Final Research Project

Periodical determination of anti-TIF1γ in adult DM patients without CAM: a new approach on the study of CAM pathogeneses and its screening

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ABSTRACT

ABREVIATIONS

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IIM – Idiopathic inflammatory myopathies

DM – Dermatomyositis

PM – Polymyositis

IBM – Inclusion body myositis

CAM – Cancer associated myositis

MSA – Myositis specific antibodies

MAA – Myositis associated antibodies

SLE – Systemic lupus erythematous

LE – Lupus erythematous

CPK – Creatine phosphokinase

VCAM – Vascular cell adhesion molecule

ICAM – Intercellular adhesion molecule

SIR – Standardized incidence ratios

SPSS – Statistical Package for the Social Science

CRP – C-reactive protein

ESR – Erythrocyte sedimentation rate

OR – Odds ratio

PBS – Phosphate-buffered saline

RT – Room temperature

ABSTRACT

Background: Dermatomyositis (DM) is the idiopathic inflammatory myopathy most associated to malignancy, being present in around 30% of the patients. Anti-TIF1γ is a myositis specific antibody (MSA) that is almost only present in DM patients. It is present in 73% of the cases of cancer-associated myositis (CAM). Nowadays, a single test of this antibody is carried out in order to detect a subset of patients diagnosed of DM who may have a higher likelihood to develop cancer, and therefore, will need accurate cancer surveillance. Some MSA have been noticed to be present in both, muscle-regenerating cells and in cancers known to be associated with CAM. It has been suggested that myositis is the consequence of a cross-react immunity, which originally targeted cancer cells, that fights against regenerating-muscle cells that originally targeted cancer cells. Since anti-TIF1γ is so highly associated with CAM in prevalence studies of the antibody, it is reasonable to think that TIF1γ may play a role in the pathogenesis of cancer in DM patients. Hence, we consider necessary to study the anti-TIF1γ behaviour along time to study its possible relationship with the tumorigenesis process. The aim of this study is to go forward on the understanding of cancer and DM pathogenesis in order to develop more specific therapies on both processes. Simultaneously, this study will provide information on the possible development of a more accurate cancer surveillance protocol in DM patients**.**

Objectives: To determine whether there are changes from a first positive anti-TIF1γ value to a negative one or vice versa by performing periodical anti-TIF1γ serum analyses in adult DM patients without CAM criteria after the disease onset. Furthermore, we will also analyse whether these changes between the first serum analysis and the following are somehow associated to a different relative risk to develop cancer compared to the patients who do not have changes in their serum values.

Methods: This study is a multicentre prospective cohort study that includes recently diagnosed DM patients without CAM criteria. Patient's recruitment will last three years. There will be four groups of patients. 14 participants are needed in the group of patients with changes form a positive anti-TIF1γ value to a negative one; 22 participants are required in the group of patients with persistent positive anti-TIF1γ; 10 participants are needed in the group of patients with a change from a negative anti-TIF1γ value to a positive one; finally, 118 participants are required in the group of patients with persistent negative anti-TIF1γ values. Participants will be evaluated for a five-year follow-up period in order to compare the cancer incidence between patients with positive values of anti-TIF1γ and patients with negative values of the antibody. Anti-TIF1γ will be analysed by ELISA in absorbance units and confirmed by immunoblot. Cancer occurrence data will be registered by each physician when it is diagnosed. The results will be expressed as percentages for categorical variables. Continuous variables will be expressed as mean +/- SD if they are normally distributed or median (quartiles) if they are not. Bivariate analysis will be performed with X^2 test and the Fisher test, and Cox regression analysis will be used to perform the multivariate analysis.

Keywords: Dermatomyositis, cancer-associated myositis, anti-TIF1γ, cancer**.**

1. BACKGROUND

1.1. Idiopathic inflammatory myopathies

1.1.1. Concept

Idiopathic inflammatory myopathies are a heterogeneous group of diseases characterized by having an important inflammatory component in the muscular biopsy. They are considered systemic diseases because even though the striated muscle is the principal organ affected, there are several other organs that can be also affected such as lungs, skin, gastrointestinal tract, joints and cardiovascular system. They are mainly divided into three different entities: dermatomyositis (DM), polymyositis (PM), and inclusion body myositis (IBM).

1.1.2. Epidemiology

IIM are considered rare diseases because of their low incidence. Different epidemiological studies done all over the world estimate that the annual incidence average of these diseases is between 2.1 to 7.7 cases per million inhabitants per year. These outcomes are similar in Spain, where the annual average incidence is between 2.2 to 10.6(1). IBM, compared to the other IIM, is more common in elderly males (from two to three times more frequent). Prevalence adjusted to age increases in the group over 50 years old where prevalence is 3.5/100,000 inhabitants, being IBM the most common acquired myopathy among this group of subjects(2,3).

However, PM and DM are more frequently present in females being 4.6 vs. 3.2 per million inhabitants in PM and 6.4 vs. 3.4 per million inhabitants in DM. In Spain, DM incidence is considered to be 4.9 inhabitants per million while PM has an incidence of 3.9 per million inhabitants. Another difference between these two diseases is the moment of diagnosis, which can be both in the childhood and adulthood in DM, unlike in PM, which is more frequently observed from the second decade of life onwards.

1.1.3. Classification

Different authors have described several classifications. Bohan and Peter's classification and Dalakas' classification are probably the most relevant. (Annex 1)

1.2. Dermatomyositis

1.2.1. Concept

DM is a type of idiopathic inflammatory myopathy defined mainly by muscular weakness and skin distinctive findings. It has a characteristic muscular inflammatory component which can be detected on the biopsy. At the same time, DM is considered a systemic disease because of the involvement of different organs: lungs, articulations, cardiovascular system and gastrointestinal tract. The cause is not yet defined though it is strongly associated to an autoimmunity component.

1.2.2. Pathogenesis and antibodies

Even though the cause of DM is unknown, it is widely associated with autoimmunity. This is supported by the presence of positive antinuclear antibodies in around 50% of the patients and nearly 20% of specific or associated myositis antibodies, as well as by the association with other autoimmune diseases and the response to immunosuppressive treatment.

DM develops in a genetically predisposed individual that interacts with physical, chemical or infectious external agents. In fact, the MHC locus has been identified as the genetic risk region with strongest impact for DM, despite the fact that other non-MHC genes may also take part in the pathogenic process(4). As mentioned above, there are two different groups of antibodies related to DM: the myositis specific

antibodies (MSA), only present in myositis, and the myositis associated antibodies (MAA), which can also be related to other diseases.

Antibodies can help with the DM diagnosis and the identification of different DM subsets that will have different clinical features, treatment responses, and prognosis. Thus, the presence of the anti-histidyl tRNA synthetase (Jo-1) antibody, the most common among anti-synthetases, is associated to the anti-synthetase syndrome. On the other hand, anti-Mi-2 appears in adult and juvenile DM with distinctive Gottron's papules, heliotrope rash and different rashes over the skin, mild muscular weakness, lower risk of interstitial pneumonia and good response to treatment. A third type, anti-MDA5, is closely associated with rapidly progressive interstitial pneumonia(5–7). (Annex 2)

The resulting substrate of the disease is the presence of microangiopathy and muscle ischemia. Capillar endothelia is the main affected place in DM, being C5b-9 membranolitic attack complex one of the first agents triggering an inflammatory infiltrate dominated by CD4$^+$T cells and B cells located in perivascular and perimisal areas. Metalloproteases, citokines, macrophages, plasmocytic dendritic cells and adhesion molecules (VCAM and ICAM) may also be present along the process.

The resulting endothelial cell necrosis, the diminished endomisial capillary number, ischemia and muscle-fiber destruction resemble micro infarcts lesions. Perifascicular atrophy and C5b9 detection at vascular endothelia by immunohistochemically techniques are pathognomonic of DM(1,3,8).

1.2.3. Clinical features

<u>Cutaneous manifestations</u>

Skin features usually appear concomitant with muscle weakness, but can also appear isolated(3,8). Thus, at an early stage, it can be easily confused with psoriasis lesions,

eczema, dye allergy or other allergies that do not respond to the antihistaminic treatment(3).

The erythematous-desquamate papules and plaques are localized on skin extension areas, articulations and bonny prominences such as elbows, knees, malleolus, etc. When found on the knuckles (interphlangeal and metacarpophalangeal articulations) it are called Gottron's papules. The purple coloured rash around the orbital area, which can sometimes be accompanied by bilateral and symmetric oedema of the upper eyelid, is known as heliotrope exanthema. The Gottron's papules and the heliotrope exanthema are considered pathognomonic (1,3,9).

Erythematous-violaceous lesions can also be present in other areas of the body that are characteristically photo-exposed, such as the cleavage or the upper part of the back and shoulders, known as the "V sign" and "shawl sign" respectively. The presence of altered hyperplasic cuticles which are thickened and irregular(8–10) and telangiectasia with dilated capillary fingernail loop and little haemorrhagic infarcts are also common in DM. A erythema with tenderness at the nailfold is the characteristic "Kveim sign"(3).

