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Official Title: Relevance of Monitoring Blood and Salivary Levels of Drugs Used in Rheumatic Autoimmune Diseases

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THEMATIC PROJECT

ASSESSMENT OF RELEVANCE OF BLOOD LEVELS OF DRUGS IN THE MONITORING RHEUMATIC AUTOIMMUNE DISEASES: SAFETY/ EFFECTIVENESS OF THERAPY, DISEASE ACTIVITY AND ADHERENCE TO THERAPY

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Summary

No drug treatment is completely free of risk and lack of response, adverse events and poor adherence may affect its effectiveness. There is also a large inter-individual variability in response to treatments with regard to efficacy and toxicity, and for many drugs, there is also a period of weeks to months to establish its efficacy. Within this context, this project aims to evaluate the importance of monitoring blood levels and salivary drug used in rheumatic autoimmune diseases in the monitoring of adherence to therapy. In addition, this project intends to use the monitoring of drug levels, based on pharmacokinetic studies and pharmacokinetics/pharmacodynamics modeling, to broaden the understanding of the possible cellular, tissue and immunological mechanisms involved in efficacy and adverse effects of these drugs with the prospect of reducing the damage and maintain therapeutic efficacy. The high-performance liquid chromatography (HPLC) coupled to mass spectrometry, which will be used to measure hydroxychloroquine, thalidomide, glucocorticoids levels, is considered the gold standard technology to qualitative and quantitative analysis of drugs in blood and its comparison with the dosage in the saliva is an improvement in terms of simplification of the process. The implementation of this methodology dedicated to research in our center, with the necessary training of human resources, will enable the standardization and availability of this advanced technology to other muldisciplinary projects

in various areas of science. For biological agents the focus will be on the understanding the loss of efficacy and the possible role of anti-TNF antibodies using ELISA capture methodology. This thematic project will be divided into four sections with their respective sub-projects according to the medications that will be studied: hydroxychloroquine, thalidomide, biologic agents and glucocorticoids.

Keywords: hydroxychloroquine, thalidomide, anti-TNF, glucocorticoids.

Introduction

No drug therapy is completely risk-free, and the values associated with non-response or adverse effects may exceed the cost of therapy. There is also great interindividual variability in the response to treatments with regard to efficacy and toxicity and, for many drugs, there is also a period of weeks to months for the establishment of this efficacy (Clark et al., 1993). On the other hand, in a recent study a gap was reported between the success rates of current treatments and those believed to be viable (Agency for Healthcare Research and Quality,2003). This difference has been attributed in part to the patient's poor adherence to the recommended treatment (Agency for Healthcare Research and Quality, 2003); Pathman et al., 1996). Poor adherence to medication is common (Osterberg & Blaschke, 2005; World Health Organization, 2003) and studies consistently showed that 20% to 30% of drug prescriptions are not adequately filled and that approximately 50% of chronic disease medications are not used as prescribed (Peterson et al., 2003; Haynes et al., 2008). This poor adherence has dramatic effects on health. In the United States, it is estimated that this factor was a direct cause of about 125,000 deaths as well as at least 10% of hospitalizations poor adherence is also related to a substantial increase in morbidity and mortality. The cost of non-adherence was estimated for the U.S. health system between $100 billion and $289 billion annually (Viswanathan et al., 2012).

Adherence is a parameter generally defined as the percentage of prescribed doses of medications that are actually taken by patients over a specified period (Haynes et al., 2002; Shishov et al., 2005). Although poor adherence has negative effects on the prognosis of the patient (Uribe et al., 2004), there is no universally accepted criterion for measuring medication adherence and, its application in daily clinical practice is generally very difficult. Assessment of adherence by tablet counting is a complex task, while electronic dosing systems are expensive. The data of the number of patients, although readily available in the clinic, as already mentioned, are prone to inaccuracy (Finney et al., 1993; Wagner et al., 2001). Several questionnaires of self-reports of adherence to medication were evaluated, among them, the CQR(Compliance - Questionnaire - Rheumatology) (Klerk et al., 2003) and the Medication Adherence Self-report Inventory (MASRI) (Walsh et al., 2002; Koneru et al., 2007). However, its use in clinical practice has been quite limited. Pharmacological drug exposure tests seem a promising solution not only to the issue of adherence but would also meet dose adjustmentand to a single specific patient, which is a growing clinical and financial concern. Alternatively, recent studies with a very limited number of drugs suggest that monitoring drug concentrations can also be performed in saliva with the advantage of determining the concentration of its free form and because due to easier and less invasive sample collection (Dwivedi et al., 2015a). With respect to this, monitoring in blood and saliva as evidenced in antiepileptic therapy proved to be of great value in order to individualize and optimize the management of drugs in patients with epilepsy (Johannessen and Landmark, 2008; Dwivedi et al., 2015b). Drugs are associated with plasma proteins such as albumin and alpha-glycoprotein in different proportions. Since the 1970s, studies have shown that the free fraction of the drug corresponds to the active form responsible for its therapeutic and toxic effects. Thus, the determination of the concentration of the drug in oral fluid may have a higher predictive value of clinical prognosis and toxicity (Ghareeb and Akhlaghi, 2015). Several conditions affecting the liver and kidneys alter the proportions between free and bound fractions, altering the therapeutic efficiency of the drugs. However, few studies have explored the potential to simultaneously monitor the free and total fraction and correlate with the clinical data of patients. The equivalence of free plasma levels of some drugs has already been demonstrated with those found in saliva

and tears matrices. The relationship between the concentration of the drug's free form in saliva and plasma presents minimal intra/inter individual variability and is independent of time and dose. Nevertheless, these matrices, other than plasma, were little explored due to the lack of sensitivity of the available methodologies. The recent development of high-performance tandem mass spectrometers has made possible to determine concentrations of the order of pg/mL, enabling the monitoring of reduced values of free drugs in reduced volumes of alternative matrices. In the area of Rheumatology, there are few studies and are restricted to blood dosage and there is only one study that used saliva for glucocorticoids dosage. Saliva dosage offers a good alternative for serum dosage, because it is less invasive and because it has, in general, a good correlation with the free drug in serum (Ruiter et al., 2012).

In systemic lupus erythematosus (SLE), tests are restricted to blood measurement and there is only one open study with 50 patients demonstrating that clinical responses with the azathioprine (AZA) seem to occur at 6-TGN levels below the target established for IDI (Cuffari et al., 2001; Goldenberg et al., 2004) and it has been reported that the dosages of metabolites of azathioprine may provide a rational approach to safety (Askanase et al., 2009). For rheumatoid arthritis (AR), it has been demonstrated that levels of the active metabolite of MTX, PG-MTX, can be measured in patients and are correlated with disease activity (DAS28),but there are no data on adherence or correlation of PG-MTX levels with adverse events (Angelis-Stoforidis et al., 1999; Dervieux et al., 2004; Dervieux et al., 2006; Stamp et al., 2009; Stamp et al., 2010).

For antimalarials, there is only one study in adults with SLE (Costedoat-Chalumeau et al., 2006) and there are no blood control studies in juvenile lupus. These drugs have an exceptional immunomodulatory role in SLE and their withdrawal is associated with disease outbreaks (Tsakonas et al., 1998). Today, there are recommendations for its continuous use in all patients with SLE, except for those who have formal contraindications, including during pregnancy. In fact, in recent years, additional beneficial effects of hydroxychloroquine on cardiovascular complications, glycemic control, dyslipidemia, thrombotic events and mortality has been demonstrated (Costedoat-Chalumeau et al., 2014).

The issue of non-adherence for drugs with continuous use in SLE is a difficult problem to measure and can reach 76% of cases, bringing serious consequences for the patient related to changes in prescriptions (Costedoat-Chalumeau et al., 2013). In this context, it is relevant that some studies have shown that plasma dosage of hydroxychloroquine is a reliable parameter for adherence because the drug has a long half-life and can be detected in blood tests.

