both MS and EAE, but clonal expansion of CD8+ T cells has been detected in both MS and EAE lesions **[134-135]**. In addition, adoptive transfer of myelin-specific CD8+ T cells can induce an EAE-like disease in recipient animals **[136]**. Myelin oligodendrocyte glycoprotein (MOG)-induced EAE is characterized by many pathophysiological processes detected in MS, including encephalitogenic T cell and demyelinating antibody responses with axonal damage that is quantitatively and qualitatively similar to that seen in MS **[137-139]**.

In addition to damage of myelin and oligodendrocytes, axonal loss and injury are also characteristic features of sclerotic plaques. Axonal loss is determined by counting processes in histological samples. Injury can be detected by the accumulation of amyloid precursor protein (APP) in swellings where axonal transport has been interrupted or the axon physically sectioned. APP accumulation is a transient phenomenon and staining for this protein can thus be taken as an index of recent axonal damage. In end-state multiple sclerosis, up to 60% of the axons present in sclerotic plaques may have disappeared **[138-144]**. This proportion of axonal loss in clinically silent 'burnt-out' plaques appears to be independent of the extent of remyelination and the number of residual oligodendrocytes **[144]**. Axonal loss is also observed in normally appearing white matter adjacent to 'burnt-out' lesions in the cervical cord **[139]**. However, axonal damage is an early event in lesion formation **[145-147]**. In fact, APP accumulation in axons is most prominent in early disease and is correlated with the extent of infiltration of T cells and macrophages into the lesions **[148]**. The extent of acute axonal damage is more pronounced in lesions in which active demyelination is proceeding, compared to inactive demyelinated lesions, consistent with a causal relationship between inflammation and axonal injury **[148]**. However, neuroaxonal damage can also be demonstrated in cortical plaques where infiltration of immune cells is less pronounced **[149]**.

A number of cellular and humoral mediators of the immune response have been shown to be capable of damaging axons, including T cells, macrophages, antibodies, nitric oxide, glutamate, and matrix metalloproteases.

Binding of T cells to neurites may be facilitated by an increased expression of MHC proteins in neurons and their processes under the influence of interferon-γ from T cells infiltrating the lesion **[150]**. These observations would be consistent with the

hypothesis that release of toxic soluble mediators from these immune cells may be instrumental in damaging the axons. Axon damage has been suggested to be mediated by perforin **[151]**, whose secretion by CD8+ T cells is up-regulated in active multiple sclerosis lesions **[151-153]**, and Fas-mediated death **[144]**. In the Theiler's virus encephalopathy model of multiple sclerosis, suppression of perforin gene expression protects mice against neuronal loss and neurological impairment, whereas demyelination is unaffected **[154]**.

Disruption of the blood brain barrier (BBB) is a hallmark feature of immune-mediated neurological disorders as diverse as viral hemorrhagic fevers, cerebral malaria and acute hemorrhagic leukoencephalitis. Recently, CNS-infiltrating antigen-specific CD8 T cells have been shown to display the capacity to initiate BBB tight junction disruption through a non-apoptotic perforin-dependent mechanism **[155]**.

Activated macrophages release nitric oxide and glutamic acid. Nitric oxide has been demonstrated to cause reversible conduction block in demyelinated axons **[156]**. Exposure to concentrations of nitric oxide likely to be generated by macrophages in multiple sclerosis lesions leads to Wallerian degeneration of electrically active axons and to damage of axonal membranes **[157]**. A role for glutamic acid-mediated citotoxicity is suggested by the observation that treatment of mice with EAE with the excitatory amino acid receptor antagonist NBQX results in considerable and significant rescue of axons **[158]**. Axonal sparing was accompanied by increased oligodendrocyte survival and an improvement of the clinical state. However, markers of inflammatory activity were not affected by treatment with NBQX.

Finally, antibodies against axonal epitopes released from B cells may also be involved in concurrence with complement. In the rat EAE model, depletion of the C6

complement protein has been shown to prevent axonal damage as well as demyelination, in spite of normal levels of T cell infiltration into the nervous system **[143]**.

4.3 RATIONALE

Families of patients with ALPS, carrying inherited defects of Fas-induced apoptosis, display increased frequency of common autoimmune diseases, including MS **[159]**. This prompted an analysis of Fas function in MS patients, which showed that a substantial proportion of them (50%) display defective Fas-induced apoptosis and that the defect was more frequent in those with progressive courses (70%). The Fas gene was not mutated, but the finding that the healthy parents of the patients displayed the same defect suggested a genetic component **[160]**. Therefore, inherited alterations involved in ALPS may also be involved in MS development. This possibility was supported by the observation that the variants of the osteopontin gene inducing production of high levels of this cytokine increased not only the risk of ALPS by 8 fold (see the Introduction section), but also the risk of MS by 1.5-fold **[67,161]**. These observation prompted the work described in the **paper 3** aimed to evaluate whether variations of the perforin gene are involved not only in ALPS (as shown in the **paper 1**), but also in MS development. This possibility was supported by studies showing that a region of chromosome 10q22.1, located near to *PRF1,* may be a susceptibility locus for MS **[162]**.

The **paper 3** shows that, indeed, variations of the perforin gene are a susceptibility factor for MS development.

4.4 RESULTS

4.4.1 *Paper 3*

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ORIGINAL ARTICLE

Variations of the perforin gene in patients with multiple sclerosis

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Perforin is involved in cell-mediated cytotoxicity and mutations of its gene (PRF1) cause familial hemophagocytic lymphohistiocytosis (FLH2). PRF1 sequencing in 190 patients with multiple sclerosis and 268 controls detected two FLH2-associated variations (A91V, N252S) in both groups and six novel mutations (C999T, G1065A, G1428A, A1620G, G719A, C1069T) in patients. All together, carriers of these variations were more frequent in patients than in controls (phenotype frequency: 17 vs 9%, P = 0.0166; odds ratio (OR) = 2.06, 95% confidence interval (CI): 1.13–3.77). Although A91V was the most frequent variation and displayed a trend of association with multiple sclerosis (MS) in the first population of patients and controls (frequency of the 91V allele: 0.076 vs 0.043, P = 0.044), we used it as a marker to confirm PRF1 involvement in MS and assessed its frequency in a second population of 966 patients and 1520 controls. Frequency of the 91V allele was significantly higher in patients than in controls also in the second population (0.075 vs 0.058%, P = 0.019). In the combined cohorts of 1156 patients and 1786 controls, presence of the 91V allele in single or double dose conferred an OR = 1.38 (95% CI = 1.10–1.74). These data suggest that A91V and possibly other perforin variations indicate susceptibility to MS.
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Keywords: *MS; perforin; autoimmune diseases*

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system.[1] Its clinical course varies; at onset, approximately 15% of patients display a primary progressive (PP) form, whereas the remainder start out with a relapsing remitting (RR) form and most of them switch to a secondary progressive (SP) form within 10–30 years.[2] Both environmental and genetic factors are involved in the development/progression of MS and several studies point to a complex inheritance involving interactions between combinations of loci that may influence the immune response.[3,4]

Demyelination is obviously a pathological hallmark of MS, but recent evidence has suggested that the clinically relevant cause of functional disability is injury to the axon.[5] This neurodegenerative model posits that demyelination is a permissive factor that creates an environment in which the axon becomes susceptible to injury mediated either by loss of axo–glial trophic interactions or immune-mediated attack of the denuded axon. The cellular effectors responsible for injuring demyelinated axons are currently unidentified. The fact that CD8$^+$ T cells are the most abundant lymphocytes within MS lesions[6] and correlate with axon injury[7] suggests that class I-restricted cytotoxic T cells (CTL) may be the culprit.

