EMJ EUROPEAN MEDICAL JOURNAL

EUROPEAN MEDICAL JOURNAL

ISSN 2397-6764 Vol 3.3 • September 2018 • europeanmedical-journal.com

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Spencer Gore, CEO

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European Medical Journal is published four times a year. For subscription details please visit: www.europeanmedical-journal.com

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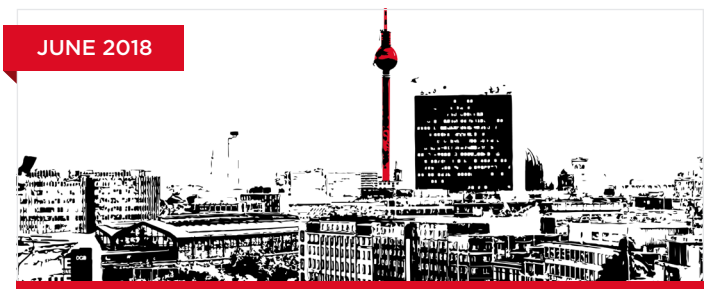
EMJ 3.3



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Welcome

Hello and welcome. It is my great pleasure to introduce to you the third edition of the *European Medical Journal* for 2018: *EMJ 3.3*. As always, our flagship journal melds articles from a variety of therapeutic disciplines together to create a truly unique and rewarding reading experience. This edition details wonderful research from many areas of interest, with rheumatology and haematology particularly well represented in the following pages.

Indeed, a haematology article has been selected as this issue's Editor's Pick by one of our Editorial Board members, Dr Emanuele Angelucci; written by Paubelle et al., the paper explores the use of chimeric antigen receptor (CAR)-engineered T cell therapy for the treatment of acute myeloid leukaemia. This thorough overview chronicles the latest developments in CAR T cell therapy, taking note of the many obstacles and breakthroughs along the way. There is still a long way to go but the future is bright for this important treatment modality.

For the nephrologists among you, this edition has an exciting article by Sifuentes-Franco et al. detailing oxidative DNA damage and apoptosis markers in acute renal graft dysfunction; the results of this study represent some of the first evidence exploring the relationship between oxidative stress, apoptosis, and graft dysfunction. This edition also features a paper by Wen and Ho discussing how to distinguish alcoholic from nonalcoholic steatohepatitis, alongside a review by Shiani et al., wherein the diagnostic accuracy of magnetic resonance cholangiopancreatography is compared to endoscopic retrograde cholangiopancreatography in the postorthotopic liver transplant population.

EMJ 3.3 is brimming with the latest clinical updates, and this is especially true for Panda and Mabalirajan's paper summarising the most recent research on corticosteroid resistance in asthma, as well as presenting some promising strategies for tackling steroid-resistant disease. With many more exciting articles within, this edition of the *European Medical Journal* is not to be missed.

Medicine is a complex field of many interconnected specialities and, as our understanding of disease grows, doctors from every background are finding themselves increasingly encouraged to connect and engage with their interdisciplinary colleagues. I am delighted to present to you the great variety of high-quality peer-reviewed papers contained within the pages of *EMJ 3.3*, and I hope they will inspire you to broaden your horizons, strike out onto paths (and pathologies!) unknown, and celebrate the wonderful complexity of the human body.

Enjoy.

Spencer Gore

Chief Executive Officer, European Medical Group

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Abbreviations CML, chronic myeloid leukaemia; CP, chronic phase; ELN, European LeukaemiaNet; MMR, major molecular response, ≤0.1% BCR-ABL1 transcripts on the international scale with ≥3,000 ABL1 assessed. MR4.5, ≤0.0032% BCR-ABL1 transcripts on the international scale with ≥30,990 ABL1 assessed; Ph+, Philadelphia chromosome-postive; TEAE, treatment-emergent adverse event.

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Foreword

Dear colleagues and friends,

I am very pleased to introduce this important new issue of the *European Medical Journal*, which contains important articles from various fields of medicine. First of all, I would like to underline that all the published articles help physicians in understanding important advances derived from translational and clinical research that will have an immediate impact on our daily clinical practice. It is hard but at the same time dutiful and fascinating to keep up with innovation, and I am convinced that this is one of the most beautiful characteristics of our jobs as medical professionals.

In *EMJ 3.3*, several issues from various medical disciplines are reported. These include research on oxidative stress and apoptosis markers in acute renal failure, analyses of different diagnostic techniques in postorthotopic liver transplant and the role of alcohol abuse on liver transplant outcomes for steatohepatitis, updates on corticosteroid resistance in asthma, the pathogenesis and treatment of rheumatoid arthritis, infection after knee surgery, and an overview of a real-world experience in the long-debated use of biosimilars in Europe.