Cutaneous alterations can be pruriginous(3), which helps differentiate this disease from subacute cutaneous LE(10). Facial erythema, when present, forces a differential diagnoses with LE, rosacea, seborrheic dermatitis and atopic dermatitis. Non-scarring alopecia may also occur mimicking a psoriasiform dermatitis(10) as well as poikilodermia, a kind of erythematous lesion at photo-exposed regions, characterized by typical skin atrophy with or without telangiectasia and pigmentation alterations. Ulcerations may also be present as a result of ischemia on the skin and subsequent necrosis.

Some authors state that other infrequent manifestation like panniculitis, flagellate eruption, vesiculobullous lesions(9,10), plaquelike mucinosis and scleromyxedema-like papular lesions(10) can also be seen in DM.

Defining the "anti-synthetase syndrome", we may find the presence of hyperkeratosis, fissures and thickening of the lateral side and palms of the hands (also defined as "mechanic hands") together with interstitial lung disease, arthritis, myositis, Raynaud phenomenon and the serum presence of anti-Jo-1, (3,9).

Dermatomyositis-sine myositis is defined as cutaneous manifestations not followed by clinical and laboratory muscle alterations in the two subsequent years.

Muscular manifestations

DM main muscular alteration is the proximal, progressive and symmetric muscle weakness of the shoulder and pelvic girdle that takes from weeks to months. This alteration leads to a difficulty or even inability to do daily activities such as brushing their hair, shaving or standing up when sitting. When flexor muscles of the neck are impaired there is impossibility of keeping the head stand, also known as "head drop". Face and ocular muscles' function are preserved. If affected, other diagnosis should be considered(11). Clinical features may also be accompanied by laboratory alterations with elevated muscular enzymes levels(3,7).

Malignancies

Idiopathic inflammatory myopathies are associated with cancer, being the overall risk for developing malignance of 2.6 (95%CI, 2.1 to 3.3)(12). Cancer may be present before, concurrently or after the IIM(13).

In 1916, the first reports suggesting certain association between dermatomyositis and cancer appeared(9). Later on, in 1992 Sigurgeirsson *et al*.(14) proved that cancer relative risk in male patients was of 2.4 (95 % CI, 1.6 to 3.6) and 3.4 in female patients (95 %CI, 2.4 to 4.7). Moreover, they found out that patients with DM had a higher mortality, twice the risk among females, being cancer the main cause of death (40%).

Several years later, in 2001 Hill *et al.(15)* performed a population-based study where demonstrated a higher association in DM than in PM. They observed that after DM diagnosis there was a three-fold increase risk of cancer, being higher in men than

women when comparing with the standardized incidence ratios (SIR). They also observed that more than the 60% of cancers were diagnosed after the DM diagnosis. Furthermore, young group of patients with DM (15-44 years) showed malignance risk but this was higher in patients who aged 45 and older. On the other side, they analyzed the strong association between the disease and cancer in particular (SIR, 95% CI): ovarian (10.5, 6.1 to 18.1), lung (5.9, 3.7 to 9.2), pancreatic (3.8, 1.6 to 9), stomach (3.5, 1.7 to 7.3) and colorectal (2.5, 1.4 to 4.4) and breast (2.2, 1.2 to 3.9) cancers and non-Hodgkin lymphomas (3.6, 1.2 to 11.2). Moreover, they affirmed that malignancy risk was higher in the first year and thereafter there was a drop on the cancer incidence. Ovarian, lung and pancreatic cancer kept higher risk within the five years after diagnosis and colorectal and pancreatic cancers even after the five years.

On the same year in Victoria, Australia, Buchbinder *et al*.(12) followed a cohort of 537 patients with biopsy positive idiopathic inflammatory myopathy. The outcomes where concordant with the other studies showing the highest malignancy risk (6.2 SIR (95% CI, 3.9 to 10)). It seemed that the early increased risk to develop cancer was not due to the immunosuppressant therapy. Nevertheless, the increased risk that was still evident after five years of disease could be due to the therapy.

There are different cancer risk factors in DM patients such as older age, male sex, cutaneous necrosis and dysphagia(13). While on the other hand, arthritis and interstitial lung disease where protective factors. Madan *et al.* suggested that the presence of a myositis specific and associated antibodies negative profile was a risk factor to develop an associated malignancy(5). Furthermore, Lu *et al*.(16) also added that, elevated ESR (>35mm/hr), higher CRP levels and anti-p155 antibody were risk factors for develop malignance in DM.

Even though the pathogenesis is still unknown Wang *et al.* set probable associations between IIM and cancer: the presence paraneoplastic conditions which produce bioactive mediators that may induce the immune actions against muscle fibers and skin; the presence of a compromised immune system which could be the origin to development both tumors and myositis; the presence of cancer due to the cytotoxic

effect of the myositis therapy; or the possible exposure to environmental hazards that originate both cancer and myositis(13).

1.2.4. Diagnosis

DM is not easily diagnosed and the differential diagnoses with several diseases must be performed. To use the Bohan and Peter criteria usually helps(1). Muscle biopsy, an invasive test, is a great pillar on the DM diagnosis. Despite in some childhood forms it can be avoided, in adults is mandatory(7). The presence of the characteristic C5bP or the perifascicular atrophy added to the presence of perivascular and perimysium inflammatory infiltrate with predominant T and B cells are typical traits of DM. However, a normal biopsy does not exclude DM as the myopathy has an spotted distribution. Thus, it is useful to perform MRI in order to guide the biopsy and also to quantify the muscle inflammation and damage(7). Among the non-invasive tests, both analyses of specific and associated antibodies may help on the diagnosis and also on the characterization of different DM subsets(7). In myositis patients, muscle enzymes such as creatine phosphokinase (CPK), aspartate, alanine aminotransferase, lactate dehydrogenase and aldolase are usually elevated. Although CPK is the most sensitive enzyme possibly being increased 50-fold when there is active disease, normal CPK outcome do not rule-out the disease(7). These tests, however, may be useful on the follow-up of myositis patients. Also useful is the capillaroscopy, a non-invasive technique that can be performed at bedside. Its applicability in the study of IIM is still being studied but it is thought that it may help on the support of DM diagnosis and maybe as a marker of occult malignancy(7).

CAM is a specific clinical scenario that needs to be evaluated. Thus, in a new onset of DM disease a conventional cancer screening or FDG-PET/CT must be performed in recently diagnosed DM. If malignancies are objectivized within a period of three years before or after the myositis onset, CAM is diagnosed. When there are no findings on the image tests and the anti-TIF1γ is negative only future cancer evaluation must be

done according to age and risk factors. When there is a positive anti-TIF1γ, annual conventional cancer screening or FDG-PET/CT is indicated to be performed for three to five years(7). More studies are required to determine how often anti-TIF1γ should be determined.

1.2.5. Management and treatment

The main two objectives of the DM therapy are: to control the disease and to keep the autonomy of the patient. The pharmacological treatment is based on the glucocorticoids, immunosuppressive drugs and intravenous immunoglobulin's administration. Specific physical therapy and exercise programs may contribute to improve therapy response in refractory cases. Furthermore, patients with severe myositis-associated ILD may need lung transplantation.

1.2.6. Prognosis

Different outcomes are found depending on the study performed. Mortality has decreased along the last years thanks to an early disease diagnosis and the administration of immunosuppressant therapy. However, nowadays increased mortality incidence is still up to 5 to 48%(17). In Spain, the fifth-year survival is around the 75%(3). Cancer, lung, cardiac complications, and infections are the most common causes of deaths in PM/DM. Being cancer the main cause (48% of deaths)(17).

1.3. Autoantibody anti-TIF1γ (anti-p155)

1.3.1. Concept

Human transcriptional intermediary factor 1 γ (TIF1γ) whose synonyms can be TRIM33, Ret-fused gene 7, PTC7 or ectodermin(18) is a member of the TIF1 family described by Targoff *et al.* to be the target antigen for the anti-p155 autoantibody(18).

Four members the TIF1 protein family: TIF1α (TRIM24), TIF1β, TIF1γ (TRIM33) and TIF1δ. These proteins are characteristically implicated on different biologic functions such as innate immunity, cell proliferation, development and apoptosis. Thus, they do share on the one hand a same N-terminal tripartite motif that allows these proteins to participate in the ubiquitination pathway to control protein degradation, localization and function while acting as E3 ligases. While on the other hand, they share the same C-terminal domain which enables them to take part in epigenetic mechanisms of transcription regulation(19).

Moreover, TIF1γ has been implicated with immune response, cell cycle regulation, DNA repair, hematopoietic differentiation and embryonic development. When focusing on molecular level, TIF1γ is implicated on chromatin remodelling and subsequent transcription modulation(20). Several authors consider TIF1γ a tumour suppressor gene which perfectly agree with the chromosomal rearrangements observed in the TIF1γ gene locus observed in carcinomas, hematopoietic neoplasia, in embryonic tumors, conjunctive tissue tumors and in nervous central system tumours(20). Furthermore, decreased TIF1γ expressions have been objectivised in different tumours including pancreatic ductal adenocarcinomas, breast tumours, non-small cell lung cancers and hepatocellular carcinomas. Pommier *et al*. observed the consequences of prolonged TIF1γ inactivation. They could observe mitotic defects accumulations leading to increased aneuploidy and chromosome rearrangements, acquiring more aggressive conditions. They also determined that in the context of low TIF1γ levels, there is an increased chromosomal instability in human tumors(20).