In adult SLE, it has been reported that HCQ blood concentrations below 1000 ng/mL are associated with disease activity, it is a strong predictor of exacerbation in the following six months (Costedoat-Chalumeau et al., 2006). It is emphasized, however, that the concentrations of HCQ in the total blood varied greatly among the patients in this study, despite the prescription of the same daily dose. In this study, however, the evaluation of lupus activity was global and did not focus on renal involvement, and it was only mentioned that 10 patients had active nephritis. Other studies are therefore necessary to define blood levels of HCQ that will be predictors of outbreaks of renal activity, whose involvement is present in more than 50% of patients and which is the greatest therapeutic challenge of the disease. In this context, it is interesting that the use of this drug is associated with higher renal survival (Fessler et al., 2005) together with a better response to standard treatment with mycophenolatote mofetil in patients with SLE (Kasitanon et al., 2006), reinforcing the importance of an adequate control of its use in this condition. It is also relevant in the context of treatment that antimalarials have a long half-life of approximately 40 days and there is a delay to reach plasma concentration with a therapeutic effect expected only after 3 months or more of treatment (Rodriguez-Caruncho et al., 2014). Monitoring the level of this medication, which was established as at least 1000 ng/mL, would certainly be very useful in determining whether a higher attack dose would allow this therapeutic effect to be achieved more quickly. In addition, monitoring HCQ levels would allow assessing whether a reduction in the weekly HCQ dose

after a prolonged period of treatment with tissue depot of the drug would be possible to avoid the most feared side effect that is retinopathy with loss of vision. This side effect is associated with the cumulative dose of an (Wolfe et al., 2010; Jallouli et al., 2015).

Ophthalmological monitoring of the early state of the retinian toxicity of chloroquine and hydroxychloroquine is critical for the prevention of vision loss, since the progression of structural and functional deficits may occur even after cessation of therapy with these drugs (Michaelides et al., 2011). The new recommendation of the American Ophthalmology Association (2011) determines that ophthalmological control of the use of antimalarial should necessarily include complete ophthalmologic examination under mydriasis and automated visual field testing Humphrey in protocol 10-2 (campimetry) and at least one more objective examination such as multifocal electroretinogram, spectral domain optical coherence tomography (SD-OCT), autofluorescence or multifocal electroretinography (mfERG) (Marmor et al, 2011). There is some evidence showing that the detection of early functional loss associated with the use of these drugs may precede structural changes (Marmor et al., 2014; Greenstein et al., 2015). However, it cannot be excluded that these observations are based on tests with false-positive results, or that any signs of retinal toxicity may not be detected, since the visual deficits in these cases result from retinal pigment epithelium damage caused by the deposit of antimalarials (Bernstein et al., 1991). New technologies, SD-OCT, with the realization of a quantitative analysis of the retinian external layers, contribute to better diagnostic accuracy (Greenstein et al., 2015). Our objectiveis to determine the mechanism of HCQ-induced injury through an evaluation of the correlation of functional and structural damage of the retina, evaluated by central visual field examination (campimetry) and SD-OCT measurements, with segmentation of the outer layer of the retina (pigment epithelium).

Another interesting aspect related to antimalarial is that the mechanism of its beneficial effect as an immunomodulator has not yet been fully clarified, but it is believed to be correlated with the blockade of toll-like receptors (TLR-7 and -9) in dendritic cells and inhibition of alpha interferon production, which plays a crucial role in the pathogenesis of SLE. In fact, an in vitro study observed that the use of hydroxychloroquine inhibits the production of interferon-alpha and TNF by plasmocytoid dendritic cells of SLE patients in european population and, apparently, this inhibition is more effective for TLR-9 than for TLR-7 (Sacre et al., 2012). However, there is still a need to determine the possible correlation between the level of antimalarial and the degree of inhibition of the two different receptors.

Thalidomide is another immunomodulatory agent used to treat the cutaneous activity of SLE and LEC, even in patients refractory to other medications (Knop et al., 1983; Kyriakis et al., 2000; Coelho et al., 2005; Cuadrado et al., 2005). Its high efficacy associated with a very rapid response of the cutaneous lupus, made thalidomide a first choice drug in patients without formal contraindication related to teratogenesis. The most common adverse effects include peripheral polyneuropathy. In a study of lupus patients treated with thalidomide (25 to 100 mg/day), peripheral polyneuropathy occurred in 27% of patients (Cuadrado et al., 2005).

Thalidomide acts mainly by inhibiting the synthesis of TNF-α and modifying the expression of the adhering molecules on the surface of endothelial cells (Nakamura et al., 2007; Kuhn et al., 2011). Reinforcing this possibility, it has been demonstrated that TNF-α and IFN-1 are increased in the skin lesion of lupus (Yu et al., 2013). In addition, thalidomide seems to inhibit keratinocyte apoptosis induced by ultraviolet B radiation (Lu et al., 2003), another relevant aspect in the pathogenesis of cutaneous lupus (Reefman et al., 2006). In order to better understand the mechanisms involved in this therapeutic response, we intend to evaluate whether plasma levels of TNF-α and tissue expression of this cytokine would be relevant parameters for the action of thalidomide in the cutaneous manifestation of SLE.

The neurotoxicity mechanism of this drug is also unclear and there is work suggesting that thalidomide could have a direct toxic effect on neurons of the posterior root ganglion (Giannini et al., 2003). In this respect, the recent description that thin fiber neuropathy is associated with anti-TNF-therapy, with clinical findings and skin biopsy suggestive of posterior dorsal root involvement in patients

with AR (Birnbaum & Bingham, 2014), reinforces this possibility. We speculate that this same mechanism would be involved in the neurotoxicity of thalidomide in SLE. This finding would have great clinical relevance, because the diagnosis of this complication still depends on electroneuromyography, which is an expensive, time-consuming, painful and operator-dependent. Skin biopsy is an interesting alternative because it is a low-cost, fast, low-risk and easily accessible procedure that would open the prospect of performing a more accurate and early diagnosis of neuropathy, besides providing histopathological characteristics of the process of injury of these fibers.

Within this same line, one of the greatest advances in the treatment of rheumatic diseases was the emergence of biological therapy using immunological TNF blockers (anti-TNF antibodies). However, throughout treatment with these biological drugs, some patients develop anti-drug antibodies (ADA), which compete with their anti-TNF action via blocking of their binding site, reducing the therapeutic effect of these biologics. This immunogenicity seems to be related to numerous clinical impacts and adverse events, including reactions related to infusion, autoantibody production and even drug-induced lupus. The clinical usefulness of detecting the presence of these antidrug antibodies concomitantly with the determination of serum levels of the biological agent in the first months of therapy was recently published in the Lancet journal in 2015 only for rheumatoid arthritis, where it was shown that the dosage at three months is predictive of therapeutic response at 12 months (Jani et al., 2015). However, the biggest challenge in the treatment of these patients is long-term management, where a significant number of patients require the exchange of biological agents after an effective initial response. In such cases, there are several dilemmas that include exchange for another biological of the same class, drug class change, reduction of the range or increase of dose. For this group of patients, which we call 'switchers', the longitudinally evaluation of antidrug antibodies seems to be even more relevant and the only study in spondyloarthritis evaluated only the first anti-TNF and did not analyze the subgroups of disease that is known to have a distinct therapeutic response characteristic, not allowing a precise conclusion about the findings. There are no data on JIS yet. On the other hand, the evaluation of antidrug antibodies (ADA) and serum levels of the biological agent anti-TNF in patients with good clinical response could also enable the reduction of the dose or increase of the interval between applications, reducing costs and the risk of toxicity.

Our Discipline has a biorepository of plasma of patients monitored since the creation of our Biological Center in 2007 with the support of a thematic project granted by FAPESP. In this Center, we have 110 patients with spondyloarthritis (AS) who presented therapeutic failure with the need for dose change or exchange of biological agent (switchers). All have biological samples obtained in the long-term follow-up within a collection protocol accompanied with clinical, laboratory and imaging data. This biorepository provides us with an opportunity to define in spondyloarthritis the relevance of antidrug antibodies in the switchers group, compared to non-switchers. It will also be possible to perform for the first time the concomitant longitudinal analysis of the production of antidrug antibodies and autoantibodies associated with rheumatologic autoimmune diseases. This would make it possible to understand whether the mechanism involved in the production of antidrug antibodies (ADA) is an immune response directed only to anti-TNF or is the result of broader polyclonal activation involving the production of autoantibodies. In addition, it was observed the concomitant use of Disease-modifying anti-rheumatic drugs (DMARDs) in RA can reduce the immunogenicity directed against anti-TNF (Krieckaert et al., 2010) and this aspect was not determined in AS.