As a haematologist, let me focus a little bit more on three haematology papers published in this issue. A biological overview of Epstein–Barr virus (EBV) infection is provided, discussing two EBV-related lymphoproliferative diseases reported in the 2017 World Health Organization (WHO) classification with different incidences in developed and less-developed countries, with both clinical and social implications evident. A second paper provides an in-depth look into the complex interplay of aplastic anaemia, myelodysplastic syndrome, and paroxysmal nocturnal haemoglobinuria; these syndromes appear on the surface to be distinct disorders but are revealed to be closely interconnected with significant overlapping features. Every one of us who has encountered this situation will immediately understand how important it is to be confident with these different but potentially interrelated diseases.

Finally, I have chosen the article by Paubelle et al. on chimeric antigen receptor (CAR)-engineered T cell therapy in acute myeloid leukaemia as my Editor's Pick. This article is important not only because it illustrates a possible new frontier for leukaemia therapy, but also because it highlights the development of this new fascinating therapeutic approach in a very clear and understandable way, which is frequently but often inaccurately reported by the media. This article is an important source of updates for all interested colleagues.

Of course, all articles have been peer-reviewed to ensure the quality and originality of content. I hope you enjoy reading as much as I did!

Dr Emanuele Angelucci

IRCCS Ospedale Policlinico, San Martino, Italy

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Chimeric Antigen Receptor-Engineered T Cell Therapy in Acute Myeloid Leukaemia

EDITOR'S PICK

This interesting, very well written article by Paubelle et al. on the use of chimeric antigen receptor (CAR) T cell therapy in acute myeloid leukaemia (AML) offers a clear, understandable, direct, and comprehensive overview of the CAR T therapy story, while highlighting the difficulties that have been encountered by researchers along the way. Once again, it is demonstrated that medical research is paved with difficulties and barriers that must be negotiated before achieving success.

CAR T therapy has so far registered impressive, although not yet definitive, advances for the treatment of lymphoid diseases; however, little has been reported on CAR T cell use in AML, a field in which clinical advances are urgently required. By reading this article, you will gain a clear idea of CAR T therapy developments, together with the future potential and problems associated with the use of the treatment for AML patients.

Dr Emanuele Angelucci

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Disclosure: The authors have declared no conflicts of interest.

Received: 03.04.18

Accepted: 19.06.18

Keywords: Acute myeloid leukaemia (AML), chimeric antigen receptor (CAR) T cells, immunotherapy.

Citation: EMJ. 2018;3[3]:11-19.

Abstract

Acute myeloid leukaemia (AML) is a disease with a very poor outcome and remains an area of significant unmet need, necessitating novel therapeutic strategies. The progress made in the field of immunotherapy, in particular chimeric antigen receptor (CAR)-engineered T cells, has given rise to many hopes for pathologies such as B cell acute lymphoblastic leukaemia and B cell lymphoma, and many studies have attempted to translate these successes to AML. This review summarises the recent advances in, and defines an ideal target for, CAR T cell therapy in AML.

INTRODUCTION

Acute myeloid leukaemia (AML) is an aggressive malignant disease affecting 2.5–3.5 per 100,000 adults each year in Western countries.[1,2] Over the last decade, considerable advances have been made in the identification of molecular markers, leading to the improvement of risk stratification, especially in AML patients with a normal karyotype.[3] However, in the past 5 years, the cure rate has been 35–40% for AML patients <60 years old and 5–15% for patients >60 years old.[4] The elderly, who are unfit to have intensive chemotherapy, have a median overall survival of 4–10 months.[2] Despite improved understanding of leukaemogenesis, AML still has poor outcomes due to high disease and treatment-related mortality. To date, allogeneic haematopoietic stem cell transplantation (alloHSCT) remains the most effective treatment for relapse prevention in non-favourable-risk patients with AML[5,6] and has remained the most successful immunotherapeutic treatment for AML patients, especially with the advances made in using alternative donors;[7] however, this therapeutic strategy has a high toxicity without guaranteeing the absence of relapse.[8] Thus, it is a public health issue that new, more effective, and less toxic treatments should emerge for the treatment of AML. In this regard, chimeric antigen receptor (CAR)-engineered T cells have been developed and used in paediatric acute lymphoblastic leukaemia (ALL), resulting in complete remission without graft versus host disease (GvHD).[9]

CAR T cells are artificial molecules comprising an extracellular antigen-binding domain, commonly derived from a monoclonal antibody in the form of a single chain fragment variable (scFv), and an intracellular signalling domain associated with ≥1 costimulatory signalling modules.[10,11] The first generation of CAR T cells were developed in ovarian metastatic carcinomas[12] and built on the model scFv-spacer-CD3ζ. Unlike the T cell receptors, they recognise the antigen in the absence of major histocompatibility complex proteins; therefore, CAR T cells can specifically target various surface antigens on malignant cells.[13]