1.3.2. Determination techniques

Immunoprecipitation (IP) from radiolabelled HeLa cell lysates is considered the reference standard in order to detect anti-p155. Labrador-Horrillo *et al*.(18) demonstrated in 2012 the concordance between anti-p155 antibodies detected by this technique and anti-TIF1γ antibodies determined by ELISA and immunoblot (IB) techniques. This finding provided a simplified technic that could be easily used in the clinical practice.

1.3.3. Clinical applications

Since first recognition in 2006 of anti-p155 or anti-p155/140 antibody by Targoff *et al.*, it was associated to DM and cancer-associated DM (18). This antibody is considered a myositis specific antibody almost only detected in DM patients but not in the other IIM or autoimmune diseases(18,21).

Nowadays several investigators have published articles that corroborate this information. In 2012 a systematic review and meta-analyses performed by Trallero-Arguás *et al.* which included 6 different studies, analysed the accuracy of anti-p155 testing for the diagnosis of cancer-associated myositis(5). These are the following outcomes of the study: the pooled estimated sensitivity and specificity were 78% (95% CI 45-94%) and 89% (95% CI 82-93%), respectively, both of them with substantial heterogeneity between the studies. In addition, the area under the SROC curve was 0.91 (95%CI 0.88-0.93); the positive predictive value was 58% while the negative predictive value was 95%. When assessing the OR which had a high heterogeneity, the overall value was 27.26 (95%CI 6.59-112.82). Even they affirm that a positive result cannot be relied to diagnose patients that will ultimately develop cancer; it could be used to identify a subset of DM patients who are in higher risk to have a malignancy occurrence who should receive more exhaustive cancer surveillance.

In accordance to the previous study, in 2015 Selva-O'Callaghan *et al.*(7) performed an algorithm to follow on the clinical practice in order to perform a proper cancer screening in patients with IMM.

FIGURE 1 from Selva-O'Callaghan *et al.*(7) Algorithm for cancer screening in patients with idiopathic inflammatory myopathy. CAM: cancer-associated myositis; FDG-PET/CT: [18F] fluorodeoxyglucose PET/computed tomography.
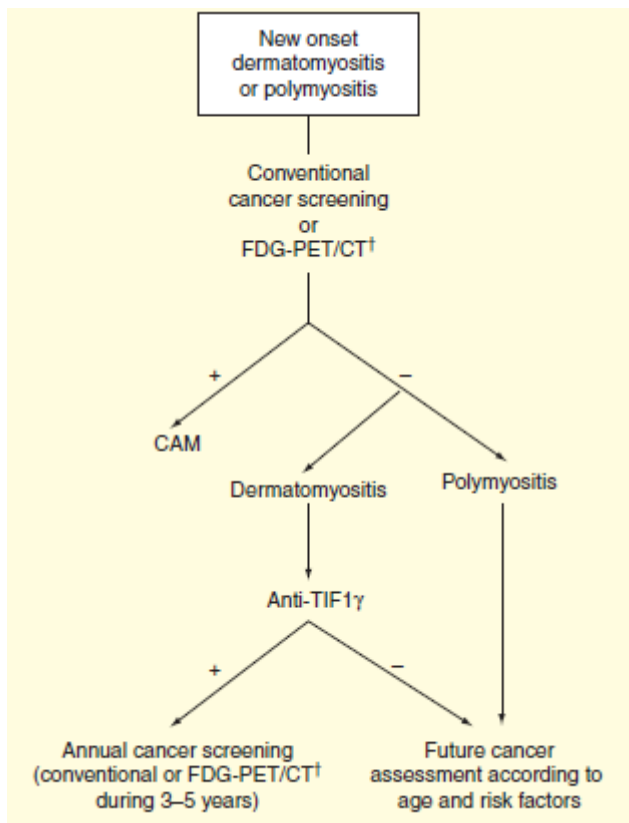
2. JUSTIFICATION

DM is the idiopathic inflammatory myopathy most associated to cancer. After the diagnosis of the disease, the risk of malignancy is three fold compared to the population without DM. Besides, cancer causes the 40% of deaths among DM patients, being thus the most common cause of death(15). The aim of this study is to take a step forward on the study of the relationship between DM, cancer and TIF1γ.

It has been suggested that DM could be a paraneoplastic autoimmune syndrome because of the improvement on DM after the cancer treatment, the high occurrence of cancer the year before and after the DM diagnosis, the reappearance of muscular weakness after cancer recurrence, and the overrepresentation of histological types of cancer such as adenocarcinomas and specific types of cancer (lung, ovarian and colorectal, and others) in DM patients(15,22,23)

Anti-TIF1γ autoantibody has been well-associated with higher risk of cancer in DM adult patients, being present in a 73% of adult cancer-associated DM(19). That leads us to wonder if TIF1γ is an epiphenomenon occurring when there is cancer or whether it is involved in the pathogenic process. Fujimoto *et al*. try to do an approximation of the relationship between TIF1γ and DM pathogeneses in an article published in 2012. They expose that large numbers of TRIM proteins are up-regulated by interferon (IFN), and at the same time, some TRIM proteins regulate IFN expression. Since it is known that IFN is involved in the DM pathogenesis and as mentioned above, TRIM are associated to cancer, researchers hypothesize that TIF1γ is the connection between cancer and IFN-mediated immunity, and therefore, DM(24).

Additionally, Fiorentino and Casciola-Rosen write an editorial on the same article(19). They expose that "even the mechanistic nature of the association between TIF1γ and cancer remains unknown, some hypothesis could be made". On one hand, they expose that the anti-TIF1γ immune response could only be a reaction against cancer and not to the DM itself because TIF proteins are overexpressed in several kinds of cancer, as well as the frequent presence of immune response against TRIM28 in colorectal carcinoma (even in patients without DM).

However, contrasting this information, they argue the fact that anti-TIF1γ antibodies are not present in other autoimmune diseases but they are in 36 % of juvenile DM, disease not associated to malignancies, which supports the idea that anti-TIF1γ is truly linked with DM itself. The arguments to consolidate this idea are based on the myositis specific antigens overexpression found in regenerating muscle cells and in cancers associated with myositis(25).

Thus, CAM could be explained as a nascent tumor being the origin of the whole process by stimulating the production of MSA. The immune system would be the responsible to start a response against the tumor antigens which, at the same time, are shared with regenerating immature muscle cells.

Non-specific muscle injuries may be produced by a virus infection, by exposition to certain toxins, trauma or even as Zambieri *et al.*(26) suggest, by the same tumor cells that produce muscle toxicity. Thus, the increased presence of regenerating muscle cells in non-specific muscle injures would be the target of the antitumor immunity response. Therefore, given that the immune system is fighting against cancer, T and B cells attack muscle tissue in cross-over immunity. Hence, certain clinical cases can be explained by this theory; when there is presence of a myositis without cancer which appears afterwards, it is likely that the immune system was successfully fighting against the tumor, but in the end, immunity fails on its attempt to keep the tumor growth. On the other hand, patients with DM who never develop cancer are those whose immunity system beats the tumor. At the same time, in several cases, when the patient with CAM receives the treatment of his/her cancer, there is an improvement in myositis clinical features, which could be explained by the MSA values below the threshold level(23).

However, even though TIF1γ is a MSA, it has not been studied yet and more investigation on this field is necessary, especially new studies analysing the presence of TIF1γ in regenerating muscle cells, normal muscle cells and in tumors known to be associated with CAM.

In this study we want to provide longitudinal evidence of the MSA anti-TIF1γ's behaviour along the time in accordance with the tumorigenic process. Trallero-Arguás *et al.* already did a meta-analysis that showed a diagnostic OR of 27.26 and a relative risk of developing cancer above 12 when having a positive anti-TIF1γ in a serum determination (calculated with their data given)(27). Indeed, it demonstrates the high association between CAM and TIF1γ.

We believe in the possibility that anti-TIF1γ levels change along time. This idea is also supported by a study from Fujimoto *et al.* where they found longitudinal changes within the serum antibody titers in 8 patients. They noticed that after cancer treatment, antibody titers measured by ELISA had decreased although IP results kept positive(24). At the same time, nowadays Selva-O'Callaghan *et al.* are also analysing periodically the serum anti-TIF1γ titers from DM patients with cancer. Although the study has not been published yet, in some patients the anti-TIF1γ behaves as a cancer marker, decreasing its titers when the cancer treatment is being performed and the cancer is not present anymore. It is of great importance to do a periodical analysis of the antibodies to assess more accurately its relationship with the malignancy occurrence; given that not all the patients who have a positive first determination of anti-TIF1γ develop cancer, and neither the totality of patients who present negative anti-TIF1 values, do not develop cancer.