Cytolytic granules of CD8$^+$ CTL and natural killer (NK) cells contain perforin and granzymes, and are released on the target cell upon its recognition by the cytotoxic cell. Perforin polymerizes on the target cell membrane and forms pores allowing entry of granzymes

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that trigger apoptosis of the target cell by cleaving caspases.[8]

Biallelic loss-of-function mutations of the perforin gene (*PRF1*) have been classically associated with about 30% of cases of familial hemophagocytic lymphohistiocytosis (FLH2), a rare life-threatening immune deficiency that occurs in infants and young adults.[9,10] FLH2 has been classically ascribed to decreased capacity of CTL and NK cells to clear viral infections; viral persistence is thought to cause the lymphoproliferative pattern. FLH2 is a recessive disease and subjects carrying heterozygous *PRF1* mutations are generally healthy. However, some heterozygous variations may favor development of autoimmune diseases. This has been initially suggested for the autoimmune lymphoproliferative syndrome (ALPS), a rare pediatric autoimmune disease due to defective function of the Fas death receptor involved in both downmodulation of the immune response and cell-mediated cytotoxicity.[11] ALPS is primarily due to mutations of the Fas gene or other genes involved in Fas function, but other genetic factors may concur. We have detected two FLH2-associated amino-acid substitutions of *PRF1* that are associated with ALPS, that is, N252S and A91V.[11] A subsequent work on patients with type 1 diabetes mellitus (T1DM) detected association with N252S, but not A91V, and a patient displayed a novel mutation causing a P477A amino-acid change decreasing NK function.[12] This work was aimed to evaluate whether *PRF1* also contributes to MS development in the light of recent studies showing that a region of chromosome 10q22.1, located near *PRF1*, may be a susceptibility locus for MS.[13–15]

Results

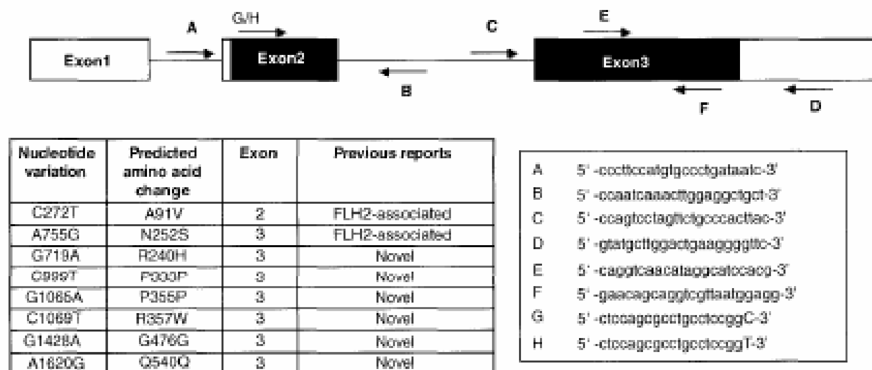
Analysis of the whole coding region of PRF1

The entire coding region of *PRF1* was sequenced in 190 MS and 268 controls to look for variations associated with FLH2 or novel variations (Figure 1). Four missense variations were detected, that is, C272T (rs35947132), A755G (rs28933375), G719A and C1069T (numerations are referred to the GenBank cDNA clone M28393, ATG = +1) causing A91V, N252S, R240H and R357W amino-acid substitutions, respectively. A91V and N252S are variations previously associated with FLH2, whereas R240H and R357W are new.

Four other novel variations, C999T, G1065A, G1428A and A1620G, were detected, but they were synonymous variations (P333P, P355P, G476G and Q540Q, respectively); analysis of their putative effect on splice sites using the Spliceview software and ESEfinder scoring matrix showed that only A1620G (Q540Q) may have an effect by creating a novel acceptor splice site (Spliceview) and a novel binding site for Ser/Arg-rich proteins (ESEfinder), a family of conserved splicing factors.

Finally, we detected the two nucleotide variations, C822T (rs885821) and T900C (rs885822), previously reported as common polymorphisms not associated with FLH2; they did not change the amino acid, nor influence the splicing sites. Their frequency was similar in the patients and the controls. Two other synonymous variations (G435A and A462G) are known to be in perfect linkage disequilibrium with N252S and were in fact only detected in the two subjects (one patient and one control) carrying this variation.[11]

A91V was detected 29 times in 26 patients (23 heterozygotes, 3 homozygotes) and 23 controls (heterozygotes); N252S in 1 patient and 1 control (heterozygotes); R240H in 2 patients (heterozygotes); R357W, P333P, P355P, and G476G in 1 patient each (heterozygotes); and Q540Q in 1 patient (homozygote). The R357W and P355P carriers were also heterozygous for A91V, and the two variations were found to be on different alleles by allele-specific PCR. All together, frequency of the FLH2-associated and novel variations was higher in patients than in controls (allele frequencies: 0.100 vs 0.045, $P = 0.0016$; phenotype frequency: 17 vs 9%, $P = 0.0166$; odds ratio (OR) = 2.06, 95% confidence interval (CI): 1.13–3.77; Table 1).



Nucleotide variation	Predicted amino acid change	Exon	Previous reports
C272T	A91V	2	FLH2-associated
A755G	N252S	3	FLH2-associated
G719A	R240H	3	Novel
C999T	P333P	3	Novel
G1065A	P355P	3	Novel
C1069T	R357W	3	Novel
G1428A	G476G	3	Novel
A1620G	Q540Q	3	Novel

A	5' -cccttccatgtgccctgataatc-3'
B	5' -ccaatcaaacttggaggctgct-3'
C	5' -ccagtcctagttctgcccacttac-3'
D	5' -gtatgcttggactgaaggggttc-3'
E	5' -caggtcaacataggcatccacg-3'
F	5' -gaacagcaggtcgttaatggagg-3'
G	5' -ctccagcgcctgcctccggC-3'
H	5' -ctccagcgcctgcctccggT-3'

Figure 1 Graphical representation (not in scale) of the *PRF1* gene, primers used for typing and the variations found in MS patients. The upper panel shows a scheme of the gene and the relative position of the primers; boxes represent the exons (the coding region is shown in black), lines the introns. Letters and arrows indicate the primers used to amplify and sequence the gene (see Materials and methods) and their sequence is shown in the lower right table. The lower left panel shows a summary of the FLH2-associated (*) and novel *PRF1* variations detected in 190 MS patients and 268 controls.

Table 1 Summary of the genotypes of 190 MS patients and 268 controls carrying *PRF1* variations

Allele 1	*Allele 2*	*MS (n = 190)*[a]	*Controls (n = 268)*[a]
A91V	A91V	3	0
A91V	R357W	1	0
A91V	P355P	1	0
Q540Q	Q540Q	1	0
A91V	wt	21	23
N252S	wt	1	1
R240H	wt	2	0
P333P	wt	1	0
G476G	wt	1	0
Total		32 (17%)	24 (9%)
		[b]OR = 2.06, 95% CI: 1.13–3.77; $P = 0.0166$	

Abbreviations: CI, confidence intervals; MS, multiple sclerosis; OR, odds ratio; wt, wild type.
[a]Number of subjects (frequency in the brackets).
[b]OR and 95% CI limits; *P*-values are two-tailed.

The PolyPhen algorithm was used to predict the functional effect of the two novel R240H and R357W missense variations and showed that both may damage the function and structure of the protein (R240H: score = 2.335; R357W: score = 2.690). Therefore, we directly assessed whether R240H affects perforin function by evaluating NK activity in the 2 patients carrying the variation and 15 controls. Results showed that, at low effector/target ratios, NK activity was defective in one patient and in the low level (that is, within the first quartile) of the normal range in the other (Figure 2). This analysis was not performed in the R357W carrier because his cells were not available. Intriguingly, both patients carrying R240H displayed an early switch from the RR to the SP course (5 and 6 years from onset, respectively) and a multiple sclerosis severity score (MSSS) of 7.65 and 7.38, respectively. By contrast, this aggressive clinical evolution was not displayed by the R357W carrier.

Search for the A91V variation in a second population of patients and controls

Although A91V was the most frequent variation and displayed a trend of association with MS in the first population of patients and controls (frequency of the 91V allele: 0.076 vs 0.043, $P = 0.044$), we used it as a marker to confirm *PRF1* involvement in MS, and assessed its frequency in a second independent population of 966 patients and 1520 ethnically and geographically matched controls. The 91V allele was carried by 138 patients (131 heterozygotes and 7 homozygotes) and 168 controls (160 heterozygotes and 8 homozygotes) and its frequency was significantly higher in patients than in controls (0.075 vs 0.058%, $P = 0.019$). In the combined cohorts of 1156 patients and 1788 controls, presence of the 91V allele in single or double dose conferred an OR = 1.38 (95% CI = 1.10–1.74; Table 2).

No differences were found between subjects carrying or not carrying A91V in terms of gender distribution, MS clinical form (RR, PP and SP) and MSSS (data not shown). Moreover, frequency of the MS susceptibility allele HLA-DR15 was not different in patients carrying A91V or not, as HLA-DR15 was carried by 32% of patients with the 91V allele, 29% of patients without it and 12% of the controls.