Prednisolone and prednisone are drugs widely used in the induction and maintenance of immunosuppressive regimens in rheumatologic diseases. Prednisolone is the active part of the drug, while prednisone is a prodrug and an inactive metabolite of prednisolone. There is only one open study that evaluated the blood level of this drug with a single dose in juvenile SLE. This study with only eight patients demonstrated that there is a great individual variability and a possible association of the dose with disease activity (Sagcal-Gironella et al., 2011). Patient adherence for these drugs is particularly difficult due to the aesthetic changes (acne, stretch marks and obesity) related to their use. However, the

efficacy and rapid onset of action related to these drugs allow the treatment of severe activity and acute exacerbations (flares), being used either for induction of remission, or as maintenance therapy in SLE and JSLE (Mukhtyar et al., 2009; Mukhtyar et al., 2009a).

Despite the established role of glucocorticoids (GCs) in controlling inflammation (Karan et al., 1995), its potential adverse effects (AEs) include cardiovascular, infectious, gastrointestinal, psychological, behavioral, endocrine, metabolic, dermatological, musculoskeletal and ophthalmological manifestations (Hoes et al., 2007; Wang et al., 2009; Carla et al., 2013; Han et al., 2013). GC is today the leading cause of damage in SLE and is responsible for more than 60% of permanent lesions in these patients (Glad man et al., 2003). In bone, it is known that, in patients using GC, fracture can occur even with normal bone mineral density assessed by densitometry, and the mechanisms of this condition have not yet been clarified. In this sense, we intend to evaluate the bone structure and volumetric density, currently possible with the use of HRpQCT, a device acquired by our group with multi-user FAPESP project. This equipment provides important additional information on bone quality (Tang et al., 2012; Tang et al., 2013). We believe that this first prospective study accompanying the introduction of glucocorticoid in these patients may provide relevant data on the mechanisms involved in bone loss, in addition to understanding the dynamics of corticoid-induced bone alteration, as well as determining the predictive factors of fractures in this population. This evaluation will be accompanied by the determination of markers of bone metabolism.

On the other hand, ocular complications are also very frequent in patients who use GC and, among these, is the carotidal thickness increase (Han et al., 2013). As this condition has been implicated in many retinal diseases, such as central serous chorioretinopathy and pachychoroid neovasculopathy (Han et al., 2013), patients undergoing GC therapy may have an increased risk of developing these conditions. Thus, one of the objectives of this study is to understand this process of injury through the characterization of the effects of CG on the chroidal thickness, which would contribute not only to the understanding of the mechanism of choroidal injury but also to the identification of patients with retinal diseases associated with chronic use of GC.

For GC-induced cataract, the mechanism involved s not yet known, but it may be associated with gene activation, non-enzymatic binding of corticosteroids to the ocular lens, altered osmotic balance, or oxidative damage (Manabeet al., 1984; McLean et al., 1995; Zhou et al., 2015). Regarding oxidative damage, serum levels of oxidative stress products have been shown to be increased in cataract associated with age (Chang et al., 2013), but there are no data for corticosteroid-induced cataract. It is interesting that, in SLE, oxidative stress is present and seems to contribute to the immunomodulation of the disease (Shahet al., 2014). In this respect, we speculate whether the detection and quantification of oxidative stress products in the blood could eventually help elucidate the pathophysiological mechanisms involved in the development of corticosteroid-induced cataract or be a predictive factor of their onset in patients with SLE on chronic corticosteroid therapy.

Therefore, this project aims to evaluate the relevance of monitoring blood and salivary levels of drugs used in rheumatologic autoimmune diseases by monitoring efficacy and adherence to therapy. In addition, this project aims to broaden the understanding of the possible mechanisms involved in the adverse effects of these drugs, aiming damage reduction, and maintaining therapeutic efficacy. High-efficiency chromatography (HPLC) coupled with tandem mass spectrometry (HPLC/MS-MS) is considered the gold standard in qualitative and quantitative analysis of drugs in biological matrices. The advantages of this methodology could be grouped into two categories: bioanalytical quality and logistics. At present, there is no other methodology that equals HPLC-MS/MS for the analytical quality of the data obtained. This technique allows the measurement of multiple drugs in a single analysis run, a fact that helps to reduce the cost and the time required to perform the analyses. The high sensitivity related to HPLC/MS-MS allows to detect compounds in very low concentrations, especially for free compounds that will be measured in saliva. The specificity obtained allows precise determinations and elimination of interference generated by metabolites of drugs and other endogenous and xenobiotic interfering factors.

In the proposed project, there is a special need to obtain high accuracy due to the toxicity of the drugs studied and the need for precise adjustment of therapeutic levels. In the logistics aspect, the methodology allows simplification in the procedures of extraction of the analytes and reduction in the acquisition time of bioanalytical data. Consequently, with smaller turn-around times there is an increase in processing capacity in the analytical stage, enabling the analysis of hundreds/thousands of samples in the time frame compatible with the proposed study. The multiplexing capacity of the determinations is an unattainable aspect regarding other quantification methods, however using HPLC/MS-MS in this project will allow the determination of the intact drug and its metabolites in a single acquisition, generating more comprehensive results that could reveal in more depth the therapeutic dynamics.

The implementation of this methodology for research and routine in our center, with the necessary training of human resources, will allow the standardization and availability of this advanced technology for other multidisciplinary projects in various areas of science. In addition, drug concentrations obtain from patients in this study will be used to establish pharmacokinetic-pharmacodynamic models for GC, thalidomide and hydroxycloroquine use in patients with SLE. Pharmacokinetic-pharmacodynamic modeling is a technique that combines two important aspects in medical therapy: pharmacokinetics and pharmacodynamics. This integration is done in a series of mathematical expressions that allows the description of the duration of the therapeutic effect in relation to the dose administered (Poggi JC et al., 2006). This thematic project will be divided into four sections with their respective subprojects, according to the medications that will be studied:

1. hydroxychloroquine;
2. thalidomide.

Project 1: HYDROXYCHLOROQUINE (1A, 1B and 1C)

Evaluation of the relevance of determining blood levels of hydroxychloroquine and its metabolites in rheumatologic autoimmune diseases: safety/efficacy of therapy, disease activity, adherence to therapy and cost-effectiveness

TITLE OF THE SUBPROJECT

Correlation of blood levels of hydroxychloroquine (HCQ) with the indices of disease activity and adherence to therapy in a cross-sectional study diseases Systemic lupus erythematosus (SLE), and Juvenile systemic lupus erythematosus (JSLE).

Background

HCQ has been used in the treatment of autoimmune rheumatologic diseases, including SLE (Ruiz-Irastorza et al., 2010). However, only one study that evaluated blood levels of HCQ in adult SLE (Costedoat-Chalumeau et al., 2006), without data on juvenile SLE. The issue of non-adherence in SLE for drugs of continuous use is difficult to measure and can reach 76% of cases, bringing serious consequences for the patient related to changes in prescriptions (Costedoat-Chalumeau et al., 2013). In this context, it is relevant that some studies have shown that plasma dosage of hydroxychloroquine is a reliable parameter of adherence, as the drug has a long half-life and is detected in blood samples.

In adult patients with SLE, it has been reported that concentrations below 1000 ng/mL of HCQ in the blood are associated with disease activity, and a strong predictor of disease exacerbation in the following six months (Costedoat-Chalumeau et al., 2006). It is emphasized, however, that the concentrations of HCQ in the total blood varied greatly among the patients in this study, despite the prescription of the same daily dosage. In this study, however, the evaluation of lupus activity was global and did not focus on renal involvement, and it was only mentioned that 10 patients had active nephritis.

Other studies are therefore necessary to define blood levels of HCQ that will be predictors of outbreaks of renal activity, whose involvement is present in more than 50% of patients and is the greatest therapeutic challenge of the disease. In this context, it is interesting that the use of this drug is associated with higher renal survival (Fessler et al., 2005) and a better response to standard treatment with mycophenolatote mofetil in patients with SLE (Kasitanon et al., 2006), reinforcing the importance of an adequate control of its use in this condition.

Alternatively, to blood measurement, recent studies with a very limited number of drugs suggest that monitoring drug concentrations can be performed in saliva, with the advantage of detecting its free form (Dwivedi et al., 2015a). In the area of Rheumatology, there are few studies and are restricted to blood measurement and there is only one study that used saliva for glucocorticoid dosage. Saliva dosage offers a good alternative to serum level, because it is less invasive and because it generally has a good correlation with the free drug in serum (Ruiter et al., 2012).