To such degree, we would be able to move closer on the investigation of the TIF1γ and its possible role in the etiopathogenical process of CAM. Definitely, as mentioned above, more research at molecular level further to this study is required. However, by understanding this process, we could reach the objective of developing more specific therapy to treat both cancer and DM by using TIF1γ as the target of the treatment. Furthermore, if we can demonstrate that anti-TIF1γ changes along time and this change is associated to a higher or lower risk of cancer occurrence, the protocol of cancer screening in DM patients could change and not only could a single determination be done, but also a periodical study of the antibody. Since an early detection of patients with cancer will help performing an early and effective cancer

treatment and also decreasing the disability produced by the myositis, this study would be important also for short-time clinical consequences.

To sum up, there are risk factors associated to higher cancer incidence, among which, the most important one is the presence of positive anti-TIF1γ in serum that allows the definition of a patient's subset with a higher risk of developing cancer. Thus, this subset will have a PET-CT annually in order to rule out the presence of malignancy.

Unfortunately, more diagnostic techniques are necessary in order to know who will finally develop cancer. Given that TIF1γ could be linked with the pathogenic process of DM and cancer, as mentioned above, it makes us wonder whether it could be a marker of the underlying tumoral process. Therefore, the periodical analysis of this antibody could help with the prediction of who will finally develop cancer.

On the other hand, when this cancer will appear is still uncertain. It would be interesting to assess the time between the presence of a first positive anti-TIF1γ in serum and the occurrence of cancer.

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4. HYPOTHESIS AND OBJECTIVES OF THE STUDY

Objectives:

General objectives

1. To determine whether there are changes from a first positive anti-TIF1γ value to a negative one or vice versa by performing periodical anti-TIF1γ serum analyses in adult DM patients without CAM criteria after the disease onset.

2. To analyse whether a change in the anti-TIF1γ outcome between the first serum determination and the following determinations in adult DM patient without CAM criteria is associated to a different relative risk to develop cancer compared to the patients who do not have changes in their serum outcomes.

Specific objectives

1. To investigate the proportion of adult DM patients without CAM with negative anti-TIF1γ in a serum first determination that can develop a change in the anti-TIF1γ concentration, showing positive anti-TIF1γ values in following serum determinations along five years of follow-up.

2. To determine the proportion of adult DM patients without CAM with positive anti-TIF1γ in a serum first determination that can develop a change in the anti-TIF1γ concentration, showing negative anti-TIF1γ values in following serum determinations along five years of follow-up.

3. To assess whether a negative anti-TIF1γ value in serum first determination that changes to a positive anti-TIF1γ value in following serum determinations is associated to a higher relative risk to develop cancer compared to the patients with negative anti-TIF1γ without changes in their serum determinations along five years of follow-up.

4. To assess whether a positive anti-TIF1γ value in serum first determination that changes to a negative anti-TIF1γ value in following serum determinations is associated to a lower relative risk to develop cancer compared to the patients with positive anti-TIF1γ without changes in their serum determinations along five years of follow-up.

Secondary objectives

1. To describe the elapsed time from the appearance of the positive anti-TIF1γ value to the occurrence of cancer in patients whose first anti-TIF1γ values were negative.

Hypothesis

1. A proportion of adult DM patients without CAM criteria and negative anti-TIF1γ in serum first determination will change to a positive anti-TIF1γ in following serum determinations within a period of five years.

2. A proportion of adult DM patients without CAM criteria and positive anti-TIF1γ in serum first determination will change to a negative anti-TIF1γ result in following determinations within a period of five years.

3. A change from a negative anti-TIF1γ in serum first determination to a positive anti-TIF1γ result in following determinations in adult DM patients without CAM criteria; is associated to a higher relative risk to develop cancer compared to the patients without changes in their serum outcomes.

4. A change from a positive anti-TIF1γ in serum first determination to a negative anti-TIF1γ result in following determinations in adult DM patients without CAM criteria; is associated to a lower relative risk to develop cancer compared to the patients without changes in their serum outcomes.

5. MATERIAL AND METHODS

5.1. Design

The study designed is a multicentre prospective cohort study.

5.2. Study period

The study consists on a cohort observation of five years. The serum analysis of the anti-TIF1γ presence will be regularly performed every four months during five years for each patient. At the same time there will be a cancer surveillance of every patient every four months.

5.3. Population definition

The reference population is composed by patients with adult DM onset without CAM criteria at the moment of the diagnosis.

The study population is composed by DM patients whose diagnosis is based on the following Bohan and Peter's criteria(28,29) where only patients with definite (at least four criteria including rash) and probable (at least three criteria including rash) disease are included:

1. Symmetrical weakness of the limb-girdle muscles and anterior neck flexors, progressing over weeks to month, with or without dysphagia or respiratory muscle involvement
2. Muscle-biopsy evidence of necrosis of type I and II fibers, phagocytosis, regeneration with basophilia, large vesicular sarcolemmal nuclei and prominent nucleoli, atrophy in perifascicular distribution, variation in fiber size, and an inflammatory exudate, often perivascular.
3. Elevation in serum of skeletal-muscle enzymes, particularly CPK and often aldolase, serum glutamate oxaloacetate and pyruvate transaminases, and lactate dehydrogenase.

4. Electromyographic triad of short, small, polyphasic motor units, fibrillations, positive sharp waves and insertional irritability, and bizarre, high-frequency repetitive dishcarges.
5. Dermatologic features including a lilac discoloration of the eyelids (heliotrope) with periorbital oedema, a scaly, erythematous dermatitis over the dorsum of the hands (especially the metacarpophalangeal and proximal interphalangeal joints, Gottron's sign), and involvement of the knees, elbows, and medial malleoli, as well as the face, neck and upper torso .

The Sontheimer criteria are used in order to diagnose amyopathic DM (29). (Annex 3)

5.4. Exclusion and inclusion criteria

Inclusion criterion

- A muscular biopsy must have been performed.

Exclusion criteria

- CAM diagnosis. CAM will be considered as cancer appearance at the time of DM diagnosis or within three years of myositis diagnose, same as Troyanov *et al.* propose(30). In order to detect these patients, a careful medical history and a physical examination will be done for every patient. After that, a whole-body PET/TC scanning using [18F] fluorodeoxyglucose (FDG-PET/TC) will be performed. In place of the FDG-PET/TC, a classical cancer screening consisting on a thoracoabdominal computed tomography, tumor marker analysis and a mammography an gynaecological examination with transvaginal ultrasonography for women could be performed (7).

- Patients who meet the criteria for myositis overlap syndrome(30), sporadic inclusion body myositis(31) or juvenile dermatomyositis(28,29) (Annex 4)

5.5. Sample and sampling method

Sample selection

The sample will be obtained by a consecutive non-probabilistic sampling as patients are diagnosed of adult DM once their inclusivity is confirmed according to the predefined eligibility criteria. On the following doctor's appointment, each patient will be given the information about the study and if they agree to participate, the informed consent will be delivered and signed.

Because it is a multicentre study, the study will be explained in the following meeting of the "Grupo para el estudio de las Enfermedades Autoinmunes Sistémicas (GEAS) de la Sociedad Española de Medicina Interna (SEMI)" and a physician of each hospital who agrees to participate will be offered to take part in the study. One month later, a course will be given in order to instruct them all as well as a folder with information about the aims of the study, the participants and the tests to be performed.

Sample size

GRANMO application has been used to determine the simple size of the study. As we are working with two main hypothesis (hypothesis 3 and 4), we need to determine the number of participants required in two big groups (one with a positive anti-TIF1γ at the first determination and one with a negative anti-TIF1γ) which at the same time are both divided into two subgroups: one composed by exposed patients and one composed by non-exposed. In order to work with the fourth hypotheses, the patients composing this big group are the ones who have a positive TIF1γ in the first serum determination. Exposed patients are considered the ones who do not have a change to a negative anti-TIF1γ in any of the following determinations. On the other hand, non-exposed patients are the ones who have a change in the serum outcome, showing a negative anti-TIF1γ in any of the following determinations.

Accepting an alpha risk of 0.05 and a beta risk inferior to 0.2 in a bilateral contrast, 22 subjects are required in the exposed group and 14 in the non-exposed group, in order

to detect a minimum relative risk of 7 whether the patients with cancer taxes at the non-exposed group is 0,1. A drop-out rate of 30% has been anticipated.

On the other hand, to work with the third hypothesis, patients with a first negative anti-TIF1γ in their first serum determination compose the second big group. This group, at the same time is divided in exposed and non-exposed patients. Exposed patients are the ones who develop a change in any the following serum samples, presenting a positive anti-TIF1γ; while non-exposed patients are defined as the ones who after different serum determinations keep presenting negative anti-TIF1γ determinations. Accepting an alpha risk of 0.05 and a beta risk inferior to 0.2 in a bilateral contrast, 10 subjects are required in the exposed group and 118 in the non-exposed group, in order to detect a minimum relative risk of 12 whether the patient taxes at the non-exposed group is 0.05. It has been anticipated a drop-out rate of 30%.

Due to the lack of studies that assess the anti-TIF1γ longitudinally, it is impossible to know the proportion of patients who may be into the exposed group and the non-exposed group. However, many researchers have done prevalence studies of the anti-TIF1γ.