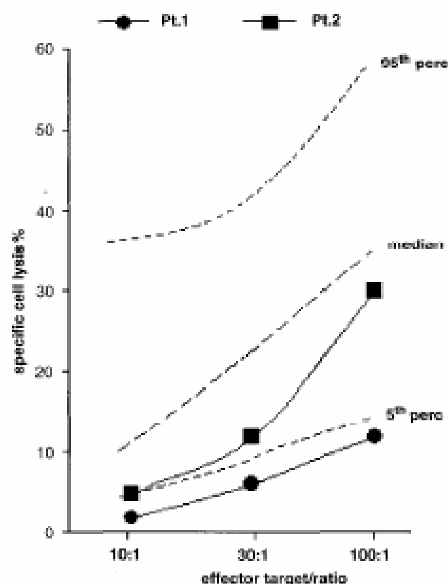


Figure 2 NK activity in PBMC of MS patients carrying the R240H perforin variations and controls. NK activity was assessed at the 100:1, 30:1, and 10:1 effector/target (E:T) ratios; continuous lines indicate patients; stripped and dotted lines indicate the median values and interquartile ranges of 15 controls.

Table 2 Genotype frequencies of A91V in the combined cohorts of MS patients ($n = 1156$) and healthy controls ($n = 1788$)

Genotypes	*MS (n = 1156)*[a]	*Controls (n = 1788)*[a]
AA	992 (0.858)	1597 (0.893)
AV	154 (0.133)	183 (0.102)
VV	10 (0.009)	8 (0.005)
AV+VV vs AA	[b]OR = 1.38, 95% CI = 1.10–1.74; $P = 0.005$	

Abbreviations: CI, confidence intervals; MS, multiple sclerosis; OR, odds ratio.
[a]Number of subjects; frequencies are shown in the brackets. Genotypic distribution did not deviate significantly from the Hardy–Weinberg equilibrium in any group (data not shown).
[b]OR and 95% CI limits; *P*-values are two-tailed.

Discussion

Multiple sclerosis is a complex disease that is probably the result of multiple genetic and environmental factors. Several genes have been involved in its development,[4] and some of them are important in the immune response. This work shows that *PRF1* may also be involved, as MS patients displayed higher frequency of *PRF1* variations

than the controls. This confirms data obtained by other authors showing that the chromosome region 10q22.1, where *PRF1* is located, contains susceptibility genes for MS development.[13–15]

The most frequent variation was A91V, as frequency of the 91V allele was increased in two independent populations of MS patients than in the respective controls, and increased the risk of MS by about 1.4-fold in the combined cohorts. By contrast, A91V did not seem to influence the disease course as MSSS was not different between patients with or without A91V. Studies based on analysis of cytotoxic lymphocytes from A91V carriers or rat basophil leukemia cells transfected with variants of the perforin cDNA have shown that A91V decreases perforin function by altering its conformation, decreasing its cleavage to the active form and increasing its degradation.[16,17] Risma *et al.*[18] classified A91V as a class 1 missense mutation with limited functional impact that allows partial maturation of the protein. Voskoboinik *et al.*[19] have recently used a complementation assay with perforin-knockout primary CTL to show that A91V reduces both the steady-state level of perforin expression in effector cells ('presynaptic' dysfunction) and its intrinsic lytic capacity on target cells, and also displays some dominant-negative effect on the wild-type protein ('postsynaptic' dysfunction).

Our previous work showed that frequency of the 91V allele was also increased in an incomplete variant of ALPS, whereas N252S was associated with the typical form of ALPS and T1DM.[11,12] The functional significance of N252S is debated, but we showed that it may be associated with decreased NK activity in the early childhood.[11,12] Although frequency of N252S was apparently not different in MS and controls, it is possible that *PRF1* variations favor development of several autoimmune diseases, with differences reflecting their effects on perforin function. A91V has also been associated with other immune diseases, such as lymphomas and acute childhood lymphocytic leukemia, and atypical (late-onset) FLH2.[20,21]

Besides A91V and N252S, we detected two new missense *PRF1* mutations in MS patients that cause R240H and R357W amino-acid substitutions. R240H occurs nearby N252S within the membrane-attack complex, a region critically involved in the pore-forming activity of perforin.[22] However, analysis of NK activity in the two R240H carriers showed that it was near the low limit of the normal range. Although both carriers were heterozygous, we suggest that R240H causes a mild decrease of perforin function without exerting a dominant-negative effect on the wild-type form. R357W is located in the same domain, but we could not evaluate its functional effect because fresh cells from the carrier were not available. However, both R357W and R240H were predicted to damage perforin function and structure by *in silico* analysis with the PoliPhen program.

Four other novel mutations were detected in MS patients, but they were synonymous (P333P, P355P, G476G, Q540Q). Q540Q may have an effect on RNA splicing, as it seems to create a new acceptor splice site. The others did not influence canonical splicing sites, but they might theoretically influence perforin expression by disturbing exonic splicing enhancers, mRNA processing and transport, efficiency of codon usage by tRNA stability of mRNA secondary structure, protein folding or interaction with microRNA.[23–28] An alternative possibility is that they do not have a direct effect, but they are linkage disequilibrium with other unknown *PRF1* mutations in the 5′ UTR. However, we could not assess perforin expression because fresh cells from the carriers were not available.

Besides A91V, the other mutations are too rare to draw conclusions about their individual association with MS, but they raise the possibility that the overall effect of *PRF1* variations on MS development may be substantially higher than that detected by A91V alone. In line with this possibility, these variations conferred a global OR = 2.06 for MS development in the first population of patients and controls whose entire *PRF1*-coding region was sequenced. It is intriguing that two patients were compound heterozygous for A91V, and R357W or P355P, respectively, and another patient was homozygous for Q540Q, which raises the possibility that the biallelic variations may have contributed to their MS. The MS association with several rare *PRF1* variations is in line with reports on systemic lupus erythematosus and inflammatory bowel disease indicating that private/rare variations as well as common polymorphisms of other genes may be important in common complex diseases.[29,30]

Perforin-mediated cytotoxicity has been classically associated with clearance of virus-infected cells. Therefore, it is possible that defects of perforin activity favor MS development by delaying virus clearance, which may favor development of crossreactions between viral and self-antigens by molecular mimicry. In this context, it is noteworthy that EBV infections are crucial in FLH2 pathogenesis, and have also been suggested to be important as triggering factors in MS.[31,32]

On the other hand, an increasing bulk of data suggests that perforin and cell-mediated cytotoxicity may also be involved in downmodulation of the immune response. This regulatory activity may involve several mechanisms including perforin-mediated killing of effector lymphocytes and antigen-presenting cells. Defective immune response switching off may favor both lymphocyte accumulation and autoimmunity.[8,33–36] It is noteworthy that involvement of inherited defects of the immune response switching off in MS development may not be limited to *PRF1*, but may also involve defective apoptosis of activated lymphocytes induced through the Fas or the activation-induced cells death (AICD) mechanisms. This possibility is suggested by our previous work showing that substantial proportions of MS patients carry inherited defects of Fas function similar to those displayed by ALPS patients.[37] Moreover, several reports detected high serum levels of osteopontin, a cytokine capable to inhibit AICD, in MS patients and we found that this is partly associated with variants of the osteopontin gene.[38–41]

In conclusion, this work suggests that *PRF1* variations may be a predisposing factor for MS by affecting either the antiviral response or the immune response switching off. Defects of both of these functions may favor development of autoimmunity by prolonging the immune response and increasing the risk of crossreactions between viral and self-antigens. Similar defects may be caused also by alterations of other genes and may be a general predisposing factor for autoimmunity.

Materials and methods

Patients

We analyzed two independent cohorts of Italian patients (391 men, 765 women; M/F: 1/1.96) with MS, diagnosed according to McDonald *et al.*'s criteria[42] and randomly selected ethnically matched healthy controls. The first population was composed of 190 patients and 268 controls, the second by 966 patients and 1520 controls.

Patients were consecutive patients enrolled from the Multiple Sclerosis Centers of the 'Amedeo Avogadro' University of Eastern Piedmont (Novara), the University of Milan, IRCCS Maggiore Policlinico Hospital (Milan), the Don Gnocchi Institute (Milan), the Santa Croce Hospital (Cuneo), the University of Rome 'La Sapienza', S Andrea Hospital (Rome) and the University of Bari (Bari). Their clinical and demographic features were similar to those of other series.[43,44] Controls were consecutive Italian donors obtained from the transfusion services of the respective hospitals. Patients and controls were unrelated, Caucasian and Italian, matched for age and gender, and analyzed as follows:[45]

1. RR: Occurrence of exacerbations, each lasting at least 24 h and separated by at least 1 month of inactivity, with full recovery or sequelae ($n = 852$).
2. PP: Steady worsening of symptoms and signs from onset for at least 6 months, whether superimposed with relapses or not, with occasional plateau and temporary minor improvements ($n = 92$).
3. SP: Initial RR course followed by steady worsening of symptoms and signs for at least 6 months, whether superimposed with relapses or not, with minor remissions and plateaux ($n = 212$).