Another interesting aspect in relation to antimalarial is that the mechanism of its beneficial effect has not yet been fully clarified, but it is believed to be correlated with the blockade of toll-like receptors (TLR7-9) and inhibition of alpha interferon production, which plays a crucial role in the pathogenesis of SLE. In fact, an in vitro study observed that the use of hydroxychloroquine inhibits the production of interferon-alpha and TNF by plasmocytoid dendritic cells of patients with SLE and, apparently, this inhibition is more effective for TLR-9 than for TLR-7 (Sacre et al., 2012). However, there is still a need to determine the possible correlation between the level of antimalarial and the degree of inhibition of the two different receptors.

Objectives primary:

- Monitoring of HCQ blood levels: possible relevance on disease activity in JSLE and renal adult SLE to inclusion and 6 months following inclusion.

Secondary:

- Evaluate the predictive value of HCQ levels for JSLE disease activity and renal disease activity in adult SLE within 6 months of inclusion.

- To verify the possible association between blood levels of HCQ with adherence to treatment assessed by a specific questionnaire.

- Evaluate the correlation between blood and salivary levels of HCQ in a subgroup of patients with SLE and JSLE and their possible association with therapeutic response and safety.

- Check whether the production of interferon-alpha and TNF by plasmocytoid dendritic cells correlates with blood HCQ levels.

NUMBER OF INDIVIDUALS

Total patients required for the study = 309, being: SLE (n= 206), SLEJ (n= 103).

STUDY DESIGN

Initially, a cross-sectional study will be conducted with patients using HCQ at a dose of 5-6.5 mg/kg/day (maximum of 400 mg/day) for at least 6 months (Clark et al., 1993). At the entrance, peripheral blood samples will be taken to determine HCQ levels and patients will be evaluated for the activity of the underlying disease in a "blind" way to HCQ concentrations.

The following indices will be used to assess disease activity every 3 months:

- SLE and SLEJ: Systemic Lupus Erythematosus Disease Activity Index (SLEDAI-2K) (Glad man et al., 2002) and active disease randomly defined asSLEDAI-2K≥ 6.

- Renal activity: blood pressure (BP), urine type I, protein and creatinine in an isolated urine sample, serum keratinize, C3, C4, anti-dsDNA, according to the recommendations of the American College of Rheumatology (ACR) (Hahnet al., 2012).

Adherence will be evaluated by the Medication Adherence Self-report Inventory (MASRI) applied by a pharmacist not directly involved in patient care (Koneru et al., 2007).

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Adherence will be evaluated by the Medication Adherence Self-report Inventory (MASRI) applied by a pharmacist not directly involved in patient care (Koneru et al., 2007).

The production of interferon-alpha and TNF-alpha by plasmocytoid dendritic cells will be evaluated in vitro using mononuclear cell preparations isolated from peripheral blood from a random sample of the same number of consecutive patients with active and inactive SLE. The cells will be stimulated with the TLR-9 agonist, Oligodeoxynucleotides CpG (ODN CpG-A) -2216, and with the TLR-7 agonist, imiquimod. The percentage of monocytes, B cells, dendritic cells and natural killers (NK) in the PBMC fraction will be determined by the phenotypic characterization of these cell subpopulations by flow cytometry using monoclonal fluorochrome-conjugated antibodies. Intracellular detection of IFN-alpha and TNF-alpha in plasmocytoid dendritic cells will also be performed by flow cytometry and specific monoclonal antibodies (Sacre et al., 2012).

TARGET POPULATION

Inclusion criteria:

- LES and LESJ: Criteria of the American College of Rheumatology (ACR) (Hochberg, 1997);

- use of HCQ at a dose of 5-6.5 mg/kg/day (maximum 400 mg/day) for at least 6 months.

Exclusion criteria:

- alcoholism;

- renal dialysis;

- concomitant infectious process;

- acute and chronic liver diseases and

- concomitant use of some drugs that interact with HCQ (cimetidine, antacids, digoxin, aminoglycosides, penicillin, neostigmine, pyridostigmine).

RELEVANT METHODS

HPLC MS/MS: The determination of blood and salivary levels of hydroxychloroquine and its metabolites bisdesetylchloroquine (BDCQ), desetilhydroxychloroquine (DHCQ) and desetylchloroquine (DCQ) will be performed by HPLC and tandem mass spectrometry. The process of extracting the analytes will be as described by Munster et al. (2002): 10 mL of blood will be collected in silanized tubes containing heparin, divided in aliquots and stored at -80ºC until analysis. After thawing, the sample will be alkalized with NaOH 10 N and supplemented with internal control (chloroquine or HCQ-D4 sulfate). The sample will then be subjected to extraction with acetonitrile and the residue obtained after evaporation will be reconstituted with the mobile phase (methanol:acidified water with formic acid) and injected into the LC-MS/MS system according to the Soichot protocol, 2014. Chromatographic separation will be obtained using hypersilgold aQ analytical column and, as mobile phase, water gradient and

acidified methanol with 0.1% formic acid. The detection of analytes will be by tandem mass spectrometry with mass spectrometer equipped with an electrospray ionization source operating in ion-positive mode. HCQ sulfate and its metabolites DHCQ, DCQ, BDCQ will be added in increasing concentrations to a blood sample of healthy individuals as standards in the elaboration of the calibration curve to be used in the quantitative calculation of the drug and its derivatives and as quality control of the procedure.

Phenotypic characterization of PBMC subpopulations: mononuclear cells will be isolated from peripheral blood in physio-Hypaque gradient, placed on plates containing RPMI 1640 culture medium supplemented with fetal serum and antibiotics. The percentage of monocytes, B cells, dendritic cells, and natural killers (NK) in this preparation will be determined by flow cytometry, using monoclonal antibodies conjugated to fluorochromes (CD123, CD20, Cd11b, CD16, CD14, HLA-DR).

Intracellular detection of IFN-alpha and TNF-alpha cytokines in plasmocytoid dendritic cells: PBMC cells will be stimulated with TLR-7 and TLR-9 agonists, imiquimod (5 μg/mL) and CpG-A ODN 2216 (5 μM) for 5 hours at 37ºC. After 3 hours of stimulation, Brefeldin A will be added to induce the accumulation of cytokines in the endoplasmic reticulum by its inibiotic action on intracellular protein transport to the Golgi complex. Phenotypic cell characterization (PBMC) and intracellular detection of cytokines will be performed by sequential membrane marking and intracellularly using specific monoclonal antibodies labeled with fuorochromes followed by evaluation by flow cytometry (Sacre et al., 2012).

WITHDRAWALS

It doesn't apply.

ORIGINALITY WITH IMPACT

Healthcare

To date, there is no study that has evaluated blood and salivary levels of hydroxychloroquine (HCQ) and its predictive value for disease activity in JSLE and adult lupus nephritis.

The expected impact with the availability of this technique in clinical practice is an improvement in therapeutic adherence and an earlier intervention with reduction of outbreaks of disease activity. The use of saliva as an alternative to this monitoring would provide a less invasive technique for this process.

ADVANCES IN MECHANISMS

The expected progress is a better understanding of the mechanism of action of HCQ on TLR-7-9, previously suggested as possible therapeutic targets for the disease.

STATISTICAL PLAN

SAMPLE SIZE

The sample size was calculated based on the HCQ levels observed in the study by Costedoat-Chalumeau et al., 2006, in patients with SLE and assuming: 95% confidence interval, α error of 5%; power of 80%; mean HCQ concentration in active patients: 694 ± 448 ng/mL; mean HCQ concentration in inactive patients: 1079 ± 526 ng/mL; aggregate standard deviation: 532 ng/mL and loss: 10%.

For the SLE group with nephritis, 16% of SLE patients in our outpatient clinic present active renal disease, then we expect 33 patients with renal activity out of the 206 patients which will be included.

For the JSLE group, 34% of our outpatient clinic patients present active, then 35 with disease activity are expected out of a total number of 103 patients to be included statistical analysis The groups of patients with and without disease activity will be compared for categorical variables by fisher's exact test and, for quantitative variables, by student and mann-whitney t tests, when applicable.

TITLE OF THE

SUBPROJECT 1B

Prospective, randomized and controlled study comparing the frequency of SLE and jSLE activity of patients with a stable dose of HCQ and patients with dose reduction of this drug and its correlation with blood levels of its metabolites diseases.