Nonetheless, first-generation CAR T cells exhibit a lack of cytokine secretion, insufficient proliferation, and low persistence *in vivo*. To improve these shortcomings, the next generation of CAR T cells involved the addition of costimulatory signalling domains, such as CD28 and 41BB or CD3ζ,[14] to the cytoplasmic tail of the CAR to provide additional signals to T cells. The addition of these costimulatory proteins limited apoptosis of CAR T cells because of the synthesis of IL-2.[15] Preclinical studies have indicated that this second generation improves the antitumour activity of T cells.[15] Recently, third-generation CAR T cells combine multiple signalling domains, such as CD3ζ–CD28–41BB or CD3ζ–CD28–OX40, to improve activity;[16] however, this improvement has not demonstrated a significant clinical impact when compared with the prior generation of CAR T cells. For example, the CAR T cell strategy is highly specific in redirecting T cells towards predefined target cells but reaches its limits when targeting solid tumours with phenotypic heterogeneity.[17] After initial tumour reduction by CAR T cells, antigen-negative cancer cells not recognised by CAR may give rise to tumour relapse. This situation might be overcome by CAR-mediated activation of T cells in the tumour, releasing inducible IL-12 that increases tumour-infiltrating T cell activation and attracts and activates innate immune cells to eliminate the antigen-negative cancer cells in the targeted lesion; these fourth-generation CAR T cells are called TRUCK T cells.[18] The evolution of CAR T cells is shown in Figure 1.

Recently, the U.S. Food and Drug Administration (FDA) gave approval for two third-generation CAR T cells targeting CD19;[19] the first was tisagenlecleucel in B cell-lineage ALL, followed by axicabtagene ciloleucel in diffuse large B cell lymphoma and B cell-lineage ALL. This therapeutic strategy may be applicable to a large number of tumoural pathologies, including AML, for which there is a need for treatment in view of the poor results observed in adults and children. This review therefore focusses on CAR T cells for the treatment of AML.

TARGETING OF CHIMERIC ANTIGEN RECEPTOR T CELLS IN ACUTE MYELOID LEUKAEMIA

CAR T cell therapy targeting CD19 has yielded remarkable outcomes in paediatric and young adult patients with relapsed or refractory

diffuse large B cell lymphoma and ALL.[20-27] Many groups have attempted to reproduce these encouraging results in other pathologies, such as AML; however, because of the lack of expression of CD19 on the surface of AML cells, CAR T cells directed against CD19 have no efficacy in this pathology.[28] An ideal target for CAR T cells in AML should be highly expressed in most of the malignant cells, including leukaemic stem cells (LSC), and should be shared by most AML patients. Unlike naïve T lymphocytes, which can signal through the T cell receptor in response to very low antigen density, CAR T cells need a higher density of antigen to be effective.[29-32] Expression of the target by normal cells may or may not be associated with high toxicity but, to prevent toxicity, the target should not be shared by vital tissues such as the heart, liver, and normal haematopoietic stem cells (HSC). An approach to remedy this pitfall is the use of an algorithm integrating a large dataset of transcriptomics and proteomics from malignant and normal tissues to identify potential targets expressed in LSC but not in normal CD34+CD38- haematopoietic cells, T cells, or vital tissues.[33] However, using CAR T cells in AML remains difficult due to the non-restricted expression of AML-associated antigens. Moreover, CAR T cells may persist in the organism for several years,[34] thus an unwise target choice may lead to prolonged aplasia. Table 1 summarises the different clinical trials currently underway employing CAR T cells in AML.[35-42]

CD33

CD33 (also known as Siglec-3) is a transmembrane receptor expressed on cells of myeloid lineage and on approximately 90% of AML cells.[43-45]