Progression of disability was assessed with the MSSS.[46] In RR patients, MSSS score was assessed in remission phase.

All patients gave their informed consent according to the Declaration of Helsinki.[47] The research was approved by the Novara ethical committee.

Amplification of PRF1 and mutation detection

Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using standard methods and exons 2 and 3 of the *PRF1*-coding region were amplified in standard PCR conditions. PCR products were purified with the EXO/SAP kit (GE Healthcare, Piscataway, NJ, USA). In the first population, the entire coding region was sequenced with the ABI PRISMR BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA) on an automatic sequencer (Applied Biosystems 3100 Genetic Analyser) according to the manufacturer's instructions. Figure 1 shows primers used for amplification, sequencing and typing. Briefly, exon 2 was amplified with primers A + B (755 bp) and sequenced with the same oligonucleotides. Exon 3 was amplified with C + D (1289 bp) and sequenced with these and with two additional internal primers (E and F). In the second population, the +272 C/T (A91V) variation was typed by sequencing (233 patients and 548 controls) or by the TaqMan 5′-allelic discrimination assay (733 patients and 972 controls; Applied Biosystems). Allelic-specific primers and probes used for discrimination have been previously described.[26] Genotyping of each sample was automatically attributed by the SDS 1.3 software for allelic discrimination. Similar results were obtained in patients typed by the two methods. All variations were confirmed twice by sequencing independent DNA samples. The genotypic distribution of the variation did not deviate significantly from the Hardy–Weinberg equilibrium in any group.

Allele-specific PCR

The wild-type (91A) and mutant (91V) alleles were separately amplified using specific PCR amplification of genomic DNA (forward primer: G or H; reverse primer: D). PCR products were typed for P355P and R357W by sequencing with the ABI PRISMR BigDye Terminator kit on the 3100 Genetic Analyser using the internal primer E.

HLADRB1 typing

Patients and controls were specifically typed for DRB1*1501 allele as previously described.[48]

Cytotoxicity assays

Natural killer activity of PBMC was assessed by a standard 4 h ^{51}Cr-release assay with K562 cells as the target. Results are expressed as specific lysis % calculated as follows: (sample ^{51}Cr release−spontaneous release)/(maximal release−spontaneous release) × 100.

Statistical analysis

Phenotype frequencies were calculated as the number of individuals carrying an allele (either homozygotes or heterozygotes) divided by the total number of individuals.

Allelic and phenotype frequencies were compared with the χ^2-test with the Yates correction. All P-values are two-tailed and the significance cutoff was $P<0.05$. Putative effect of the variation on splicing sites was evaluated using the SpliceView program on the WebGene website (http://www.itb.cnr.it/sun/webgene/) and the ESEfinder scoring matrix (http://www.rulai.cshl.edu/tools/ESE). Putative functional significance of the missense variations was evaluated with the PolyPhen program (http://genetics.bwh.harvard.edu/pph).

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Disclosure

The authors report no conflict of interest.

References

1 Compston A, Coles A. Multiple sclerosis. *Lancet* 2002; **359**: 1221–1231.
2 Weinshenker BG. The natural history of multiple sclerosis. *Neurol Clin* 1995; **13**: 119–146.

3 Sospedra M, Martin R. Immunology of multiple sclerosis. *Annu Rev Immunol* 2005; **23**: 683–747.
4 Steinman L, Martin R, Bernard C, Conlon P, Oksenberg JR. Multiple sclerosis: deeper understanding of its pathogenesis reveals new targets for therapy. *Annu Rev Neurosci* 2002; **25**: 491–505.
5 Howe CL, Rodriguez M. Remyelination as neuroprotection. In: Waxman SG (ed). *Multiple Sclerosis as a Neuronal Disease*. Elsevier Academic Press: San Diego, 2005, pp 389–419.
6 Babbe H, Roers A, Waisman A, Goebels N, Hohlfeld R, Friese M *et al*. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J Exp Med* 2000; **192**: 393–404.
7 Bitsch A, Schuchardt J, Bunkowski S, Kulnann T, Brück W. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. *Brain* 2000; **123**: 1174–1183.
8 Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* 2002; **2**: 735–747.
9 Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA *et al*. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* 1999; **286**: 957–959.
10 Clementi R, zur Stadt U, Savoldi G, Varoitto S, Conter V, De Fusco C *et al*. Six novel mutations in the *PRF1* gene in children with haemophagocytic lymphohistiocytosis. *J Med Genet* 2001; **38**: 643–646.
11 Clementi R, Chiocchetti A, Cappellano G, Cerutti E, Ferretti M, Orilieri E *et al*. Variations of the perforin gene in patients with autoimmunity/lymphoproliferation and defective Fas function. *Blood* 2006; **108**: 3079–3084.
12 Orilieri E, Cappellano G, Clementi R, Cometa A, Ferretti M, Cerutti E *et al*. Variations of the perforin gene in patients with type 1 diabetes. *Diabetes* 2008; **57**: 1078–1083.
13 Goertsches R, Villoslada P, Comabella M, Montalban X, Navarro A, de la Concha EG. A genomic screen of Spanish multiple sclerosis patients reveals multiple loci associated with the disease. *J Neuroimmunol* 2003; **143**: 124–128.
14 Goertsches R, Comabella M, Navarro A, Perkal H, Montalban X. Genetic association between polymorphisms in the ADAMTS14 gene and multiple sclerosis. *J Neuroimmunol* 2005; **164**: 140–147.
15 Goertsches R, Baranzini SE, Morcillo C, Nos C, Camiña M, Oksenberg JR *et al*. Evidence for association of chromosome 10 open reading frame (C10orf27) gene polymorphisms and multiple sclerosis. *Mult Scler* 2008; **14**: 412–414.
16 Trambas C, Gallo F, Pende D, Marcenaro S, Moretta L, De Fusco C *et al*. A single amino acid change, A91V, leads to conformational changes that can impair processing to the active form of perforin. *Blood* 2005; **106**: 932–937.
17 Voskoboinik I, Thia MC, Trapani JA. A functional analysis of the putative polymorphisms A91V and N252S and 22 missense perforin mutations associated with familial hemophagocytic lymphohistiocytosis. *Blood* 2005; **105**: 4700–4706.
18 Risma KA, Frayer RW, Filipovich AH, Sumegi J. Aberrant maturation of mutant perforin underlies the clinical diversity of hemophagocytic lymphohistiocytosis. *J Clin Invest* 2006; **116**: 182–192.
19 Voskoboinik I, Sutton VR, Ciccone A, House CM, Chia J, Darcy PK *et al*. Perforin activity and immune homeostasis: the common A91V polymorphism in perforin results in both pre- and post-synaptic defects in function. *Blood* 2007; **110**: 1184–1190.
20 Mehta PA, Davies SM, Kumar A, Devidas M, Lee S, Zamzow T *et al*. Perforin polymorphism A91V and susceptibility to B-precursor childhood acute lymphoblastic leukaemia: a report from the Children's Oncology group. *Leukemia* 2006; **20**: 1539–1541.
21 Clementi R, Locatelli F, Dupré L, Garaventa A, Emmi L, Bregni M *et al*. A proportion of patients with lymphoma may harbor mutations of the perforin gene. *Blood* 2005; **105**: 4424–4428.
22 Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. *Nat Rev Immunol* 2006; **6**: 940–952.
23 Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet* 2002; **3**: 285–298.
24 Lavner Y, Kotlar codon bias as a factor in regulating expression via translation rate in the human genome. *Gene* 2005; **345**: 127–138.
25 Kotlar D, Lavner Y. The action of selection on codon bias in the human genome is related to frequency, complexity, and chronology of amino acids. *BMC Genomics* 2006; **7**: 67.
26 Shabalina SA, Ogurtsov AY, Spiridonov NA. A periodic pattern of mRNA secondary structure created by the genetic code. *Nucleic Acids Res* 2006; **34**: 2428–2437.
27 Komar AA. Genetics. SNPs, silent but not invisible. *Science* 2007; **315**: 466–467.
28 Martin MM, Buckenberger JA, Jiang J, Malana GE, Nuovo GJ, Chotani M *et al*. The human angiotensin II type 1 receptor +1166 A/C polymorphism attenuates microRNA-155 binding. *J Biol Chem* 2007; **282**: 24262–24269.
29 Lee-Kirsch MA, Gong M, Chowdhury D, Senenko L, Engel K, Lee YA *et al*. Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 are associated with systemic lupus erythematosus. *Nat Genet* 2007; **39**: 1065–1067.
30 Lesage S, Zouali H, Cézard JP, Colombel JF, Belaiche J, Almer S *et al*. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. *Am J Hum Genet* 2002; **70**: 845–857.
31 Lunemann JD, Munz C. Epstein–Barr virus and multiple sclerosis. *Curr Neurol* 2007; **7**: 253–258.
32 Chuang HC, Lay JD, Hsieh WC, Su IJ. Pathogenesis and mechanism of disease progression from hemophagocytic lymphohistiocytosis to Epstein–Barr virus-associated T-cell lymphoma: nuclear factor-kappaB pathway as a potential therapeutic target. *Cancer Sci* 2007; **98**: 1281–1287.
33 Su MW, Pyarajan S, Chang JH, Yu CL, Jin YJ, Stierhof YD *et al*. Fratricide of CD8+ cytotoxic T lymphocytes is dependent on cellular activation and perforin-mediated killing. *Eur J Immunol* 2004; **34**: 2459–2470.
34 Badovinac VP, Hamilton SE, Harty JT. Viral infection results in massive CD8+ T cell expansion and mortality in vaccinated perforin-deficient mice. *Immunity* 2003; **18**: 463–474.
35 Zhou S, Ou R, Huang L, Moskophidis D. Critical role for perforin-, Fas/FasL-, and TNFR1-mediated cytotoxic pathways in down-regulation of antigen-specific T cells during persistent viral infection. *J Virol* 2002; **76**: 829–840.
36 de Saint Basile G, Fischer A. Defective cytotoxic granule-mediated cell death pathway impairs T lymphocyte homeostasis. *Curr Opin Rheumatol* 2003; **15**: 436–445.
37 Comi C, Leone M, Bonissoni S, DeFranco S, Bottarel F, Mezzatesta C *et al*. Defective T cell Fas function in patients with multiple sclerosis. *Neurology* 2000; **55**: 921–927.
38 Chiocchetti A, Comi C, Indelicato M, Castelli L, Mesturini R, Bensi T *et al*. Osteopontin gene haplotypes correlate with multiple sclerosis development and progression. *J Neuroimmunol* 2005; **63**: 172–178.
39 Hur EM, Youssef S, Haws ME, Zhang SY, Sobel RA, Steinman L. Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells. *Nat Immunol* 2007; **8**: 74–83.
40 Comabella M, Pericot I, Goertsches R, Nos C, Castello M, Blas Navarro J *et al*. Plasma osteopontin levels in multiple sclerosis. *J Neuroimmunol* 2005; **158**: 231–239.
41 Vogt MH, Lopatinskaya L, Smits M, Polman CH, Nagelkerken L. Elevated osteopontin levels in active relapsing-remitting multiple sclerosis. *Ann Neurol* 2003; **53**: 819–822.
42 McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD *et al*. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. *Ann Neurol* 2001; **50**: 121–127.