We have chosen a Spanish study performed in 2010 from a historical cohort of patients from the Hospital Universitari Vall d'Hebron(32). In this case, they studied the prevalence of anti-TIF1γ and the incidence of CAM in patients with PM and DM. We expect that the anti-TIF1γ will have the same behaviour as in our study. To calculate the expected number of patients who will present first positive anti-TIF1γ titers, we consider that the antibody prevalence will be the same as in the study mentioned above. The same exercise has been done in order to calculate the proportion of patients who first present with a negative anti-TIF1γ determination. When talking about the subgroups with a first negative anti-TIF1γ, we take into consideration that the proportion of patients who may be in the exposed group is similar to the proportion of patients who present a cancer along time in the reference study. On the contrary, we estimate that the proportion of patients who may be in the non-exposed group is approximately the same as the proportion of patients who do not present

cancer on the reference study. In order to calculate the proportion of patients in the two subgroups who present a first positive anti-TIF1γ, we did the same exercise as we the above mentioned. We estimate that the non-exposed group has a similar proportion to the patients who showed a positive anti-TIF1γ in the reference study but did not present cancer. On the contrary, to calculate the proportion of patients in the exposed group, we estimate that it is close to the proportion of patients who present cancer.

When talking about the expected cancer incidence in the four groups, it is just an approximation because no data is available yet. Our estimation is only based on clinical experience; the knowledge of the TIF1γ cancer and DM association; and the high relative risk (12.4 when calculated with the data given) presented on the meta-analysis done by Trallero-Arguás *et al*.(27). Since it is the first time that anti-TIF1γ is being studied longitudinally when a cancer has not presented yet, we consider our research to be a pilot study.

We assume a DM incidence rate of 4.9 patients per million inhabitants every year(33) in Spain. The Instituto Nacional de Estadística(34) estimates a Spanish population of 46.4 million people, which means a DM incidence of approximately 227 patients per year in Spain. If we exclude the patients, who may be in other departments different from Internal Medicine and the ones diagnosed with CAM criteria, we expect to reach a population of 100 incidence DM patients per year. In consequence, three years of patients' recruitment will be needed. The following scheme explains how many patients we need and how many patients we may have in every subgroup.

TABLE 1. Sampling method scheme

DM patients without CAM **100 px**	Spanish population = 46,40 milion people DM incidence in Spain = 4,9 per milion inhabitant per year Spanish population X DM incidence in Spain = 227 px Sample = 100 px (227 px - px in other departments apart from Interna Medicine - px with CAM at diagnosis)		
TIF1γ + Prevalence <u>15%</u> (10^i/65) ▲ Our sample prevalence = 15% X 100 px = **15 px** ^{i} 10 = 4 px with no cancer + 6 px with cancer No cancer = 4 px / 10 px = 40% ▲ Cancer = 6 px / 10 px = 60 % ▲	+ ⟶ + Exposed Minimum expected cancer incidence = 70% 60 % of 15 = 9 px (per year) 9 px X 3 years = 27px **GRANMO CALCULATION = 22 px** + ⟶ - Non exposed Maximum expected cancer incidence = 10 % 40% of 15 = 6 px (per year) 6px X 3 year = 24 px **GRANMO CALCULATION = 14 px**		
TIF1γ - Prevalence <u>77%</u> (50^i/65) ▲ Our sample prevalence = 77% X 100 px = **77 px** ^{i} 50 = 65 px - 15 px with TIF1γ + No cancer = 100%-8% = 92% ▲ Cancer = 4px / 50 px = 8% ▲	- ⟶ - Non exposed Maximum expected cancer incidence = 5 % 92 % of 77 = 71 px (per year) 71 px X 3 years =213 px **GRANMO CALCULATION = 118 px** - ⟶ + Exposed Minimum expected cancer incidence = 80% 8 % of 77 = 6 px (per year) 6px X 3 years = 18 px **GRANMO CALCULATION = 10 px**		
px = Patients DM = Dermatomyositis CAM = Cancer Associated Myositis ▲ Trallero - Arguás *et al* " *Cancer-Associated Myositis and Anti-p155 Autoantibody in a Series of 85 Patients With Idiopathic Inflammatory Myopathy* " (65 px with DM)			

5.6. Variables and methods of measurement

Independent variable: Anti-TIF1γ.

- Analysed by ELISA (absorbance units) and confirmed by immunoblot.

- Positive anti-TIF1γ value is established from a cut-off point on ELISA at 0,209 absorbance units which is later confirmed by a positive immunoblotting result.(18)

- Negative anti-TIF1γ value is given by an ELISA value below 0.209 or an ELISA value above 0.209 which later has a negative immunoblotting result(18).

- Change form a positive anti-TIF1γ value to a negative anti-TIF1γ is considered when a patient has a positive anti-TIF1γ value in a serum sample determination but in any of the following determinations shows up a negative anti-TIF1γ value.

- Change form a negative anti-TIF1γ value to a positive anti-TIF1γ is considered when a patient has a negative anti-TIF1γ value in a serum sample determination but in any of the following determinations shows up a positive anti-TIF1γ value.

- Persistent positive anti-TIF1γ values are considered when a patient has positive anti-TIF1γ values persistently along the periodical determinations.

- Persistent negative anti-TIF1γ values are considered when a patient has negative anti-TIF1γ values persistently along the periodical determinations.

Expert nurses from each hospital will obtain blood sample. In case the patient uses a health care centre other than Hospital Universitari Sant Pau, the serum sample will be taken at his/her health care centre and send under the appropriate ambient conditions (packed into a dry ice kit –solid Carbon Dioxide-CO_2) sent by urgent messenger service to the Department of Immunology laboratory of Hospital Universitari Sant Pau where the analysis will be carry out. The serum extraction will be done every four months since the moment of inclusion in the study. It will be performed until cancer appears or

during five years if there is no cancer occurrence. However, the serum will be stored at -80º C in the Hospital Universitari Sant Pau and the analysis will be performed every six month in order to make the most of the 96-well ELISA plates.

The antibody presence will be analysed at the laboratory from the immunologic department by home-made ELISA technique and confirmed by immunoblot technic (IB) using commercial recombinant TIF1γ as antigen. These techniques have been proved to have excellent agreement with immunoprecipitation (IP), the gold standard(18). (Annex 5)

Dependent variable: cancer occurrence: presence of cancer / no presence of cancer.

- *When the patient has negative anti-TIF1γ values on the first serum analysis*: The assessment of cancer will be performed as it is done in the clinical practice. The patient will be set to date every four month to his/her physician where a detailed anamnesis and physical examination will be performed. A week before the appointment with his/her physician, she/he will have an appointment with the nurse in order to have some blood extraction that will be taken in the hospital. Thus, the physician will be able to assess the blood analysis results just a week after. The blood analysis will include a full blood account, biochemistry profile, ESR, CRP and creatine phosphokinase. However, other tests should be performed if there is any evidence suggesting the presence of cancer that requires to be assessed.

- *When the patient has positive anti-TIF1γ values on the first serum analysis:* the assessment of cancer will be performed following the same protocol as the mentioned above but a whole-body PET/TC scanning using [18F] fluorodeoxyglucose (FDG-PET/TC) will be performed annually during the five years of follow-up. A classical cancer screening consisting on a thoracoabdominal computed tomography, tumor marker analysis and a mammography an gynaecological examination with transvaginal

ultrasonography for women can be performed in place of the FDG-PET/TC(7). However, other tests should be performed if there is any evidence suggesting the presence of cancer that needs to be assessed.

Co-variables or Confusion variables:

At baseline, the identification and sociodemographic data will be collected by a questionnaire: name, date of birth, gender (male/female), home address and contact telefon number.

- *Variables that may be associated to have a higher risk of cancer in IID*(22) *and clinical characterization variables:*

    1. Skin lesions: heliotrope rash, Gottron's papules, perionychia erythema, truncal erythema, cutaneous vasculitis, cutaneous necrosis, shawl sign, V sign. (Present/ not present).

    2. Immunosuppressive medications: taken/not taken.

    3. Erythrocyte sedimentation rate: mm/h.

    4. Creatine kinase: IU/L.

    5. Age at myositis onset: years.

All the mentioned variables will be assessed by the physician of each patient and will be evaluated every four months when the patient is set to date with his physician.

    6. Capillary damage evident on muscle biopsy: (present/not present). Physicians from the anatomic-pathologic laboratory from each hospital where the biopsy has been performed will assess it.

7. Time from the appearance of a positive anti-TIF1γ value to the occurrence of cancer in patients whose first anti-TIF1γ values were negative: each physician will collect this data once cancer occurs. It will be assessed in days.

All of the following variables will be assessed by means of the clinical interview performed on the baseline date by each physician:

- *General cancer associated variables*(8)*:*

1. Alcohol use: (not a hazardous use/hazardous use) obtained by the three first questions of the AUDIT questionnaire(35). Hazardous alcohol abuse considered ≥ 8 score. (Annex 6)

2. Tobacco use: measured in pack year. It will be calculated by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked.

3. Age: it will be measured in years.

4. Obesity: considering obese when BMI ≥ 30 kg/m^2/ not obese when BMI <30 kg/m^2(8). Height and weight will be measured wearing only underwear and without shoes.