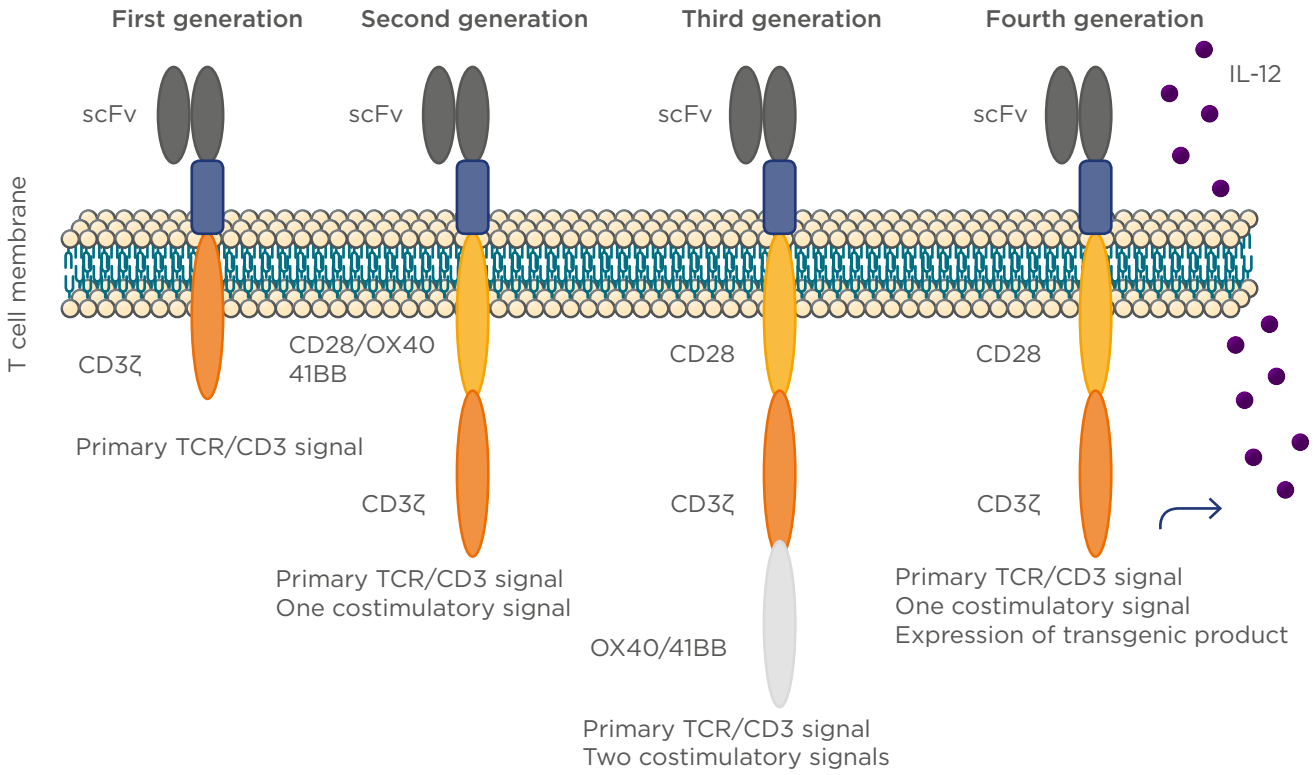

Figure 1: The evolution of chimeric antigen receptor T cells.

First-generation CAR T cells consist of a single-chain fragment of variable region antibody for target binding linked by a spacer domain to the transmembrane and intracellular signalling domain of CD3ζ derived from the T cell receptor. Addition of a costimulatory domain, mostly of the CD28 family, makes up second-generation CAR T cells. Third-generation CAR T cells contain two costimulatory domains. Fourth-generation CAR T cells (also known as TRUCK T cells) are additionally modified with a constitutive or inducible expression cassette for a transgenic protein, for instance a cytokine, which is released by the CAR T cell to modulate the tumour T cell response.

CAR: chimeric antigen receptor; scFv: single-chain fragment variable region; TCR: T cell receptor.

Table 1: Current clinical trials using chimeric antigen receptor T cells for acute myeloid leukaemia.

Study number	AML type	Target	Costimulation domain	Dosage	Associated conditioning	Clinical phase
NCT03473457[35]	R/R	CD38/CD33/ CD56/CD123/ CD117/CD133/ CD34/Mucl-	N/A	0^6–10^7/kg	N/A	II
NCT03222674[36]	R/R	Muc1/CD33/ CD38/CD56/ CD117/CD123	N/A	N/A	N/A	I/II
NCT03114670[37]	Relapsed after alloHSCT	CD123	41BB-CD3ζ	N/A	N/A	I/II
NCT03126864[38]	R/R	CD33	N/A	$5x10^8$–$5x10^{10}$/kg	Fludarabine–cyclophosphamide	I
NCT03190278[39]	R/R	CD123	N/A	$6.25x10^5$–$6.25x10^6$/kg	N/A	I
NCT02159495[40]	All AML	CD123	CD28-CD3ζ	N/A	Fludarabine–cyclophosphamide	II
NCT01864902[41]	R/R	CD33	CD137-CD3ζ	N/A	N/A	I
NCT01716364[42]	MM, AML, MDS	LeY	CD28	N/A	N/A	I

ALL: acute lymphoblastic leukaemia; alloHSCT: allogeneic haematopoietic stem cell transplantation; AML: acute myeloid leukaemia; LeY: Lewis antigen Y; MDS: myelodysplastic syndrome; MM: multiple myeloma; N/A: not applicable; R/R: relapsed/refractory.