43 Weinshenker BG, Bass B, Rice GP, Noseworthy J, Carriere W, Baskerville J *et al*. The natural history of multiple sclerosis: a geographically based study: I. Clinical course and disability. *Brain* 1989; **112:** 133–146.
44 Trojano M, Avorio C, Mannari C, Calò A, De Robertis F, Serio G *et al*. Multivariate analysis of predictive factors of multiple sclerosis course with a validated method to assess clinical events. *J Neurol Neurosurg Psychiatry* 1995; **58**: 300–306.
45 Lublin FD, Reingold SC. Defining the clinical course of multiple sclerosis: results of an international survey. *Neurology* 1996; **48**: 907–911.
46 Roxburgh RH, Seaman SR, Masterman T, Hensiek AE, Sawcer SJ, Vukusic S *et al*. Multiple sclerosis severity score: using disability and disease duration to rate disease severity. *Neurology* 2005; **64**: 1144–1151.
47 International Committee of Medical Journal Editors. Protection of patients' rights to privacy. *BMJ* 1995; **311**: 1272.
48 D'Alfonso S, Bolognesi E, Guerini FR, Barizzone N, Bocca S, Ferrante D *et al*. A sequence variation in the MOG gene is involved in multiple sclerosis susceptibility in Italy. *Genes Immun* 2008; **9**: 7–15.

REFERENCES

1. Masson D, Tschopp J. Isolation of a lytic, pore-forming protein (perforin) from cytolytic T-lymphocytes. **J Biol Chem** 1985;260:9069-9072.
2. Young JD, Podack ER, Cohn ZA. Properties of a purified pore-forming protein (perforin 1) isolated from H-2-restricted cytotoxic T cell granules. **J Exp Med**;164:144-155.
3. Burkhardt JK, Hester S, Argon Y. Two proteins targeted to the same lytic granule compartment undergo very different posttranslational processing. **Proc Natl Acad Sci U S A** 1989;86:7128-7132.
4. Kupfer A, Singer SJ, Dennert G. On the mechanism of unidirectional killing in mixtures of two cytotoxic T lymphocytes. Unidirectional polarization of cytoplasmic organelles and the membrane-associated cytoskeleton in the effector cell. **J Exp Med** 1986;163:489-498.
5. Ortaldo JR, Winkler-Pickett RT, Nagashima K, et al. Direct evidence for release of pore-forming protein during NK cellular lysis. **J Leukoc Biol** 1992;5:483-488.
6. Tschopp J, Schäfer S, Masson D, et al. Phosphorylcholine acts as a Ca2+-dependent receptor molecule for lymphocyte perforin. **Nature** 1989;337:272-274.
7. Ménasché G, Feldmann J, Fischer A, et al. Primary hemophagocytic syndromes point to a direct link between lymphocyte cytotoxicity and homeostasis. **Immunol Rev** 2005;203:165-179.
8. Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. **Nat Rev Immunol** 2006;6:940-952.

9. Mullbacher A, Hla RT, Museteanu C, et al. Perforin is essential for control of ectromelia virus but not related poxviruses in mice. **Journal of Virology** 1999;73:1665–1667.

10. Kagi D, Seiler P, Pavlovic J, et al. The roles of perforin- and Fas dependent cytotoxicity in protection against cytopathic and noncytopathic viruses. **European Journal of Immunology** 1995;25:3256–3262.

11. Franco MA, Tin C, Rott LS, et al. Evidence for CD8+ T-cell immunity to murine rotavirus in the absence of perforin, Fas, and gammainterferon. **Journal of Virology** 1997;71:479–486.

12. Gebhard JR, Perry CM, Harkins S, et al. Coxsackievirus B3-induced myocarditis: perforin exacerbates disease, but plays no detectable role in virus clearance. **American Journal of Pathology** 1998;153:417–428.

13. Usherwood EJ, Brooks JW, Sarawar SR, et al. Immunological control of murine gammaherpesvirus infection is independent of perforin. **Journal of General Virology** 1997;78:2025–2030.

14. Smyth MJ, Thia KY, Street SE, et al. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. **J Exp Med** 2000;192:755-760.

15. Lichtenheld MG, Olsen KJ, Lu P, et al. Structure and function of human perforin. **Nature** 1988;335:448–451.

16. Uellner R, Zvelebil MJ, Hopkins J, et al. Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. **EMBO Journal** 1997;16:7287–7296.

17. Voskoboinik I, Thia MC, Fletcher J, et al. Calcium-dependent plasma membrane binding and cell lysis by perforin are mediated through its C2 domain: A critical

role for aspartate residues 429, 435, 483, and 485 but not 491. **J Biol Chem** 2005;280:8426-8434.

18. Masson D, Peters PJ, Geuze HJ, et al. Interaction of chondroitin sulfate with perforin and granzymes of cytolytic T-cells is dependent on pH. **Biochemistry** 1990;29:11229–11235.

19. Metkar SS, Wang B, Aguilar-Santelises M, et al. Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. **Immunity** 2002;16:417–428.

20. Balaji KN, Schaschke N, Machleidt W, et al. Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. **Journal of Experimental Medicine** 2002;196:493–503.

21. Arico M, Janka G, Fischer A, et al. Haemophagocytic lymphohistiocytosis: report of 122 children from the International Registry. **Leukemia** 1996;10:197–203.

22. Clementi R, Emmi L, Maccario R, et al. Adult onset and atypical presentation of hemophagocytic lymphohistiocytosis in siblings carrying PRF1 mutations. **Blood** 2002;100:2266–2267.

23. Henter JI, Arico M, Elinder G, et al. Familial hemophagocytic lymphohistiocytosis. Hematology/Oncology. **Clinics of North America** 1998;12:417–433.

24. Komp DM, McNamara J, Buckley P. Elevated soluble interleukin-2 receptor in childhood hemophagocytic histiocytic syndromes. **Blood** 1989;73:2128.