5. First-degree relative (parent, child or sibling) with cancer antecedents: yes/no.

6. Personal cancer antecedent earlier than three years before DM diagnose: yes/no.

- *Specific cancer associated variables:*

• Variables associated with lung cancer:

1. Asbestos exposition: yes/no.

- Variables associated with breast and/or ovary cancer:

  1. Menarche: ≤12 years old/ >12 years old.

  2. Parity: nulliparous /first full-term pregnancy by age 18 years/ first full term pregnancy by age older than 18 years.

  3. Menopause: ≤ 42 years old/ between 42 and 52 years old / ≥ 52 years old.

  4. Hormone replacement therapy: received/ not received.

- Variables associated to stomach cancer:

  1. Actual or present *H. pylori* infection: present / not present. It will be evaluated by anamneses. In case the patient presents dyspepsia symptoms, an urea breath testing(8) will be performed on the following medical appointment in order to do the possible diagnoses.

- Variables associated to colorectal cancer:

  7. Inflammatory bowel disease (ulcerative colitis or Crohn's disease): yes/no. The physician must check the personal antecedents on the clinical history to corroborate the information.

Nurses and physicians will introduce all the data in a common database.

5.7. Statistical analysis

Univariate descriptive analysis

The results will be expressed as percentages for categorical variables. Continuous variables will be expressed as mean +/- SD if they are normally distributed or median (quartiles) if they are not.

Bivariate analysis

Proportions will be compared with X^2 and Fisher test.

Multivariate analysis

It will be stratified by age and sex using a Cox regression.

All analyses will be performed using Statistical Package for the Social Sciences (SPSS Statistics) 22.0 version

5.8. Study limitations and strengths

Among the study limitations it is of great importance that we are dealing with a rare disease with a low incidence. At the same time, DM is a systemic disease that implicates the involvement of several medical specialities for the patient management. It can occur that patient may not be admitted in the Internal Medicine Department but he may in other departments such as the Neurologic or Dermatologic Departments. All these facts lead to a major difficulty to obtain the required sample size. In order to solve this problem we perform a multicentre study. Even though we will only diagnose patients who are admitted in the Internal Department, we will have enough population to demonstrate our hypotheses. As we are dealing with a rare disease there will be heterogeneity on the disease knowledge between the different researchers. In order to overcome this weakness a course aimed to train all the professionals who will take part on the study will be performed in the following month after the GEAS Annual

Meeting. Moreover, every year there will be an in-person meeting among all the participant researchers in order to solve problems that may materialize so as to increase the internal validity of the study.

Another important weakness of the study is the sample calculation. Our study has never been performed before so we are working with different groups of exposed and non-exposed patients whose proportion we ignore. However, we made an accurate calculation and estimation by using the anti-TIF1γ prevalence from a Spanish study performed in Hospital Universitary Vall d'Hebron. Thus, the patients composing the sample will probably have similar anti-TIF1γ prevalence on the first determination of the antibody and similar exposition to the mentioned study. The handicap produced by the lack of similar studies to perform the sample calculation reinforces our choice to perform this study as it will be the first time anti-TIF1γ is studied longitudinally. The design of this pilot study will show evidence of the antibody behaviour towards the cancer occurrence giving more information of its possible causality relationship.

Since our study takes five years of follow up in a big proportion of the patients (once cancer occurrence appears no more anti-TIF1γ will be needed) we expect that a significant number of patients will be missing. To avoid any selection bias, missed patients and patients who do not agree to participate on the study will be analysed afterwards.

Finally, many confusion variables may not be controlled because cancer is associated with a large number of variables. As ours is a pilot study, we consider it important to assess the relationship between the dependent and the independent variable controlling some of the confusion variables, encouraging the following studies to improve our variables weaknesses.

6. ETHICAL CONSIDERATIONS

Clinical Research Ethics Committee (CEIC) of each participating center will evaluate the project and approval must be obtained. This study is designed in accordance with the medical ethics requirements defined on the WMA Declaration of Helsinki of Ethical Principles for Medical Research Involving Human Subjects (last revision in October 2013)(36).

The information will be confidential, and the anonymity of the patients participating in the study will be protected under the Spanish legal regulations on confidentiality of personal data (Ley Orgánica 15/1999 de 13 de Diciembre de Protección de Datos de Carácter Personal [LOPD]).This study will also be protected under the Ley 14/2007, de 3 de julio, de investigación biomédica.

Every participant will be informed and given the signed consent when they agree to participate in the study, authorizing the use of their personal data for further investigations. (Annex 7)

7. CLINICAL IMPACT

If our hypotheses are demonstrated, it will be justified to perform periodical analysis of the anti-TIF1γ which will allow us to predict more accurately which patients will finally develop the cancer and when.

8. WORK PLAN

8.1. Research group

The group will be composed by two principal researchers (PR) Albert Selva O'Callaghan form Hospital Universitari Vall d'Hebron, and Anna Maura Prat, medical student from University of Girona. Additionally, there will be collaborating researchers (CR), represented by physicians who accept to participate in the study from different hospital of Spain. A monitor (M) will be the responsible to coordinate the different investigators. Moreover, there will be the Laboratory staff (LS) from the Hospital Universitari Sant Pau and the nursery team (N) from each hospital. Finally there will be a statistical specialist (SS).

8.2. Chronogram

Task 1: coordination phase, development of theoretical framework

- Literature review (PR)

- Study research proposal design (PR): The protocol of the study will be written down.

- Evaluation of the study research protocol (PR): The study will be evaluated by the CEIC of the main center, Hospital Universitari Vall d'Hebron.

- Recruitment of collaborating researchers (PR and CR): The PR will present the study protocol to the "Grupo para el estudio de las Enfermedades Autoinmunes Sistémicas (GEAS) de la Sociedad Española de Medicina Interna (SEMI)" and a physician of each hospital will be offered to participate in the study.

TASK 2: data collection

- In-person meeting (PR, CR and M): a course will be given in order to instruct all the collaborating researchers, as well as a folder with information about the aims of the study, the participants and the tests to be performed.

- Patient's recruitment (PR and CR): non-probabilistic consecutive sampling. Patients will be recruited until the sample size of each group is achieved. The time expected is supposed to take three years.

- Data collection (PR, CR, N and LS): the data is going to be collected until the cancer occurrence or, if it does not happen, once five years of follow-up have passed.

- Computer processing data (PR, CR and N): data entry.

- In-person meeting (PR, CR and M): They will be performed yearly to solve problems which may materialize so as to increase the internal validity of the study.

- Evaluation of correct data collection (M): the monitor will assess that each of the researchers of the study performs the data collection properly.

TASK 3: STATISTICAL ANALYSIS

- In-person meeting (PR and SS)

- Statistical data analysis (SS)

- Inspection of statistical analysis (SS and PR)

TASK 4: ANALYSIS OF THE RESULTS

- In-person meeting (PR, SR and SS): presentation and discussion of the statistical analysis.

- Statistical analysis of the results (PR)

- Discussion (PR)

- Conclusion (PR)

TASK 5: FINALITZATION AND RESULSTS PUBLICATION

- Final report elaboration (PR)

- Presentation in GEAS Annual meeting and National congresses (PR)

Gantt Chart

Month columns per year group: 2015 = S O N D; 2016/2017/2018/2019-2023/2024 = J F M A M J J A S O N D.

ACTIVITIES	2015 (S O N D)	2016 (J F M A M J J A S O N D)	2017 (J F M A M J J A S O N D)	2018 (J F M A M J J A S O N D)	2019 -2023 (J F M A M J J A S O N D)	2024 (J F M A M J J A S O N D)
TASK 1: COORDINATION PHASE, DEVELOPMENT OF THEORETICAL FRAMEWORK						
1. Literature review	■ ■ ■ ·					
2. Study research proposal desing	· · ■ ■					
3. Evaluation of the study research protocol	· · · ■					
4. Recruitement of collaborating researchers	· · · ■					
TASK 2: DATA COLLETION						
5. In-person meeting	· · · ■					
6. Patients recuitment		■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■	■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■	■ ■ ■ ■ ■ ■ ■ ■ ■ ■ · ·		
7. Data collecting		■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■	■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■	■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■	■ ■ ■ ■ ■ ■ ■ ■ ■ ■ · ·	
8. Computer procesing data		■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■	■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■	■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■	■ ■ ■ ■ ■ ■ ■ ■ ■ ■ · ·	
9. In-person meeting		· · · · · · ■ · · · · ·	· · · · · · ■ · · · · ·	· · · · · · ■ · · · · ·	· · · · · · ■ · · · · ·	
10. Evaluation of correct data colletion		■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■	■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■	■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■ ■	■ ■ ■ ■ ■ ■ ■ ■ ■ ■ · ·	
TASK 3: STATISTICAL ANALYSIS						
11. In-person meeting						■ · · · · · · · · · · ·
12. Statistical datal analysis						■ ■ · · · · · · · · · ·
13. Inspection of statistical analysis						· · ■ ■ · · · · · · · ·
TASK 4: ANALYSIS OF THE RESULTS						
14. In-person meeting						· · · ■ · · · · · · · ·
15. Statistical analysis of the results						· · · · ■ · · · · · · ·
16. Discusion						· · · · · ■ · · · · · ·
17. Conclusion						· · · · · · ■ · · · · ·
TASK 5 : FINALIZATION AND RESULTS PUBLICATION						
18. Final report elaboration						· · · · · · · ■ · · · ·
19. Presentation in National congresses						· · · · · · · · ■ ■ ■ ■

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9. BUDGET

Although we already have most of the needed infrastructure for the correct development of the study, we will need to analyse the anti-TIF1γ (30 €) periodically every four months during a maximum of 5 years per patient:

- Anti-TIF1γ: 15€ x 3 times per year x 5 years =225 €

- MRW messenger services: 20 € (mean) x 3 times per year x 5 years = 300 €

- Dry ice kit (solid Carbon Dioxide-CO_2) of 2 kg = 25€ x 3 times per year x 5 years = 375 €

- Total per patient = 900 €

- Total = 900 x 118 patients = 106,200€ (after cancer occurrence no more analysis of the anti-TIF1γ will be done. Thus, the money left will be given back).