SLE AND jSLE

Background

It takes at least 6 months of HCQ use to achieve its best efficacy (Clark et al., 1993). In SLE, regular use of HCQ promotes a decrease in inflammatory outbreaks of the disease (Ruiz-Irastorza et al., 2010) and its withdrawal is associated with worsening activity remission (Tsakonas et al., 1998 - The Canadian Hydroxychloroquine Study Group). Its action is due to a modulating effect on the immune response, influencing the presentation of antigens and production of pro-inflammatory cytokines, which are probably mediated mainly by inhibition of Toll-like receptors-7 and -9 (Wallace et al., 2012). Adequate serum levels of HCQ metabolites are related to a better efficacy in the treatment of the cutaneous form of lupus (Francès et al., 2012) and HCQ concentrations in the blood lower than 1,000 ng/mL determined by HPLC have been associated with higher inflammatory activity of SLE (Costedoat-Chalumeau et al., 2006). Monitoring of HCQ in the blood would allow assessing whether, after a prolonged period of treatment, which causes a tissue deposit of the drug in the skin, a reduction in the weekly dose would be possible. In addition, the measurement of HCQ in saliva could offer a good alternative for blood levels, because it is less invasive, as previously demonstrated with other drugs (Ruiter et al., 2012). The goal for reduced HCQ dose is to avoid retinopathy with vision loss, without risk of increased outbreaks of disease activity. Retinopathy is the most feared side effect of HCQ use and is associated with the cumulative dose of antimalarial (Wolfe et al., 2010). The new recommendation of the American Ophthalmology Association of 2011 determines that ophthalmological control of the use of antimalarials must necessarily include campimetry and at least one more objective examination such as multifocal electroretinogram, optical coherence tomography (SD-OCT) (Marmor et al., 2011). Our objective is to determine the mechanism of HCQ-induced injury through an evaluation of the correlation of functional and structural retinal damage, evaluated by central visual field examination (campimetry) and SD-OCT measurements, with segmentation of the outer layer of the retina (pigment epithelium). The proposed acquisition of a campimeter dedicated to the research intends to validate the isolated use of campimetry, limited to the 10-2 Humphrey field, as the gold standard of this evaluation.

In addition, it is extremely important to evaluate whether the reduction of the weekly dose of HCQ after a long period of use of this therapy would be able to maintain inhibition of TLR-7 and -9.

Objectives primary:

- Compare the frequency of outbreaks of disease activity (SLE and jSLE) in patients using standard dose (HCQ 400 mg/day) vs. patients with reduced dose (HCQ 400 mg/3X week) and its correlation with blood and saliva levels of the drug. Secondary: - Compare frequency of retinopathy in patients using standard dose (HCQ 400 mg/day) vs. patients with reduced dose (HCQ 400 mg/3X week) and its correlation with HCQ blood levels.

- Verify a possible longitudinal correlation of HCQ concentrations with the production of interferon-alpha and TNF by plasmocytoid dendritic cells.

- Correlate blood and salivary levels of HCQ and its metabolites in patients with SLE and JSLE with adherence to this treatment.

- Correlate functional and structural damage of the retina, evaluated by central visual field examination (campimetry) and SD-OCT measurements, with segmentation of the outer layer of the retina (pigmental epithelium).

- Establishment of a pharmacokinetic-pharmacodynamic model of hydroxychloroquine dose reduction in patients with SLE.

NUMBER OF INDIVIDUALS

Total number of patients required for the study= 256 (SLE and JSLE).

STUDY DESIGN

Randomized controlled prospective study of non-inferiority of one year of follow-up of patients with SLE and JSLE in remission with HCQ use (stable dose of 400 mg/day for at least five years).

Patients will be randomized into two groups: no daily dose reduction group (HCQ 400 mg/day) and reduction group (HCQ 400 mg/day - 3X week). Patients will be evaluated for the activity of the underlying disease in a "blind" way. HCQ concentrations will be measured at the entrance, 6 months and 12 months after.

The groups will be compared for episodes of disease activity (clinical and laboratory parameters). The production of interferon-alpha and TNF by plasmocytoid dendritic cells will be evaluated in the same period in a subgroup of patients.

The following indexes will be used to assess disease activity:

- SLE and jSLE: SLEDAI-2K (Gladman et al., 2002) and active disease randomly defined as SLEDAI-2K≥ 6.

- Renal activity: BP, urine type I, protein and creatinine in an isolated urine sample, serum creatinine, C3, C4, anti-dsDNA, according to the recommendations of the ACR (Hahn et al., 2012).

Adherence will be assessed by the MASRI questionnaire applied by a pharmacist not directly involved in patient care (Koneru et al., 2007).

The following clinical, laboratory and treatment data will be evaluated in the groups (active disease and inactive disease): age, sex, race, weight, height, BMI, ideal weight; disease time, time of HCQ use, adherence to treatment; smoking; use and dose of prednisone, methotrexate, azathioprine, mycophenolate mofetil, cyclophosphamide, non-hormonal anti-inflammatory drugs; urinary protein/creatinine ratio; serum creatinine; blood concentrations of HCQ by HPLC; specific adverse events.

- Complete ophthalmologic assessment (at the entrance and at the end of the study), including campimetry and SD-OCT measurements, with segmentation of the outer layer of the retina (pigmentary epithelium).

- The production of interferon-alpha and TNF-alpha by plasmocytoid dendritic cells will be evaluated in vitro using mononuclear cell preparations isolated from peripheral blood from a random sample of the same number of consecutive patients with active and inactive SLE. The cells will be stimulated with the TLR-9 agonist, Oligodeoxynucleotide S CpG (ODN CpG-A)-2216 and with the TLR-7 agonist, imiquimod. The percentage of monocytes, B cells, dendritic cells and natural killers (NK) in the PBMC fraction will be determined by the phenotypic characterization of these cell subpopulations by flow cytometry using monoclonal fluorochrome-conjugated antibodies. Intracellular detection of IFN-alpha and TNF-alpha in plasmocytoid dendritic cells will also be performed by flow cytometry and specific monoclonal antibodies (Sacre et al., 2012).

- Establishment of a pharmacokinetic-pharmacodynamic model of hydroxychloroquine use with two groups of 5 patients: one with 5mg/kg/day daily and another 5mg/Kg/day 3 times a week. On inclusion both will be on hydroxychloroquine use for at least 6 months and should have the stable level of the drug (> 1000ng/mL).

Outcomes: assessment on days 0 and 120 days.

a) Worsens no SLEDAI -2K. Change in SLEDAI-2K evaluating activity criteria from 0-105 in 9 organs and 24 items (clinical and laboratorial). The increase represents clinical/laboratory worsening of the disease.

b) Cytokine measurement. Plasma levels of TNF-α and IFN-1 will be determined by Luminex MAP (EMD Millipore Corporation®). Plasma samples will be stored at -80oC until use.

TARGET POPULATION

Inclusion criteria:

- LES and jSLE (ACR criteria) (Hochberg, 1997);

- use of HCQ at a dose of 400 mg/day for at least 3 years.

Exclusion criteria:

- alcoholism;

- renal dialysis;

- concomitant infectious process;

- acute and chronic liver diseases;

- concomitant use of some drugs that interact with HCQ (cimetidine, antacids, digoxin, aminoglycosides, penicillin, neostigmine, pyridostigmine).

RELEVANT METHODS - Ophthalmological assessment: central visual field examination (campimetry) and SD-OCT measurements, with segmentation of the outer layer of the retina (pigmentary epithelium).

- Blood levels of HCQ will be determined by HPLC MS/MS (Munster et al., 2002).

- Pharmacokinetic analysis: The pharmacokinetic parameters of hydroxychloroquine will be calculated in the 24 h dose range based on the total plasma concentration versus time curves. The Winnonlin program, version 4.0 (PharsightCorp., Mountain View, California, USA) and theNon-linear mixed effect level(NONMEN) program will be employed. The area under the plasma concentration curve versus time will be calculated in the 24-h dose interval by the trapezoid method on days 0 and 120. The apparent total clearance (ClT/F) will be obtained through the equation: ClT/F= Dose/AUC0-24. The apparent distribution volume (Vd/F) will be calculated by the standard software equation. The hydroxychloroquine-free concentration will be determined by the dosage in saliva. The PK-PD analysis will be performed using as pharmacokinetic parameters the total and free plasma concentrations of hydroxychloroquine and, as a pharmacodynamic parameter, we will use the clinical/laboratory indices obtained by the evaluation of SLEDAI-2K (American College of Rheumathology). The PK-PD model will be developed using the non-linear mixed effect level (NONMEM).