25. Henter, JI, Ekinder G, Soder O, et al. Hypercytokinemia in familial hemophagocytic lymphohistiocytosis. **Blood** 1991;78:2918–2922.

26. Akashi, Hayashi S, Gondo H, et al. Involvement of interferon-gamma and macrophage colony-stimulating factor in pathogenesis of haemophagocytic lymphohistiocytosis in adults. British **Journal of Haematology** 1994;87:243–250.

27. Osugi Y, Hara J, Tagawa S. Cytokine production regulating Th1 and Th2 cytokines in hemophagocytic lymphohistiocytosis. **Blood** 1997;89:4100–4103.

28. Takada H, Ohga S, Mizuno Y, et al. Oversecretion of IL-18 in haemophagocytic lymphohistiocytosis: a novel marker of disease activity. **British Journal of Haematology** 1999;106:182–189.

29. Takada H, Takahata Y, Nomura A, et al. Increased serum levels of interferon-gamma-inducible protein 10 and monokine induced by gamma interferon in patients with haemophagocytic lymphohistiocytosis. **Clinical Experimental Immunology** 2003;133: 448–453.

30. Henter JI, Carlson LA, Soder O, et al. Lipoprotein alterations and plasma lipoprotein lipase reduction in familial hemophagocytic lymphohistiocytosis. **Acta Pediatrica Scandinavica** 1991;80:675–681.

31. Fadeel B, Orrenius S, Henter, JI. Induction of apoptosis and caspase activation in cells obtained from familial haemophagocytic lymphohistiocytosis patients. **British Journal of Haematology** 1999;106:406–415.

32. Ohadi M, Lalloz MR, Sham P, et al. Localization of a gene for familial hemophagocytic lymphohistiocytosis at chromosome 9q21.3–22 by homozygosity mapping. **American Journal of Human Genetics** 1999;64:165–171.

33. Goransdotter Ericson K, Fadeel B, Nilsson-Ardnor S, et al. Spectrum of perforin gene muations in familial hemophagocytic lymphohistiocytosis. **American Journal of Human Genetics** 2001;68:590–597.

34. Stepp SE, Dufourcq-Lagelouse R, Le Deist, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. **Science** 1999;286:1957–1959.

35. Feldmann J, Callebaut I, Raposo G, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). **Cell** 2003;115:461–473.

36. Bryceson YT, Rudd E, Zheng C, et al. Defective cytotoxic lymphocyte degranulation in syntaxin-11 deficient familial hemophagocytic lymphohistiocytosis 4 (FHL4) patients. **Blood** 2007;110:1906-1915.

37. Kogawa K, Lee SM, Villanueva J, et al. Perforin expression in cytotoxic lymphocytes from patients with hemophagocytic lymphohistocytosis and their family members. **Blood** 2002;99:61–66.

38. Sullivan K, Delaat CA, Douglas SD et al. Defective natural killer cell function in patients with hemophagocytic lymphohistiocytosis and in first degree relatives. **Pediatric Research** 1998;44:465–468.

39. Busiello R, Adriani M, Locatelli F, et al. Atypical features of familial hemophagocytic lymphohistiocytosis. **Blood** 2004;103:4610–4612.

40. Molleran Lee S, Villanueva J, Sumegi J, et al. Characterisation of diverse PRF1 mutations leading to decreased killer cell activity in North American families with haemophagocytic lymphohistiocytosis. **Journal of Medical Genetics** 2004;41:137–144.

41. Ueda I, Morimoto A, Inaba T, et al. Characteristic peforin gene mutations of haemophagocytic lymphohistiocytosis in Japan. **British Journal of Haematology** 2003;121: 503–510.

42. Clementi R, zur Stadt U, Savoldi G, et al. Six novel mutations in the PRF1 gene in children with haemophagocytic lymphohistiocytosis. **Journal of Medical Genetics** 2001; 38:643–646.

43. Suga N, Takada H, Nomura A, et al. Perforin defects of primary haemophagocytic lymphohistiocytosis in Japan. **British Journal of Hematology** 2002;116:346–349,

44. Voskoboinik I, Thia MC, Trapani JA. A functional analysis of the putative polymorphisms A91V and N252S and 22 missense perforin mutations associated with familial hemophagocytic lymphohistiocytosis. **Blood** 2005;105:4700-4706.

45. Clementi R, Locatelli F; Dupre L, et al. A proportion of patients with lymphoma may harbor mutations of the perforin gene. **Blood** 2005; 105:4424-4428.

46. Clementi R, Dagna L, Dianzani U, et al. Inherited perforin and Fas mutations in a patient with autoimmune lymphoproliferative syndrome and lymphoma. **New Eng J Med** 2004; 351:1419-1424.

47. Solomou EE, Gibellini F, Stewart B, et al. Perforin gene mutations in patients with acquired aplastic anemia. **Blood** 2007;109:5234-5237.

48. Cannella S, Santoro A, Bruno G, et al. Germline mutations of the perforin gene are a frequent occurrence in childhood anaplastic large cell lymphoma. **Cancer** 2007;109:2566-2571.

49. Rieux-Leucat F, Le Deist F, Hivroz C, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity**.** **Science** 1995;268:1347-1349.

50. Dianzani U, Bragardo M, DiFranco D, et al. Deficency of the Fas apoptosis pathway without Fas gene mutations in pediatric patients with autoimmunity/lymphoproliferations. **Blood** 1997; 89: 2871-2879.

51. Nagata S. Apoptosis by death factor. **Cell** 1997;88:355-365.

52. Krammer PH. CD95's deadly mission in the immune system. **Nature** 2000; 407:789-795.

53. Nagata S, Suda T. Fas and Fas ligand: *lpr* and *gld* mutations. **Immunol Today** 1995;16:39-43.

54. Fisher GH, Rosenberg FJ, Straus SE, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmunelymphoproliferative syndrome. **Cell** 1995;81:935-946.

55. Le Deist F, Emile JF, Rieux-Laucat F, et al. Clinical, immunological, and pathological consequences of Fas-deficient conditions. **Lancet** 1996 ;348:719-723.

56. Drappa J, Vaishnaw AK, Sullivan KE, et al. Fas gene mutations in the Canale-Smith syndrome, an inherited lymphoproliferative disorder associated with autoimmunity. **N Engl J Med** 1996;335:1643-1649.

57. Canale VC, Smith CH. Chronic lymphadenopathy simulating malignant lymphoma. **J Pediatr** 1967;70:891-899.

58. Straus SE, Sneller M, Lenardo MJ, et al. An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative sindrome. **Ann Int Med** 1999;130:591-601.

59. Sneller MC, Dale JK, Straus SE. Autoimmune lymphoproliferative syndrome. **Curr Opin Rheum** 2003;15:417-421.

60. Marlies A, Udo G, Juergen B, et al. The expanded double negative T cell populations of a patient with ALPS are not clonally related to CD4+ or to CD8+ T cells. **Autoimmunity** 2007;40:299-301.

61. Bide`re N, Su HC, Lenardo MJ. Genetic disorders of programmed cell death in the immune system. **Annu Rev Immunol** 2006;24**:**321–352.

62. Rieux-Laucat F, Le Deist F, Fischer A. Autoimmune lymphoproliferative syndromes: genetic defects of apoptosis pathways. **Cell Death Differ** 2003;10:124–133.

63. Wu J, Wilson J, He J, et al. Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. **J Clin Invest** 1996;98:1107–1113.

64. Bi L, Pan G, Atkinson TP, et al. Dominant inhibition of Fas ligand-mediated apoptosis due to heterozygous mutation associated with ALPS type Ib. **BMC Med Genet** 2007; 8: 41.

65. Del Rey M., Ruiz-Contreras J, Bosque A, et al. A homozygous Fas ligand gene mutation in a patient causes a new type of autoimmune lymphoproliferative syndrome. **Blood** 2006;108:1306–1312.

66. Oliveira J B, Bide`re N, Niemela JE, et al. NRAS mutation causes a human autoimmune lymphoproliferative syndrome. **Proc Natl Acad Sci USA** 2007; 104:8953–8958.

67. Chiocchetti A, Indelicato M, Bensi T, et al. High levels of osteopontin associated with polymorphisms in its gene are a risk factor for development of autoimmunity/lymphoproliferation. **Blood** 2004;103:1376-1382.

68. Poppema S, Maggio E, van den Berg A. Development of lymphoma in Autoimmune Lymphoproliferative Syndrome (ALPS) and its relationship to Fas gene mutations. **Leuk Lymphoma** 2004;45:423-431.