It is usually considered myeloid-specific but can also be found on lymphoid cells and non-haematological tissues.[45] Gemtuzumab ozogamicin (Mylotarg®, Pfizer, New York City, New York, USA) is an antibody-targeted chemotherapy agent consisting of a humanised murine anti-CD33 monoclonal antibody (clone P67.6) linked to a calicheamicin Y1 derivative via a hydrolysable bifunctional linker.[46] Gemtuzumab ozogamicin induces cell death in CD33-positive cells by internalisation of the calicheamicin drug and then cleavage of double-stranded DNA,[47] and has shown promising results in AML.[48,49] Using an antiCD33 scFv, CAR T cells targeting CD33 have been developed for the treatment of AML, and promising results of murine AML models[50,51] and humans[52] have been reported. Several clinical trials are currently ongoing in AML with CAR T cells directed against CD33.

CD123

CD123 is the alpha chain of the IL-3 receptor and is a cluster-like protein of differentiation encoded by *IL3RA* on the human X chromosome. It is expressed on the surface of several leukaemic cells, including cells of AML, plasmacytoid,[53] hairy cell leukaemia,[54] and Hodgkin's lymphoma,[55] thus constituting a potential target for the treatment of these diseases. Although CD123 presents low expression levels on normal haematopoietic cells,[56] it may be a possible target for AML.[57] CAR T cells directed against CD123 in AML mouse models resulted in a reduction of leukaemic burden but showed contrasting results on normal haematopoiesis.[58-62] Modifying the scFv directed against CD123 may be able to reduce myelotoxicity in an AML murine model,[62] and clinical studies are underway in AML patients with CAR T cells targeting CD123.

Lewis Y Antigen

The Lewis antigen system is a human blood group system based on genes of chromosome 19. The Lewis antigen Y (LeY) is an oligosaccharide overexpressed on most epithelial cancer cells and haematological malignant cells, including AML cells,[63,64] but is weakly expressed by healthy tissues.[65,66] A Phase I study with second-generation CAR

T cells directed against LeY in four AML patients reported two cytogenetic remissions without Grade 3 or 4 side effects and with long-term persistence of the CAR T cells.[67] Moreover, *in vivo* models have shown promising results of CAR T cells targeting LeY in epithelial cancers without significant toxicity.[68]

FMS-Like Tyrosine Kinase 3

FMS-like tyrosine kinase 3 (FLT-3/CD135) is a receptor-type tyrosine-protein kinase, or fetal liver kinase-2, that belongs to the receptor tyrosine kinase Class III family of cytokine receptors and binds the FLT-3 ligand. It is expressed on the surface of many haematopoietic progenitor cells and its signalling is important for the normal development of HSC and progenitor cells. The *FLT3* gene is one of the most frequently mutated genes in AML;[69] high levels of wild-type FLT-3 have been reported for blast cells of some AML patients without *FLT3* mutations. These high levels of wild-type FLT-3 may be associated with worse prognosis.[70] It was recently reported that CAR T cells directed against FLT-3 in *in vivo* and *in vitro* models had a potent reactivity against AML cell lines and primary AML blasts expressing either wild-type FLT-3 or FLT-3 with internal tandem duplication. However, as anticipated, FLT-3-CAR T cells recognised normal HSC *in vitro* and *in vivo* and disrupted normal haematopoiesis in colony-formation assays, suggesting that adoptive therapy with FLT-3-CAR T cells will require subsequent CAR T cell depletion and alloHSCT to reconstitute the haematopoietic system.[71]

Folate Receptor β

Folate receptor β (FOLR2) is a protein encoded by the *FOLR2* gene,[72] and is expressed on myeloid lineage cells and frequently upregulated in AML cells.[73] Preclinical models using CAR T cells directed against FOLR2 have shown promising results on AML cells while preserving normal HSC; the concomitant use of all-trans retinoic acid, which allows an upregulation of FOLR2, improved efficiency against AML.[74]

Hyaluronate Receptor

The hyaluronate receptor (CD44) is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion, and migration, and is expressed on many mammalian cell types. CD44 variant domain 6 (CD44v6) is a variant isoform expressed in multiple myeloma[75] and AML.[76] CAR T cells directed against CD44v6 have shown antileukaemic effects in mouse models, although at the cost of leukopenia.[77] Further steps may involve adding a suicide gene to this construct.[78]

CD38

CD38, also known as cyclic ADP ribose hydrolase, is a glycoprotein[79] found on the surface of many immune cells (specifically white blood cells), including T, B, and natural killer lymphocytes. CD38 also functions in cell adhesion, signal transduction, and calcium signalling.[80] CD38 is expressed on AML cells but not on normal HSC.[81,82] As for FOLR2, all-trans retinoic acid enhances CD38 expression and its action is synergistic with CAR T cells targeting CD38.[83]