- Others, the same as described in subproject 1A.

Safety

Monitoring of outbreaks of inflammatory activity(flare) and identification of retinopathy.

WITHDRAWALS

Signs of retinopathy.

ORIGINALITY WITH IMPACT on

Healthcare

To date, there is no study demonstrating that the reduction of HCQ dosage after tissue depot caused by a prolonged time of use of the drug can be safely performed in SLEJ and SLE, without increasing the number of outbreaks of inflammatory activity.

The expected care impact is a reduction of severe ophthalmologic event with the reduction of the HCQ dose and the expansion of the time of use of the drug that has a direct impact on the control of disease activity.

ADVANCES IN MECHANISMS Advances in understanding the role of cutaneous HCQ depose acquired after prolonged previous treatment in maintaining drug-induced TLRs-7 and -9 inhibition would justify reducing the therapeutic dose based on the drug's immunomodulatory mechanisms of action.

SAMPLE SIZE

The sample size was calculated based on the difference in Reactivation of SLE/SJS in one year after the removal or maintenance of hydroxychloroquine, as observed in the study by Tsakonas et al., 1998 and assuming: 95% confidence interval, α error of 5%; power of 80%; expected success rate after 1 year in the group that kept HCQ at the standard dose (Tsakonas et al., 1998)- 84%; expected success rate after 1 year in the group that fully removes HCQ (Tsakonas et al., 1998)- 59%; no-inferiority margin- 50% of the difference between maintaining chloroquine or removing it completely, i.e., delta of 12%; losses- 10%; n in each group- 128; total number of patients to be recruited: 256 (for SLE/JSLE).

statistical analysis The comparison of the risk of inflammatory activity of the disease in patients using standard dose (HCQ 400 mg/day) vs. patients with reduced dose (HCQ 400mg/day - 3X week) will be made through a survival curve by the log-rank test. The categorical variables will be evaluated by fisher's exact or chi-square test and quantitative variables by student and mann-whitney t tests, when applicable.

TITLE OF THE SUBPROJECT 1C

Prospective study of the pharmacokinetics of hydroxychloroquine in patients with Systemic Lupus Erythematosus using attack dose diseases SLE and jSLE.

Justification

Currently, it is recommended that all patients with SLE should be treated with antimalarials unless specific contraindications (Wallace et al., 2012; van Vollenhoven et al., 2014). Its use is justified because of the demonstrated effectiveness in the treatment of the disease (Ruiz-Irastorza et al., 2010), with a decrease in inflammatory episodes (flares) (Clark et al., 1993). Furthermore, it was demonstrated that HCQ withdrawal increases the risk of disease reactivation (The Canadian Hydroxychloroquine Study Group,1991; Tsakonas et al., 1998). There is a modulating effect on the immune response with its use, promoting a change in the production of pro-inflammatory cytokines, and these effects are probably mediated mainly by inhibition of receptor-7 and -9 Toll-like (Wallace et al., 2012). The most commonly used dosage is 400 mg/day of HCQ. It is also relevant in the context of treatment that antimalarials have a long half-life of approximately 40 days and there is a delay to reach plasma concentration with a therapeutic effect expected only after 3 months or more of treatment (Rodriguez-Caruncho et al., 2014). Monitoring the level of this medication, which was established as at least 1000 ng/mL, would certainly be very useful for determining whether a higher attack dose would allow this therapeutic effect to be achieved more quickly. Lower blood concentrations of HCQ (< 1,000 ng/mL) determined by HPLC MS/MS are associated with higher inflammatory activity of SLE (Costedoat-Chalumeau et al., 2006). Adequate serum levels of the metabolites of this drug are related to a better efficacy in the treatment of the cutaneous form of lupus (Francès et al., 2012), but even using the same daily dose of the drug, there is a great individual variability among patients (Munster et al., 2002). Therefore, in rheumatoid arthritis, it

has been suggested that an attack dose in the first weeks of treatment is related to an increase in response in this period (Emery, 1999; Furst et al., 1999). There are no studies in SLE that assess how long after the start of treatment is necessary to achieve adequate HCQ blood level, nor whether a higher initial dose (attack) could anticipate these levels in order to provide an earlier clinical/laboratory response.

Objectives Primary objective:

- Compare the time required to reach the level of 1000 ng/mL of HCQ in patients with SLE, using standard dose vs. attack dose.

Secondary objective:

- Evaluate the clinical/laboratory response and adverse events in both groups.

- Verify a possible correlation of HCQ concentrations in blood and saliva with interferon-alpha and TNF production levels by plasmocytoid dendritic cells and disease activity parameters.

- Correlate blood and salivary levels of HCQ and its metabolites in Patients with SLE with adherence to this treatment.

NUMBER OF INDIVIDUALS

Total patients needed for the study = 96 (SLE).

STUDY DESIGN

Prospective randomized study controlled for a period of six months of patients with SLE who will start the use of HCQ.

Patients will be randomized randomly into two groups: traditional group - without increasing the daily dose (HCQ 400 mg/day); attack dose group - with increased daily dose (HCQ 800 mg/day for 6 months). Patients will be evaluated for the activity of the underlying disease in a "blind" way. HCQ concentrations will be assessed at the entrance and at the end of the study.

The groups will be compared for episodes of disease activity (clinical and laboratory parameters), inflammatory cytokines, and expression of TLR-7 and TLR-9 receptors.

Blood concentrations of HCQ and metabolites will be determined in both groups by HPLC (Munster et al., 2002).

The following indexes will be used to assess disease activity:

- SLE: SLEDAI-2K (Gladman et al., 2002) and active disease randomly defined as SLEDAI-2K$\geq$ 6.

- Renal activity: BP, urine type I, protein and creatinine in an isolated urine sample, serum creatinine, C3, C4, anti-dsDNA, according to the ACR Guidelines (Hahnet al., 2012).

Adherence will be assessed by the MASRI questionnaire applied by a pharmacist not directly involved in patient care (Koneru et al., 2007).

The following clinical, laboratory and treatment data will be evaluated in the groups (traditional dose and attack dose): age, sex, race, weight, height, BMI, ideal weight; disease time, time of HCQ use, adherence to treatment; smoking; use and dose of prednisone, methotrexate, azathioprine, mycophenolate mofetil, cyclophosphamide, non-hormonal anti-inflammatory drugs; urinary protein/creatinine ratio; serum creatinine; HCQ blood concentrations by HPLC MS/MS; general- gastrointestinal and cutaneous adverse events.

- The production of interferon-alpha and TNF-alpha by plasmocytoid dendritic cells will be evaluated in vitro using mononuclear cell preparations isolated from peripheral blood from a random sample of the same number of consecutive patients with active and inactive SLE. The cells will be

stimulated with the TLR-9 agonist, Oligodeoxynucleotides CpG (ODN CpG-A)-2216 and the TLR-7 agonist, imiquimod. The percentage of monocytes, B cells, dendritic cells and natural killers (NK) in the PBMC fraction will be determined by the phenotypic characterization of these cell subpopulations by flow cytometry using monoclonal fluorochrome-conjugated antibodies. Intracellular detection of IFN-alpha and TNF-alpha in plasmocytoid dendritic cells will also be performed by flow cytometry and specific monoclonal antibodies (Sacre et al., 2012).

TARGET POPULATION

Inclusion criteria:

- SLE (ACR criteria) (Hochberg, 1997);

- without using HCQ at a dose of 400 mg/day for at least 6 months.

Exclusion criteria:

- alcoholism;

- renal dialysis;

- concomitant infectious process;

- acute and chronic liver diseases;

- concomitant use of drugs that interact with HCQ (cimetidine, antacids, digoxin, aminoglycosides, penicillin, neostigmine, pyridostigmine).

RELEVANT METHODS

See subproject 1A.

Safety. Monitoring of adverse events and outbreaks of inflammatory activity (flare).

ORIGINALITY WITH IMPACT ON ASSISTANCE

There are no studies in SLE that assess how long after the start of treatment is necessary to achieve adequate HCQ blood and salivary level, nor whether a higher initial dose (attack) could anticipate these levels to provide an earlier clinical/laboratory response without increasing adverse effects.

The expected impact would be that the dosage of HCQ levels would allow to perform a control of the inflammatory activity of the disease earlier and safely.

ADVANCES IN MECHANISMS To compare the inhibitory action of different doses of HCQ in TLRs -7 and -9 and its relevance to the immunomodulatory action of the drug.