69. Straus SE, Jaffe ES, Puck JM, et al. The development of lymphomas in families with autoimmune lymphoproliferative syndrome with germline Fas mutations and defective lymphocyte apoptosis. **Blood** 2001;98:194-199,.

70. Muschen M, Rajewsky K, Kronke M, et al. The origin of CD95-gene mutations in B-cell Lymphoma. **Trends Immuno** 2002; 23:75-80.

71. Davidson WF, Giese T, Fredrickson TN. Spontanous development of plasmacytoid tumors in mice with defective Fas-Fas ligand interactions. **J Exp Med** 1998;187:1825-1838.

72. Lowy DR, Androphy EJ. Warts. In: Freedberg IM, Eisen AZ, Wolff K, et al. 6th edn. New York: Mc Graw-Hill, 2003: 2119–2131.

73. Pereira de Oliveira WR, Carrasco S, Neto CF, et al. Nonspecific cell-mediated immunity in patients with epidermodysplasia verruciformis. **J Dermatol** 2003; 30:203–209.

74. Oliveira WRP, Neto CF, Rady PL, et al. Clinical aspects of epidermodysplasia verruciformis. **JEADV** 2003;17: 394–398.

75. Orth G. Genetics of epidermodysplasia verruciformif:insights into host defense against papillomaviruses. **Semin Immunol** 2006;18:362-374.

76. Lewandowsky F, Luiz W. Ein Fall einer bisher nicth beschiriebenen Hauterkrankung (Epidermodyspalsia verruciformis). **AMA Arch Derm Syphililol** 1922;141:193-203.

77. Sehgal VN, Luthra A, Bajaj P. Epidermodysplasia verruciformis: 14 members of a pedigree with an intriguing squamous cell carcinoma transformation. **Int J Dermatol** 2002; 41: 500–503.

78. Iraji F, Faghihi G. Epidermodysplasia verruciformis: association with isolated Ig M deficiency and response to treatment with acitretin. **Clin Exp Dermatol** 2000; 25: 41–43.

79. Glinki W, jablonska S, Langer A. Cell-mediated immunity in epidermodyspalsia verruciformis. **Dermatologica** 1976; 153:218-227.

80. Majeski S, Jablonska S. Epidermodysplasia verruciformis as a model of human papillomavirus induced genetics cancers: the role of local immunosurveilance. **Am J Med Sci** 1992; 304:174-179.

81. Azzimonti B, Mondini M, De Andrea M, et al. CD8+ T-cell lymphocytopenia and lack of EVER mutations in a patient with clinically and virologically typical epidermodysplasia verruciformis. **Arch Dermatol** 2005;141:1323–1325.

82. Notarangelo L, Casanova JL, Fischer A, et al. Primary immunodeficiency diseases: an update. **J Allergy Clin Immunol** 2004; 114:677–687.

83. Ramoz N, Taich A, Rueda LA, et al. Evidence for a nonallelic heterogeneity of epidermodyspalsia verruciformis with two susceptibility loci mapped to chromosome regions 2p21-p24 and 17q25. **J Invest Dermatol** 2000; 114: 1148-1153.

84. Ramoz N, Rueda LA, Bouadjar B, et al. Mutations in two adjacent novel genes are associated with epidermodysplasia verruciformis. **Nat Genet** 2002;32:579–581.

85. Ya-Gang Zuo , Donglai Ma , Yunpeng Zhang et al. Identification of a novel mutation and a genetic polymorphism of EVER1 gene in two families with epidermodysplasia verruciformis**. Journal of dermatological Sciences** 2006;44:153-159.

86. Zuo YG, Ma D, Zhang Y, et al. Identification of a novel mutation and a genetic polymorphism of EVER1 gene in two families with epidermodysplasia verruciformis. **J Dermatol Sci** 2006;44:153-159.

87. Kurtzke JF. MS epidemiology world wide. One view of current status. **Acta Neurol Scand** 1995;161(suppl):23–33.

88. Nicoletti A, Patti F, Lo Fermo S, et al. Possible increasing risk of multiple sclerosis in Catania, Sicily. **Neurology** 2005;65:1259-1263.

89. Pugliatti M, Riise T, Sotgiu MA, et al. Increasing incidence of multiple sclerosis in the province of Sassari, northern Sardinia. **Neuroepidemiology** 2005;25:129-134.

90. Compston A, Sawcer S. Genetic analysis of multiple sclerosis. **Curr Neurol Neurosci Rep** 2002;2:259 –266.

91. Gale CR, Martyn CN. Migrant studies in multiple sclerosis**. Prog Neurobiol** 1995;47:425– 448.

92. Weinshenker BG. The natural history of multiple sclerosis. **Neurol Clin** 1995;13: 119–146.

93. **S**adovnick AD. Familial recurrence risks and inheritance of multiple sclerosis. **Curr Opin Neurol Neurosurg** 1993;6:189–194.

94. Robertson NP, Fraser M, Deans J, et al. Age-adjusted recurrence risks for relatives of patients with multiple sclerosis. **Brain** 1996;119: 449–455.

95. Carton H, Vlietinck R, Debruyne J, et al. Risks of multiple sclerosis in relatives of patients in Flanders, Belgium. **J Neurol Neurosurg Psychiatry** 1997;62:329–333.

96. Ebers GC, Sadovnick AD, Risch NJ. A genetic basis for familial aggregation in multiple sclerosis. **Nature** 1995;377:150–151.

97. Sadovnick AD, Ebers GC, Dyment DA, et al. Evidence for genetic basis of multiple sclerosis. **Lancet** 1996;347:1728–1730.

98. Ebers GC, Yee IM, Sadovnick AD, et al. Conjugal multiple sclerosis: population based prevalence and recurrence risks in offspring. **Ann Neurol** 2000; 48:927–931.

99. Willer CJ, Dyment DA, Sadovnick AD, et al. Timing of birth and risk of multiple sclerosis: population based study. **BMJ** 2005;330:120.

100. Hupperts R, Broadley S, Mander A, et al. Patterns of disease in concordant parent–child pairs with multiple sclerosis. **Neurology** 2001;57:290–295.

101. Ebers GC, Sadovnick AD, Dyment DA, et al. Parent-of-origin effect in multiple sclerosis: observations in half-siblings. **Lancet** 2004;363 :1773–1774.

102. Kantarci OH, Barcellos LF, Atkinson EJ, et al. Men transmit MS more often to their children vs women: the Carter effect. **Neurology** 2006;67:305-310.

103. Brassat D, Azais-Vuillemin C, Yaouanq J, et al. Familial factors influence disability in MS multiplex families. **Neurology** 1999; 52:1632–1636.

104. Barcellos LF, Oksenberg JR, Green AJ, et al. Genetic basis for clinical expression in multiple sclerosis. **Brain** 2002 ;125:150–158.

105. Hensiek AE, Seaman SR, Barcellos LF, et al. Familial effects on the clinical course of multiple sclerosis. **Neurology** 2007;68:376–383.

106. Deluca GC, Ramagopalan SV, Herrera BM, et al. An extremes of outcome strategy provides evidence that multiple sclerosis severity is determined by alleles at the HLA-DRB1 locus. **Proc Natl Acad Sci USA** 2007; 104: 20896–20901.

107. Mumford CJ, Wood NW, Kellar-Wood H, et al. The British Isles survey of multiple sclerosis in twins. **Neurology** 1994; 44:11–15.

108. Willer CJ, Dyment DA, Risch NJ, et al. Twin concordance and sibling recurrence rates in multiple sclerosis. **Proc Natl Acad Sci USA** 2003;100:12877–12882.

109. Hansen T, Skytthe A, Stenager E, et al. Concordance for multiple sclerosis in Danish twins: an update of a nationwide study. **Mult Scler** 2005;11 :504–510.

110. Islam T, Gauderman WJ, Cozen W, et al. Differential twin concordance for multiple sclerosis by latitude of birthplace. **Ann Neurol** 2006; 60:56–64.

111. Ioannidis JP, Trikalinos TA, Khoury MJ. Implications of small effect sizes of individual genetic variants on the design and interpretation of genetic association studies of complex diseases. **Am J Epidemiol** 2006;164:609–614.

112. Ioannidis JP, Trikalinos TA, Ntzani EE, et al. Genetic associations in large versus small studies: an empirical assessment. **Lancet** 2003;361:567–571.

113. Yang Q, Khoury MJ, Friedman J, et al. How many genes underlie the occurrence of common complex diseases in the population? **Int J Epidemiol** 2005;34:1129–1137.