Natural Killer Group 2D

Natural killer group 2D (NKG2D) is a transmembrane protein belonging to the CD94/NKG2 family of C-type lectin-like receptors.[84] NKG2D is highly expressed in AML tissue but weakly on healthy tissue.[85] Several CAR T cells directed against NKG2D have been clinically developed, leading to complete remissions without neurotoxicity, cytokine release syndrome (CRS), or treatment-related mortality.[86]

CD7

CD7 is a transmembrane protein and a member of the immunoglobulin superfamily. This protein is found on thymocytes and mature T cells and plays an essential role in T cell interactions and in T cell–B cell interaction during early lymphoid development.[87] In AML, its expression correlates with chemoresistance.[88] CAR T cells directed against CD7 have shown potent efficiency *in vitro* and in murine AML models with low toxicity.[89]

C-type Lectin Domain Family 12 Member A

C-type lectin domain family 12 member A (*CLEC12A*) is a member of the C-type lectin/C-type lectin-like domain superfamily. Members

of this family share a common protein fold and have diverse functions, such as cell adhesion, cell–cell signalling, glycoprotein turnover, and roles in inflammation and immune response. The protein encoded by this gene is a negative regulator of granulocyte and monocyte function[90] and is overexpressed on the surface of AML cells; CLEC12A could therefore serve as a target for CAR T cells to negate minimal residual disease[91] or target LSC.[92]

TOLERANCE OF CHIMERIC ANTIGEN RECEPTOR-ENGINEERED T CELLS IN ACUTE MYELOID LEUKAEMIA

Off-Target Side Effects and Haematological Tolerance

Given the lack of specificity of the target antigens for the tumour cells, CAR T cells may have an action on normal tissues expressing these antigens. In addition, a number of these target antigens could be expressed by normal HSC and therefore myelosuppression would be expected as a consequence. However, the target selection process and the representability of the murine models made it possible to avoid major haematological toxicity in clinical trials with CAR T cells targeting CD33[52] and LeY.[67]

Cytokine Release Syndrome

By linking target antigen-expressing malignant cells to cytotoxic T cells, T cell-engaging therapies harness the cell-mediated immune response and direct it against malignant cells, bypassing the major histocompatibility complex. Clinical trials using CAR T cells have demonstrated high complete response rates;[10,26,89] however, complete remissions have been associated with CRS, the intensity of which ranges from mild to life-threatening.[9,34,93] Mild CRS symptoms are characteristic of flu-like syndrome, with fevers and myalgias, while some patients experience a severe inflammatory syndrome, including vascular leak, hypotension, pulmonary oedema, and coagulopathy, resulting in multiorgan system failure. The pathophysiology of CRS is related to a significant increase in the release of cytokines, such as IL-10 and IL-6, due to the activation and proliferation of T lymphocytes.[94] Among the cytokine storm, IL-6 appears to be the central mediator of CRS[94] and clinical studies have shown that the use of corticosteroids or an IL-6 receptor antagonist antibody (tocilizumab) allows CRS control induced by CAR T cells.[9] Other approaches, such as adding a suicide gene and kill-switch strategies,[15] are possible. Due to the increased experience of corticosteroid and tocilizumab use, a consensus is emerging for the management of CRS.[94] CRS does not spare AML patients treated with CAR T cells and has been reported after the use of CAR T cells against CD33[52] and CD123.[95]

Other Toxicities

The development of neurologic toxicities, including confusion, delirium, myoclonus, aphasia, and seizure, has been reported in patients receiving CD19-specific CAR T cells.[23,24] However, these side effects are also found with blinatumomab[96] and seem specific to CD19, so should therefore not be found in AML. Anaphylaxis can also occur because CAR T cells contain antigen-recognition domains derived from murine antibodies[97] and efforts are ongoing to humanise the components of expressed proteins. The risk of insertional oncogenesis in human cells has also been established in the context of gene therapy of HSC.[98] Insertion of a transgene into T cells may induce malignant transformation; however, no cases of transformation have been reported following the infusion CAR T cells.