SAMPLE SIZE

The sample size was calculated considering that the faster the 1000 ng/mL blood level is reached, the greater the improvement in lupus activity and the lower the recurrence rate in the groups is observed, 6 months after the onset of HCQ treatment. In the study by Tett et al., 2000, in patients with AR, in the group that started HCQ at a dose of 400 mg/d, the mean seric level was 870.3 ± 329.3 ng/mL, that is, about 34% of patients reached > 1000 ng/mL blood level after 6 months. It is estimated that more than 20% of patients will reach this value with twice the attack dose, we calculate (based on: 95% confidence interval, error α 5%; power of 80%) expected frequency of patients with 1000 ng/mL > level after 6 months in the 400 mg/d group: 34%; expected frequency of patients with 1000 ng/mL > level after 6 months in the 800 mg/d group: 55 %; losses: 10%; n = 96.

STATISTICAL PLAN

The comparison of the time required to reach the level of 1000 ng/mL of HCQ in patients with SLE and JSLE, using survival curve by log-rank test. Categorical variables will be evaluated by fisher's exact or chi-square test and quantitative variables by Student and Mann-Whitney t tests, when applicable.

Project 2: THALIDOMIDE

EVALUATION OF THE TREATMENT OF CUTANEOUS LUPUS ERYTHEMATOSUS WITH THALIDOMIDE: CLINICAL, LABORATORY AND HISTOLOGICAL FACTORS ASSOCIATED WITH CLINIC RESPONSE AND ADVERSE EFFECTS

Diseases

Systemic lupus erythematosus with cutaneous involvement (LEC).

Justification

Thalidomide is another immunomodulatory agent used to treat the cutaneous activity of SLE and LEC, even in patients refractory to other medications (Knop et al., 1983; Kyriakis et al., 2000; Coelho et al., 2005; Cuadrado et al., 2005). Its high efficacy associated with a very rapid response of the cutaneous picture, made this drug one of the first choices in patients without contraindication. Cuadrado et al. demonstrated in a retrospective study with 48 patients with SLE and skin lesions refractory to treatment with antimalarials, prednisone, azathioprine, cyclosporine and cyclophosphamide a high partial or complete clinical response rate (81%) with doses of 25, 50 or 100 mg/day of thalidomide (Cuadrado et al., 2005). Thus, thalidomide is very useful as an agent inducing remission of skin activity of SLE (Cuadrado et al., 2005). In this study, the treatment time was around one year. Thrombotic events were not observed, although some patients had positive antiphospholipid antibodies. The most common adverse effects were dizziness, abdominal pain, and peripheral polyneuropathy. Peripheral polyneuropathy occurred in 27% of patients, including 18.8% with neurological symptoms and alterations in ENMG, 4.2% only with findings at ENMG and 4.2% with neurological symptoms despite normal MGS. Regarding the time of disease, this effect seems to occur around 14 months of treatment (Briani et al., 2004).

In Brazil, thalidomide is indicated for use in SLE, subacute cutaneous LE and discoid LE (Ministry of Health, 2013). However, due to its known teratogenicity when used during pregnancy, it is recommended for male patients (who agree to use condoms throughout the treatment period and up to 30 days after the end of the same, even if they have already undergone vasectomy, as its possible excretion in the seminal fluid is not known) and to women without fertility potential (i.e., who have been in the postmenopausal phase for at least one year, or who have undergone tubal ligation or hysterectomy) and who agree to use it according to the Term of Responsibility/Clarification (Ministry of Health, 2013; System for Thalidomide Education and Prescribing Safety, Zeldis et al., 1999).

Thalidomide acts mainly by inhibiting the synthesis of TNF-α (Nakamura et al., 2007; Kuhn et al., 2011). In order to better understand the mechanisms involved in this therapeutic response, we intend to evaluate whether plasma levels and tissue expression of TNF-α at the beginning of treatment would be relevant parameters as predictors of response.

In addition to blood monitoring, salivary drug dosage has been suggested as a practical and low-invasive method to individualize and optimize drug management in patients with chronic diseases such as epilepsy (Johannessen and Landmark, 2008; Dwivedi et al., 2015b). There are no studies in the literature to monitor levels of thalidomide in saliva in patients with SLE.

In addition, it would be important to determine whether the longitudinal evaluation of these inflammatory markers and thalidomide concentration would play a role in monitoring the efficacy of this therapy in SLE. Reinforcing this possibility, it has been demonstrated that TNF-α and IFN-1 are increased in the skin lesion of lupus (Yu et al., 2013). In addition, thalidomide seems to inhibit keratinocyte

apoptosis induced by ultraviolet B radiation (Lu et al., 2003), another relevant aspect in the pathogenesis of cutaneous LE (Reefman et al., 2006).

The mechanism of neurotoxicity of thalidomide is also unclear and there is work suggesting that this drug could have a direct toxic effect on neurons of the posterior root ganglion (Giannini et al., 2003). In this respect, the recent description that thin fiber neuropathy is associated with anti-TNF-therapy, with clinical findings and skin biopsy suggestive of posterior dorsal root involvement in patients with AR (Birnbaum & Bingham, 2014), reinforces this possibility. We speculate that this same mechanism would be involved in the neurotoxicity of thalidomide in SLE. This finding would have great clinical relevance, because the diagnosis of this complication today depends on electroneuromyography, which is an expensive, time-consuming, painful and operator-dependent examination. Skin biopsy is an interesting alternative because it is today a low-cost, fast, not painful and easily accessible procedure that would open the prospect of performing a more accurate and early diagnosis of neuropathy, besides providing histopathological characteristics of the process of injury of these fibers.

Objectives primary:

Evaluate in SLE patients with cutaneous involvement (SLE):

- The possible combination of plasma levels of TNF-, αIFN-1, BAFF (B-cell activator factor) and thalidomide levels in the blood and saliva with the therapeutic response and the adverse effects.

Secondary:

- Evaluate the possible association between TNF-levels, adjoin molecules, apoptosis markers and growth factors in skin biopsy samples with efficacy and adverse effects.

- Evaluate the presence of fine fiber neuropathy in skin biopsy specimens.

- Establishment of a pharmacokinetic-pharmacodynamic model of thalidomide dose reduction in patients with SLE.

NUMBER OF INDIVIDUALS

40 patients with lupus (SLE) and active cutaneous involvement will be included.

STUDY DESIGN

A prospective study for a period of 12 months with adult patients with SLE (according to the classification criteria of the American College of Rheumatology- ACR) (Hochberg, 1997) with active skin lesions at the entrance regardless the use of hydroxychloroquine and/or dapsone and/or prednisone and/or immunosuppressants, and who are eligible for the use of thalidomide (see inclusion criteria) (Ministry of Health, 2013; System for Thalidomide Education and Prescribing Safety, Zeldis et al., 1999).

The initial dose of thalidomide will be 100 mg/day for 3 months and reduced to 50 mg/day and 25 mg/day, at any time depending on the therapeutic response until complete 12 months of treatment.

All patients will be treated simultaneously with hydroxychloroquine, unless there is contraindication for use of this medication.

The other drugs used for SLE control will be maintained during the study, including prednisone (with subsequent gradual dose decrease in case of improvement of skin lesions).

Patients will be clinically evaluated at baseline, then 1 month, 6 months and 12 months after inclusion for:

- the global activity of the disease through the SLEDAI-2K (Systemic Lupus Erythematosus Disease Activity Index 2000) (Gladman et al., 2002;

- the index of activity and skin severity (CLASI) (Albrecht et al., 2005);

- possible adverse effects and adherence by the Medication Adherence Self-report Inventory (MASRI) applied by a pharmacist not directly involved in patient care (Koneru et al., 2007);

- quality of life by the visual analog scale and the SF36 questionnaire (Mosca et al., 2010).

Plasma levels of TNF-, IFN-1, BAFF, transforming growth factor-beta (TGF-β) and thalidomide levels in the blood and saliva will be performed at zero time, at 30 days, 3 months, 6 months, 9 months and 12 months.

The clinical evaluation of peripheral neuropathy will be performed at each consultation by anamnesis and through THE ENMG at baseline, at 6 months and at 12 months.

Skin biopsy of an active lesion will be performed at baseline for histological evaluation, including the study of immunohistochemistry expression of TNF-, TGFαβ (Kuhn et al., 2010; Yu et al., 2013) and plasmacytoid and pro-apoptotic dendritic cell markers such as caspase 3, CD25, CD35, CD21, CD36, CD68, CD31, detection of IgG, IgM, C3 and T and B cell markers (Bălănescu et al., 2010).