114. Dyment DA, Ebers GC, Sadovnick AD. Genetics of multiple sclerosis. **Lancet Neurol** 2004;3:104-110.

115. Mc Farland HF. Twin studies and multiple sclerosis. **Ann Neurol** 1992; 32:722-723.

116. Brynedal B, Duvefelt K, Jonasdottir G, et al. HLA-A confers an HLA-DRB1 independent influence on the risk of multiple sclerosis. **PLoS ONE** 2007 25;2:e664.

117. Yeo TW, De Jager PL, Gregory SG, et al. A second major histocompatibility complex susceptibility locus for multiple sclerosis. **Ann Neurol** 2007;61:228-236.

118. D'Alfonso S, Bolognesi E, Guerini FR, et al. A sequence variation in the MOG gene is involved in multiple sclerosis susceptibility in Italy. **Genes Immun** 2008;9:7-15.

119. International Multiple Sclerosis Genetics Consortium, Hafler DA, Compston A, Sawcer S,et al. Risk alleles for multiple sclerosis identified by a genomewide study. **N Engl J Med** 2007;357:851-862

120. Weber F, Fontaine B, Cournu-Rebeix I, et al. IL2RA and IL7RA genes confer susceptibility for multiple sclerosis in two independent European populations. **Genes Immun** 2008;9:259-263.

121. Alcina A, Fedetz M, Ndagire D, et al. The T244I variant of the interleukin-7 receptor-alpha gene and multiple sclerosis. **Tissue Antigens** 2008;72:158-61.

122. Kurtzke JF. Epidemiologic evidence for multiple sclerosis as an infection. **Clin Microbiol Rev** 1993;6:382– 427.

123. Kurtzke JF, Heltberg A. Multiple sclerosis in the Faroe Islands: an epitome. **J Clin Epidemiol** 2001;54:1–22.

124. Sriram S, Stratton CW, Yao S, et al. *Chlamydia pneumoniae* infection of the central nervous system in multiple sclerosis. **Ann Neurol** 1999; 46:6-14.

125. Pucci E, Taus C, Cartechini E, et al. Lack of Chlamydia infection of the central nervous system in multiple sclerosis. **Ann Neurol** 2000; 8:399-400.

126. Challoner PB, Smith KT, Parker JD, et al. Plaque-associated expression of human herpesvirus 6 in multiple sclerosis. **Proc Natl Acad Sci U S A** 1995;92:7440-7444.

127. Moore FG, Wolfson C. Human herpes virus 6 and multiple sclerosis. **Acta Neurol Scand** 2002;106:63-83.

128. Serafini B, Rosicarelli B, Franciotta D, et al. Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. **J Exp Med** 2007;204:2899-2912.

129. Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors. **Ann Neurol** 2007 ;61:504-513.

130. Sriram S, Mitchell W, Stratton C. Multiple sclerosis associated with Chlamydia pneumoniae infection of the CNS. **Neurology** 1998;50:571-572.

131. Hickey WF, Hsu BL, Kimura H. T-lymphocyte entry into the central nervous system. **J Neurosci Res** 1991;28:254–260.

132. Wekerle H, Sun D, Oropeza-Wekerle RL, et al. Immune reactivity in the nervous system: modulation of T-lymphocyte activation by glial cells. **J Exp Biol** 1987;132:43–57.

133. Tsunoda I, Kuang LQ, Libbey JE, et al. Axonal injury heralds virus-induced demyelination. **Am J Pathol** 2003;162:1259–1269.

134. Babbé H, Roers A, Waisman A, et al. Clonal expansions of CD8+ T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. **J Exp Med** 2000;192:393–404.

135. Jacobsen M, Cepok S, Quak E, et al. Oligoclonal expansion of memory CD8+ T cells in cerebrospinal fluid from multiple sclerosis patients. **Brain** 2002; 125:538–550.

136. Huseby ES, Liggitt D, Brabb T, et al. A pathogenic role for myelin-specific CD8+ T cells in a model for multiple sclerosis. **J Exp Med** 2001;194:669–676.

137. Iglesias A, Bauer J, Litzenburger T, et al. T- and B-cell responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and multiple sclerosis. **Glia** 2001; 36: 220–234.

138. Linington C, Berger T, Perry L, et al. T cells specific for the myelin oligodendrocyte glycoprotein mediate an unusual autoimmune inflammatory response in the central nervous system. **Eur J Immunol** 1993;23:1364–1372.

139. Tsunoda I, Fujinami RS. Two models for multiple sclerosis:experimental allergic encephalomyelitis and Theiler's murine encephalomyelitis virus. **J Neuropathol Exp Neurol** 1996;55:673–686.

140. Lovas G, Szilagyi N, Majtenyi K, et al. Axonal changes in chronic demyelinated cervical spinal cord plaques. **Brain** 2000;123:308–317.

141. Lucchinetti, C, Bruck W, Parisi J, et al. Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination. **Ann Neurol** 2000; 47:707–717.

142. Matysiak M, Jurewicz A, Jaskolski D, et al. TRAIL induces death of human oligodendrocytes isolated from adult brain. **Brain** 2000;125:2469–2480.

143. Mead RJ, Singhrao SK, Neal JW, et al. The membrane attack complex of complement causes severe demyelination associated with acute axonal injury. **J Immunol** 2002;68:458–465.

144. Medana IM,Gallimore A,Oxenius A, et al. MHC class I-restricted killing of neurons by virus-specific CD8 + T lymphocytes is effected through the Fas/FasL, but not the perforin pathway. **Eur J Immunol** 2000;30:3623–3633.

145. Mews I, Bergmann M, Bunkowski S, et al. Oligodendrocyte and axon pathology in clinically silent multiple sclerosis lesions. **Mult Scler** 1998;4:55–62.

146. Bitsch A, Schuchardt J, Bunkowski S, et al. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. **Brain** 2000;123:1174–1183.

147. Ferguson B,Matyszak MK, Esiri MM, et al. Axonal damage in acute multiple sclerosis lesions**. Brain** 1997;120:393–399.

148. Kuhlmann T, Lingfield G, Bitsch A, et al. Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. **Brain** 2002;125:2202–2212.

149. Peterson JW, Bo L,Mork S, Chang A, et al. Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions**. Ann Neurol** 2001;50:389–400.

150. Neumann H, Schmidt H, Cavalie A, et al. Major histocompatibility complex (MHC) class I gene expression in single neurons of the central nervous system: differential regulation by interferon (IFN)-gamma and tumor necrosis factor (TNF)-alpha. **J Exp Med** 1997 185:305–331.

151. Pouly S, Antel JP. Multiple sclerosis and central nervous system demyelination. **J Autoimmun** 1999;13:297–306.

152. Vergelli M, Hemmer B, Muraro PA, et al. Human autoreactive CD4 + T cell clones use perforin- or Fas/Fas ligand-mediated pathways for target cell lysis. **J Immunol** 1997;158:2756–2761.

153. Rubesa G, Podack ER, Sepcic J, et al. Increased perforin expression in multiple sclerosis patients during exacerbation of disease in peripheral blood lymphocytes. **J Neuroimmunol** 1997;74:198–204.

154. Murray PD, McGavern DB, Lin X, et al. Perforin-dependent neurologic injury in a viral model of multiple sclerosis. **J Neurosci** 1998;18:7306–7314.

155. Suidan GL, Mcdole JR, Chen Y, et al. Induction of blood brain barrier tight junction protein alterations by CD8 T cells. **PLoS ONE** 2008;3:e303.

156. Redford EJ, Kapoor R, Smith KJ. Nitric oxide donors reversibly block axonal conduction: demyelinated axons are especially susceptible. **Brain** 1997;120:2149–2157.

157. Smith KJ, Kapoor R, Hall SM, et al. Electrically active axons degenerate when exposed to nitric oxide. **Ann Neurol** 2001;49:470–476.

158. Pitt D, Werner P, Raine CS. Glutamate excitotoxicity in a model of multiple sclerosis. **Nat Med** 2000;6:67–70.

159. Ramenghi U, Bonissoni S, Migliaretti G, et al. Deficiency of the Fas apoptosis pathway without Fas gene mutations is a familial trait predisposing to development of autoimmune diseases and cancer. **Blood** 2000 15;95:3176-3182.

160. Comi C, Leone M, Bonissoni S, et al. Defective T cell fas function in patients with multiple sclerosis. **Neurology** 2000;55:921–927.

161. Chiocchetti A, Comi C, Indelicato M, et al. Osteopontin gene haplotypes correlate with multiple sclerosis development and progression. **J Neuroimmunol** 2005;163:172-178.

162. Goertsches R, Baranzini SE, Morcillo C, et al. Evidence for association of chromosome 10 open reading frame (C10orf27) gene polymorphisms and multiple sclerosis. **Mult Scler** 2008;14:412-414.