- A statistician is needed to analyse all data. He will be paid 35/hour 30x35=1,050 €

- The monitor is estimated to cost 2,000 € x 8 years = 16,000 €

- Meeting-travelling expenses 250 € x 15 researchers x 8 meetings = 30,000 €

TOTAL = 153,250 €

10. ANNEXES

Annex 1

<u>Bohan and Peter's classification</u>(28)

1. Idiopathic polymyositis

2. Idiopathic dermatomyositis

3. Juvenile dermatomyositis or polymyositis

4. Dermatomyositis or polymyositis associated to cancer

5. Dermatomyositis and polymyositis associated to other connective tissue disease or overlap syndrome.

<u>Dalakas' histopathologic classification</u>(3)

1. Dermatomyositis

2. Polymyositis

3. Inclusion body myositis

<u>Idiopathic inflammatory myopathy's clinical classification</u>(9)

- PM

- DM

- DM sine myositis

- Juvenile PM and DM

- IBM

- CAM

- Connectivopathy associated myositis

- Eosinophilic myositis

- Granulomatous myositis

- Focal or nodular myositis

- Ocular or orbital myositis

Annex 2

Table from Gunawardena *et al.* (6)

Autoantibodies	Target autoantigen and function	Clinical phenotype	Autoantibody frequency, %	
			Adult IIM	JDM
Anti-ARS	ARS—intracytoplasmic protein synthesis	ASS	30–40	1–3
Anti-Jo-1	Histidyl			
Anti-PL-7	Threonyl	Myositis, mechanic's hands, Gottron's papules, arthritis,		
Anti-PL-12	Alanyl	fever, RP, high frequency of interstitial pneumonia		
Anti-EJ	Glycyl			
Anti-OJ	Isoleucyl			
Anti-KS	Asparaginyl			
Anti-Ha	Tyrosyl			
Anti-Zo	Phenylalanyl			
Anti-SRP	SRP—intracytoplasmic protein translocation (six polypeptides and RNP 7SLRNA)	Acute onset necrotizing myopathy (severe weakness, high CK); may be refractory to treatment	5	<1
Anti-Mi-2	Helicase protein—nuclear transcription (forms the NuRD complex)	Adult DM and JDM (hallmark cutaneous disease, milder muscle disease with good response to treatment)	<10	<10
Anti-p155/140	TIF1-γ (p155)—nuclear transcription + cellular differentiation	CAM in adult DM; severe cutaneous disease in adult DM and JDM	13–21	23–29
Anti-p140	Likely to be NXP-2—nuclear transcription + RNA metabolism	JDM with calcinosis	NA	23
Anti-SAE	SAE—post-translational modification (targets include transcription factors)	Adult DM; may present with CADM first	5	NA
Anti-CADM-140	Intracytoplasmic MDA5—innate immune responses against viral infections	CADM; rapidly progressive interstitial pneumonia	Overall—unknown	NA

SRP: signal recognition particle; NuRD: nucleosome remodelling histone deacetylase; TIF1-γ: transcriptional intermediary factor 1-gamma; NXP-2: nuclear matrix protein NXP-2; SAE: small-ubiquitin-like modifier activating enzyme; MDA5: melanoma-differentiation associated gene 5; CAM: cancer-associated myositis; NA: not applicable/no data.

Annex 3

Sontheimer's criteria

Amyopathic DM (37)
Subset of DM patients characterized by biopsy-confirmed hallmark cutaneous manifestations of classical DM occurring for 6 months or longer with no clinical evidence of proximal muscle weakness and no serum muscle enzyme abnormalities. If more extensive muscle testing is carried out, the results should be within normal limits (if such results are positive/abnormal, the patient can be classified as having ''hypomyopathic dermatomyositis''). Exclusion criteria for amyopathic DM include: 1. Treatment with systemic immunosuppressive therapy for two consecutive months or longer within the first six months after skin disease onset (such therapy could prevent the development of clinically significant myositis). 2. Use of drugs known to be capable of producing isolated DM-like skin changes (e.g., hydroxyurea) at the onset of cutaneous changes

Annex 4

Troyanov *et al.* criteria(30)

Overlap myositis: Myositis with at least one clinical overlap feature and/or an overlap autoantibody*
*Overlap autoantibodies encompass antisynthethases (Jo-1, PL-7, PL-12, OJ, EJ, KS), SSc-associated autoantibodies (SSc-specific antibodies: centromeres, topo I, RNA-polymerases I or III, Th; and antibodies associated with SSc in overlap: U1RNP, U2RNP, U3RNP, U5RNP, Pm-Scl, Ku), and other autoantibodies (SRP, nucleoporins).

Bohan and Peter's criteria (28,29)

Childhood dermatomyositis (or polymiositis) associated with vasculitis: which also follows the Bohan and Peter's criteria. This category is justified because of the widespread vasculitis that may occur with involvement of gastrointestinal tract, skin and subcutaneous tissues, and other sites.

Criteria of Needham *et al.* (31)

Inclusion body myositis: Characteristic features *Clinical features* • Duration of illness >6 months. • Age at onset >30 years. • Slowly progressive muscle weakness and atrophy: selective pattern with early involvement of quadriceps femoris and finger flexors, although can be asymmetric. • Dysphagia is common.

Laboratory features

- Serum creatine kinase concentration might be high but can be normal.
- Electromyography: myopathic or mixed pattern, with both short and long duration motor unit potentials and spontaneous activity.

Muscle biopsy

- Myofiber necrosis and regeneration.
- Endomysial mononuclear cell infiltrate (of variable severity).
- Mononuclear cell invasion of non-necrotic fibers: predominately CD8+ T cells.
- MHC class I expression in otherwise morphologically healthy muscle fibers.
- Vacuolated muscle fibers (rimmed vacuoles).
- Ubiquitin-positive inclusions and amyloid deposits in muscle fibers.
- Nuclear and/or cytoplasmic 16–20 nm filamentous inclusions on electron microscopy.
- COX-negative fibers (excessive for age).

Associated disorders

Inclusion body myositis usually occurs in isolation, but can be associated with:

- Other autoimmune disorders or connective tissue diseases.
- Occasional: HIV, HTLV-I, and hepatitis C infection.
- Rare: toxoplasmosis, sarcoidosis, post-poliomyelitis, amyotrophic lateral sclerosis.

Diagnostic categories

- Definite inclusion body myositis :
    - Characteristic clinical features, with biopsy confirmation: inflammatory myopathy with auto aggressive T cells, rimmed vacuoles, COX-negative fibers, amyloid deposits or filamentous inclusions and up-regulation of MHC-I expression. The presence of other laboratory features are not mandatory if the biopsy features are diagnostic
    - Atypical pattern of weakness and atrophy but with diagnostic biopsy features.

- Probable inclusion body myositis:
  - Characteristic clinical and laboratory features but incomplete biopsy criteria—eg, features of necrotizing inflammatory myopathy with T cell invasion of muscle fibers but absence of rimmed vacuoles, amyloid deposits, filamentous inclusions, and COX negative fibers.

- Possible inclusion body myositis
  - Atypical pattern of weakness and incomplete biopsy criteria.

Annex 5

Anti-TIF1γ ELISA:

Briefly, 96-well ELISA plates (NUNC, Kamstrup, Denmark) will be coated with 100 ng/well of purified recombinant protein encoding the longest TIF1γ isoform (OriGene, Rockville, Maryland, USA) diluted in phosphate-buffered saline (PBS) and left to stand overnight at 4ºC. Wells will be incubated for 1 h at room temperature (RT) with blocking buffer (10% non-fat dry milk in PBS). Then, Plates will be washed (HRP Wash, INOVA Diagnostic Inc, San Diego, California, USA), human serum samples diluted 1:100 in blocking buffer will be added in duplicate and plates will be incubated at RT for 1 h. After washing, horsearadish peroxidase-labelled goat anti-human IgG antibody (INOVA Diagnostic Inc) will be added to each well, and plates will be incubated for 1 h at RT and washed again. Colour development used TMB chromogen reagent for peroxidase (INOVA Diagnostic INC, SA), and absorbance at 450 nm will be determined and expressed as optical density units. The same high and low positive serum samples will be used as references in each essay(18).