Skin biopsies of uninjured skin will be obtained from the thigh area, at baseline and at 6 months for the fine fiber neuropathy assessment through immunostaining with PGP 9.5 (Birnbaum & Bingham, 2014).

- Pharmacokinetic-pharmacodynamic model of use of thalidomide will be performed in two groups of 5 patients with different doses (50 mg/day and 100 mg/day).

Outcomes: evaluations on days 0 and 30.

a) Improvement in skin symptoms and severity scores [(Cutaneous Lupus Area and Severity Activity Index score (CLASI)]. Change in CLASI score assessing the activity/severity criteria from 0 to 70 will be performed. The reduction represents an improvement in skin injury related to lupus in terms of activity and/or severity. CLASI evaluates erythema and scar/hypertrophy/atrophy in 13 anatomical areas. Membrane lesions and alopecia are also part of this index.

b) Cytokine dosage. Plasma levels of TNF-α and IFN-1 will be determined by Luminex MAP (EMD Millipore Corporation®). Plasma samples will be stored at -80oC until use.

TARGET POPULATION

Inclusion criteria:

- Adult patients with SLE (according to the classification criteria of the American College of Rheumatology) (Hochberg, 1997) with active skin lesions at the entrance to the research despite the use of hydroxychloroquine and/or prednisone and/or immunosuppressants who are eligible for thalidomide use.

- Male patients (who agree to use condoms during the entire period of treatment with thalidomide and up to 30 days after the end of it, even if they have already undergone vasectomy*) and women without fertility potential [i.e., who are in the postmenopausal phase (with amenorrhea > 1 year and confirmed by estradiol, FSH and LH levels), or who have undergone tubal ligation or hysterectomy] and who agree to the use of this drug according to the Term of Responsibility/Clarification to the introduction of thalidomide and each renewal of the prescription (Ministry of Health, 2013).

- Wish to participate in the study according to the signed TCLE.

- NORMAL ENMG at study entry.

Exclusion criteria:

Alcoholism.

- The patient's desire not to participate or leave the study at any time (without any harm to their care).

- History of peripheral neuropathy.

- Previous history of thrombophilia or presence of positive antiphospholipid antibodies.

- Renal and/or central nervous system and/or hematological activity (hemolytic anemia and/or platelet to < 50,000 platelets) concomitantly.

*Condoms will be provided by researchers (Ministry of Health, 2013).

RELEVANT METHODS

Laboratory determinations

HPLC MS/MS: The determination of blood and salivary levels of thalidomide will be performed by HPLC and tandem mass spectrometry (Teo et al., 2004).

- Pharmacokinetic analysis: The pharmacokinetic parameters of thalidomide will be calculated in the 24 h dose range based on the total plasma concentration versus time curves. The Winnonlin program, version 4.0 (PharsightCorp., Mountain View, California, USA) and the Non-linear mixed effect (NONMEN) program will be employed. The area under the plasma concentration versus time curve will be calculated in the 24-h dose interval by the trapezoid method on days 0 and 30. The apparent total clearance (ClT/F) will be obtained through the equation: ClT/F= Dose/AUC0-24. The apparent volume of distribution (Vd/F) will be calculated by the standard software equation. The free concentration of thalidomide will be determined by the dosage in saliva. The PK-PD analysis will be performed using as pharmacokinetic parameters the total and free plasma concentrations of thalidomide and, as a pharmacodynamic parameter, we will use the clinical/laboratory indices obtained by the evaluation of SLEDAI-2K (American College of Rheumathology). The PK-PD model will be developed using the non-linear mixed effect (NONMEM).

Cytokines. Plasma levels of TNF-α,IFN-1, BAFF and TGF-β will be determined by Luminex MAP (EMD Millipore Corporation®) technology. Plasma samples will be stored at -80oC until use.

thalidomide. Heparinized plasma samples will be immediately transferred to ice bath and acidified with citric acid 0.2 M pH 1.5 (v/v), to prevent spontaneous hydrolysis of thalidomide and stored at -80ºC until use (Eriksson, 1992). Plasma thalidomide levels will be determined by LC-MS (Bai,2013). For this, internal reference standard, deutered thalidomide (d4-thalidomide), will be added to the samples, prior to the addition of the mixture of methanol:ammonium acetate (50:50%, v/v) containing 0.2% formic acid. The extraction stage will be made by adding a mixture of ether-dichloromethane (3:2) and, after centrifugation, the organic phase will be collected and subjected to evaporation. The residue will be reconstituted in a mobile phase and injected into the LC-MS/MS system for analysis, using analytical column C18 (with particles of 5 μm) and under isocratic elution with mobile phase consisting of methanol-ammonium acetate-formic acid (60:40:0.04, v/v/v). Mass spectrometry with electrospray-type ionization source will be operated in positive mode. Plasma blank samples will be used for the calibration step of the HPLC MS/MS procedure by adding the calibrator (thalidomide) at various concentrations and processed similarly.

Histological analyses

Histological preparations will be obtained by skin biopsy using a punch of up to 6 mm or spindle excision. The skin specimens will then be fragmented. One fragment will be immersed in Michel's medium for direct immunofluorescence examination and the other fixed in pH buffered formalin 7.4 for histological and immunohistochemistry techniques.

Skin specimens fixed in formalin will be submitted to routine histological techniques, hematoxylin-eosin and periodic Schiff acid. All specimens will be evaluated for confirmation of the diagnosis of lupus erythematosus (Ackerman, 1978).

For immunohistochemistry techniques, the primary antibodies and their origins will be as follows: CD3, CD25, CD21, CD35, CD36, CD31, CD68, CD123, TNF-α, TGF-β1, caspase 3 cleaved and PGP 9.5.

Histological sections submitted to immunohistochemistry techniques to demonstrate cellular and cytokine elements will be digitized by the Pannoramic Scan (3D Histech, Hungary) scanner to obtain virtual histological slides. The reading of the material and capture of the images to be analyzed will be carried out with the program Pannoramic Viewer version 1.15.2.21080 (3D Histech, Budapest, Hungary). The quantification of immune tagged cellular elements will be performed with the aid of image analysis program Image Pro Plus version 4.5.0.29 (MediaCybernetics Inc. , Rockville, MD, USA).

The quantification of fine nerve fibers will be performed by counting intraepidermal nerve fibers per millimeter of dermal epidermal junction and the relationship between area of marked subepidermal nerve fibers and total area of papillary dermis examined (Rodrigues Júnior, 2011).

Safety

Patients who fit the safety recommendations of the Ministry of Health will be included.

Clinical (with special attention to symptoms of peripheral polyneuropathy) and laboratory of adverse effects (including leukocyte count) will be performed at the entrance into the study and each medical consultation.

The ENMG will be performed to include in the study, at 6 months and at the end of the study.

If there is no clinical response within 2 months of the use of thalidomide, or in the clinical suspicion or enmg of peripheral neuropathy at any time, thalidomide will be suspended (out of the study) and follow-up of the patient will be continued in the outpatient clinics of the Rheumatology and Dermatology Services of HC-FMUSP.

WITHDRAWALS

See item Security.

ORIGINALITY WITH CARE IMPACT

There are no studies correlating tissue expression of cytokines with blood and salivary levels of thalidomide and its possible role as a predictor of response and adverse effects in patients in skin lesions of SLE. In addition, there are no studies on the value of skin biopsy in the diagnosis of thalidomide-induced neuropathy.

The expected impact with the availability of blood and salivary dosage of thalidomide in clinical practice is that this parameter may be a predictor of clinical response and neuropathy.

ADVANCES IN MECHANISM

The expected advance is a better understanding of the role of thalidomide in the expression of cytokines in the cutaneous lesion of SLE.

In addition, we hope to determine whether the mechanism of thalidomide-induced neuropathy would target thin nerve fiber injury to histological skin analysis of patients treated with this medication.

SAMPLE SIZE

This is a convenience sample.

STATISTICAL PLAN

Patients with vs. without therapeutic response to thalidomide and vs. without signs of peripheral polyneuropathy will be compared for categorical variables by fisher's exact or chi-square test and, as for

quantitative variables, by t-Student or Mann-Whitney tests, when applicable. The influence of the different variables on disease activity and polyneuropathy will be evaluated by multivariate analysis.

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