CONCLUSION AND REMAINING CHALLENGES

The progress of immunotherapy brings new hope for cancer treatment. Like in ALL, CAR T cells provide much hope for the treatment of AML. However, unlike in ALL, there is no target antigen such as CD19 or CD22 in AML, and the target choice can lead to off-target effects. CD123 and CD33 are the major targets, either separately or on constructions targeting both at the same time,[99] and results of the numerous ongoing clinical trials need to be awaited to weigh the potential benefits versus side effects of these new strategies. Moreover, CAR T cells are currently reserved for relapsed or refractory AML patients; therefore this new strategy will have to find its place in a therapeutic arsenal that already contains

alloHSCT as well as new targeted therapies. In order to reduce off-target toxicities, several strategies may be used. The identification of novel leukaemia-associated antigens or neoantigens could provide more specific targets and transcriptomic and proteomic analyses are ongoing to fully characterise the AML 'surfaceome'. Furthermore, dual-targeting or sequential approaches could improve treatment specificity while relying on combinations of AML antigens. In addition, the understanding and prevention of the escape mechanisms of CAR T cells in AML remains a challenge. The strategy to address the relapses due to antigen escape involves designing CAR T cells able to target multiple antigens simultaneously or sequential strategies. Moreover, relapses in AML are caused by the persistence of LSC, which do not necessarily express the same antigens as the bulk cells, and therefore LSC could persist despite the use of CAR T cells. The exorbitant cost of these new strategies will also be a societal challenge, but the democratisation of CAR T cells may lead to lower costs. Therefore, CAR T cells could competitively challenge the prominent place of alloHSCT for the treatment of AML.

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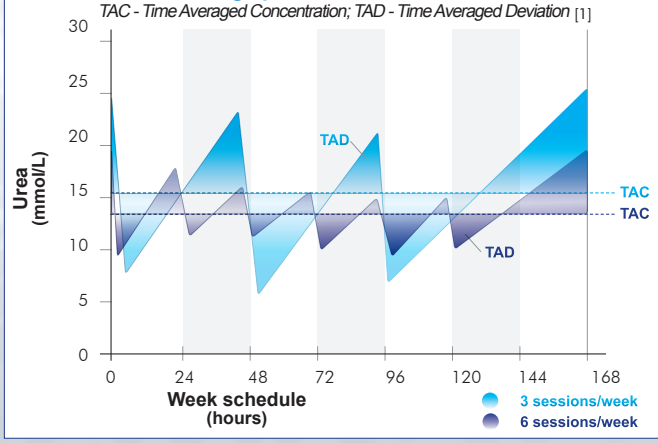

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Cardio-vascular system	
Hypertension [2,3]	↘
Antihypertensive treatment [2]	↘↘
Left ventricular hypertrophy [4,5]	↘
Phospho-calcic metabolism	
Phosphorus [6,7]	↘
Phosphate binders dose [6]	↘↘
PTH [6]	↘
Quality of life	
Quality of life (SF36) [8]	↗
Recovery time after dialysis session [8]	↘↘
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Adherence During Early Allergen Immunotherapy and Strategies to Motivate and Support Patients

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Disclosure: Prof Dr Novak has received grants and personal fees from ALK-Abelló and personal fees from Bencard Allergy Therapeutics, HAL Allergy, LETI Pharma, Lofarma, and Stallergenes Greer. Prof Dr Buhl has received personal fees from ALK-Abelló, MEDA Pharma, and Novartis and non-financial support from Thermo Fisher Scientific. Prof Dr Pfaar has received grants and personal fees from ALK-Abelló, Allergopharma, Anergis SA, ASIT Biotech Tools SA, Bencard Allergie GmbH/Allergy Therapeutics, HAL Allergy Holding BV/HAL Allergie GmbH, Laboratorios LETI/LETI Pharma, Lofarma, and Stallergenes Greer; grants from Biomay, Circassia, GlaxoSmithKline, and Nuvo; and personal fees from Indoor Biotechnologies, MEDA Pharma, Mobile Chamber Experts (a GA2LEN Partner), Novartis Pharma, and Pohl-Boskamp.

Acknowledgements: Writing assistance was provided by Paul Scutt, Ascend, Manchester, UK.

Support: The publication of this article was funded by ALK. The views and opinions expressed are those of the authors and not necessarily those of ALK.

Received: 03.07.18

Accepted: 13.08.18

Keywords: Adherence, allergen immunotherapy (AIT), allergic rhinitis (AR), quality of life (QoL), subcutaneous immunotherapy (SCIT), sublingual immunotherapy (SLIT).

Citation: EMJ. 2018;3[3]:21-29.

Abstract

Allergic rhinitis is one of the most common chronic inflammatory conditions, affecting up to 30% of people in Europe. Allergen immunotherapy (AIT) is the only treatment for allergic rhinitis and asthma that has a disease-modifying effect, and it is recommended in European guidelines for use in conjunction with patient education, specific allergen avoidance, and symptomatic pharmacotherapy. Reported AIT adherence rates vary widely but are often low in real-world settings. Factors known to affect adherence are patient, treatment, or physician-related, and vary between healthcare settings. Misconceptions or a lack of AIT knowledge among patients with regard to efficacy and side effects may contribute to high rates of discontinuation observed during the first year of AIT treatment. Interventions to improve patient adherence are multifaceted and should focus on patient education, particularly the provision of accurate information regarding adverse effects of AIT and when to expect an improvement in symptoms, patient-support programmes, and the use of regular

eHealth reminders via a telephone call, text message, or social media. Serum-based biomarkers also have the potential to play a role in evaluating early response to AIT and in monitoring treatment adherence in clinical practice. In this review, the authors explore barriers to continuation with AIT and discuss initiatives to motivate and support patients through the challenging early months of treatment, prior to the onset of clinical effect and when side effects are most common, to encourage long-term adherence to therapy and achieve optimal patient outcomes.