Anti-TIF1γ immunoblot (IB):

Briefly, 5μg of purified recombinant protein encoding the longest isoform of TIF1γ (OriGene, Rockville, Maryland, USA) will be run on 4-12% polyacrylamide-sodium dodecyl sulphate minigels with 3-(N-morpholino) propanesulfonic acid running buffer, and western blotting will be performed using the Invitrogen NuPAGE (Carlsbad, California, USA) electrophoresis system over a nitrocellulose membrane. TIF1γ – transferred nitrocellulose will be vertically cut into strips and incubated for 1 h at RT in PBS with 0.05% Tween containing 3% non-fat dry milk (blocking fufer). Each strip will be incubated with a human serum sample diluted 1:100 in blocking buffer for 1h at RT. After washing, phosphate alkaline-labelled goat anti-human IgG antibody (1:2000; Dako, Glostrup, Denmark) will be added to each strip and strips will be incubated for 1h at RT. Colour development used phosphatase reagent (BCIP/NBT, Sigma-Aldrich, St. Louis, Missouri, USA). Based on signal intensity and blinded to knowledge of the ELISA results, immunoblots will be graded as negative, weak positive, or positive(18).

Annex 6

Questionnaire by Babor et al.(35)

The Alcohol Use Disorders Identification Test: Interview Version

Read questions as written. Record answers carefully. Begin the AUDIT by saying "Now I am going to ask you some questions about your use of alcoholic beverages during this past year." Explain what is meant by "alcoholic beverages" by using local examples of beer, wine, vodka, etc. Code answers in terms of "standard drinks". Place the correct answer number in the box at the right.

1. How often do you have a drink containing alcohol?

   (0) Never [Skip to Qs 9-10]
   (1) Monthly or less
   (2) 2 to 4 times a month
   (3) 2 to 3 times a week
   (4) 4 or more times a week

2. How many drinks containing alcohol do you have on a typical day when you are drinking?

   (0) 1 or 2
   (1) 3 or 4
   (2) 5 or 6
   (3) 7, 8, or 9
   (4) 10 or more

3. How often do you have six or more drinks on one occasion?

   (0) Never
   (1) Less than monthly
   (2) Monthly
   (3) Weekly
   (4) Daily or almost daily

   Skip to Questions 9 and 10 if Total Score for Questions 2 and 3 = 0

4. How often during the last year have you found that you were not able to stop drinking once you had started?

   (0) Never
   (1) Less than monthly
   (2) Monthly
   (3) Weekly
   (4) Daily or almost daily

5. How often during the last year have you failed to do what was normally expected from you because of drinking?

   (0) Never
   (1) Less than monthly
   (2) Monthly
   (3) Weekly
   (4) Daily or almost daily

6. How often during the last year have you needed a first drink in the morning to get yourself going after a heavy drinking session?

   (0) Never
   (1) Less than monthly
   (2) Monthly
   (3) Weekly
   (4) Daily or almost daily

7. How often during the last year have you had a feeling of guilt or remorse after drinking?

   (0) Never
   (1) Less than monthly
   (2) Monthly
   (3) Weekly
   (4) Daily or almost daily

8. How often during the last year have you been unable to remember what happened the night before because you had been drinking?

   (0) Never
   (1) Less than monthly
   (2) Monthly
   (3) Weekly
   (4) Daily or almost daily

9. Have you or someone else been injured as a result of your drinking?

   (0) No
   (2) Yes, but not in the last year
   (4) Yes, during the last year

10. Has a relative or friend or a doctor or another health worker been concerned about your drinking or suggested you cut down?

   (0) No
   (2) Yes, but not in the last year
   (4) Yes, during the last year

Record total of specific items here

If total is greater than recommended cut-off, consult User's Manual

Annex 7

ESTUDIO PERIODICO DEL ANTICUERPO ANTI-TIF1γ EN PACIENTES ACABADOS DE
DIAGNOSTICAR DE DM SIN CRITERIOS DE CAM

INFORMACIÓN PARA EL PACIENTE

La Dermatomiositis es un tipo de miopatía inflamatoria idiopática caracterizada por la presencia de inflamación en el tejido muscular y debilidad muscular y una afectación cutánea. Ésta está caracterizada por la presencia de un exantema periorbitario en heliotropo y la presencia de placas eritematovioláceas en los nudillos de los dedos (llamados nódulos de Gottron) y en el resto de áreas de extensión de las articulaciones, como las muñecas, los codos, las rodillas y otras zonas de foto exposición como el escote o la espalda. La dermatomiositis se considera una enfermedad sistémica ya que los pacientes afectos de ella también pueden tener comprometidos otros órganos como el tracto digestivo, el sistema respiratorio, las articulaciones o el sistema cardiovascular. Desafortunadamente, la dermatomiositis también está muy asociada una mayor aparición de cáncer, siendo aproximadamente tres veces más frecuente que en la población normal.

El anticuerpo anti-TIF1γ aparece prácticamente exclusivamente en pacientes con dermatomiosistis. Se ha encontrado una importante asociación entre este anticuerpo y la presencia de cáncer en pacientes con dermatomiositis. Los pacientes con un resultado negativo en el test tienen muy pocas probabilidades de tener cáncer. De modo que es útil en el diagnóstico de diferentes subgrupos de pacientes que van a tener un mayor riesgo de cáncer y otro con un riesgo muy bajo. En España, es posible analizar el valor de este anticuerpo mediante técnicas de laboratorio llamadas ELISA e immunoblot en el laboratorio del Departamento de Inmunología del Hospital Universitari Sant Pau de Barcelona.

Le invitamos a participar en nuestro estudio cuyo objetivo es analizar periódicamente los valores del anti-TIF1γ   en pacientes con dermatomiositis para estudiar dichos valores a lo largo de la enfermedad y observar su cambio a lo largo del tiempo para así conocer más específicamente su relación con el cáncer. Para estudiar su elegibilidad en este estudio se van a realizar las mismas pruebas que se llevarían a cabo en un inicio para realizar una práctica clínica normal, en la que se descarta la presencia de cáncer. Así, los participantes de nuestro estudio deberán ser pacientes con dermatomiositis acabada de diagnosticar sin tener presencia de cáncer en el momento del diagnóstico o en los tres años previos.

La duración de este estudio será de un máximo de cinco años. El procedimiento adicional al de la práctica clínica será el estudio de sus valores de anti-TIF1γ con una periodicidad de cuatro meses. De modo que en las extracciones periódicas de sangre para realizar los análisis generales de sangre se le recogerá   también, parte de la

muestra en otro recipiente para que así pueda ser analizado. Esta tarea se realizará en este mismo centro. Además tendrá un seguimiento periódico con su médico responsable una semana posterior a cada extracción de sangre. En estas visitas se valorará al paciente y se le informará de los resultados de los análisis generales de sangre realizados y si el paciente lo prefiere, se la informará también de los sus valores de anti-TIF1γ. La extracción de sangre es una prueba muy poco invasiva.

El principal beneficio en participar en este estudio es la posibilidad de obtener un mejor conocimiento de la relación entre la dermatomiosistis, del cáncer y del anticuerpo en cuestión. De este modo obtendremos más información sobre el posible uso de este anticuerpo como marcador de cáncer en estos pacientes. También dará más conocimiento de la fisiopatogenia de estas dos enfermedades que en un futuro podrán ayudar en el desarrollo de medicamentos dirigidos específicamente a su tratamiento.

Este estudio ha estado aprobado por el Comité de Ética e Investigación Clínica de cada cetro. Está diseñado respetando los requisitos éticos médicos definidos por la AMM en la Declaración de Helsinki de los principios éticos para las investigaciones médicas en seres humanos (última versión, diciembre del año 2013). El uso de sus datos personales será de forma confidencial y anónima, así, este estudio está amparado por la Ley Orgánica 15/1999 de 13 de Diciembre de Protección de Datos de Carácter Personal [LOPD]).También está emparado por la Ley 14/2007, de 3 de julio, de investigación biomédica.

El/la paciente tiene la total libertad de renunciar a la participación o continuación del estudio sin que esta decisión tenga ningún tipo de perjuicios en su seguimiento médico ni en ningún otro ámbito.

El/la paciente tiene total la total libertad de realizar todas las preguntas que le sean necesarias o consultar otro profesional siempre que lo desee.

Puede contactar con el equipo de investigación siempre que le sea conveniente contactando el correo electrónico y/o número de teléfono que le proporcionará su médico.

CONSENTIMIENTO INFORMADO

El Sr./La Sra.:……………………………………………………………………………………………ha estado informado de las finalidades e implicaciones de este estudio, ha podido realizar las preguntas que ha creído convenientes, y acepta participar en este estudio permitiendo que los investigadores conserven su material biológico para futuras investigaciones.

Como prueba de su conformidad firma a……………………………………………., el…… de……….. de 201…

Declaración del profesional de la salud médica de que ha informado debidamente al participante del estudio.

Nombre:………………………………………………………….
Firma:…………………………………………………………………