INTRODUCTION

Allergic rhinitis (AR) is characterised by multiple symptoms involving the upper airways, nose, and eyes.[1] It is one of the most common chronic inflammatory conditions, affecting approximately 20–40% of the global population and 15–30% of people in Europe,[2-6] and frequently coexists with asthma.[7] AR can impair sleep and mood, negatively impacting quality of life (QoL), daily activities, social functioning, productivity, and work performance, and represents a considerable burden on public health.[3,8]

Allergen immunotherapy (AIT) is the only treatment for AR and asthma that has a disease-modifying effect.[1] Guidelines recommend that the management of AR combines AIT with patient education, specific allergen avoidance, and symptomatic pharmacotherapy.[9] AIT involves the repeated administration of allergens over a period of years (optimally 3–5 years)[1,10] to reduce clinical and immunological responses to the allergens and induce allergen-specific immunological tolerance.[11-14] AIT is typically administered either as monthly injections of subcutaneous immunotherapy (SCIT) or daily oral doses of sublingual immunotherapy (SLIT) as tablets or liquid drops. SCIT requires visits to the clinic for dosing, while SLIT can be administered easily at home following the initial dose.[13,14]

EFFICACY AND SAFETY OF ALLERGEN IMMUNOTHERAPY

AIT has proven long-term efficacy in reducing allergic symptoms and the need for symptomatic medication,[1,11,12,14-18] and has also been shown to reduce the risk of progression from AR to the development of asthma symptoms and/or the use of asthma medication in children.[19,20] It is important to note that an improvement, not complete resolution, of symptoms should be expected with AIT.[1] The manufacturers of all AIT products within Europe must follow the European Medicines Agency (EMA) guidance for the manufacturing and quality control of allergen products of biological origin, which provides an assurance of quality and effectiveness.[1,14,18]

Large clinical trials have demonstrated significant improvements to the QoL of patients with grass pollen AR treated with SLIT or SCIT.[10,22-25] However, there remains a need for further real-world studies examining QoL in patients receiving AIT. Both SCIT and SLIT demonstrate favourable safety and tolerability when administered in an appropriate setting and according to current guidance.[1,14,15]

COST-EFFECTIVENESS OF ALLERGEN IMMUNOTHERAPY

A systematic review of 24 studies conducted in Europe or North America, encompassing a range of perennial and seasonal allergic conditions due to house dust mite, grass or ragweed pollen, or a mixture of various allergens, demonstrated that AIT was associated with cost savings relative to symptomatic treatment.[26] Of the 6 studies that compared SLIT with SCIT, 4 found cost savings for SLIT and 2 for SCIT.[26] A health-technology assessment conducted in the UK that examined the relative costs of SLIT and SCIT showed that, when compared with symptomatic treatment, both therapies may become cost-effective at a threshold of £20,000–30,000 (equivalent to approximately €24,000–36,000 at the time of study publication in December 2013) per quality-adjusted life-year approximately 6 years after treatment initiation, or 5 years for SCIT compared with SLIT.[27] However, these estimates were based on limited data and a number of assumptions.[27] Since patients self-administer SLIT at home after the first dose, there is the potential for considerable time savings for both the patient and healthcare

provider, thereby improving cost-effectiveness compared with SCIT.[14] An economic evaluation of patients with AR found that when both direct and indirect costs were considered, 3-year expenditures per patient were €684 and €1,004 for SLIT and SCIT, respectively.[28]

Table 1: Differences in adherence and persistence rates with allergen immunotherapy in selected real-world studies.

Study	Key findings
Allam et al.,[32] 2018 Retrospective cohort study using prescription renewal rates for SQ-T grass pollen SLIT (n=2,429) or SCIT (n=2,109) among children, adolescents, and adults in Germany.	› Overall proportion of persistent patients having prescriptions in Year 3 was similar in the SLIT and SCIT subgroups (30% versus 31%, respectively; p=0.51). › Among those continuing treatment at Year 2, proportionally fewer SLIT-treated patients discontinued treatment in the third year. › The proportion of persistent patients was comparable between SLIT and SCIT within age groups. › The highest persistence was observed in the 5–14 years age group (34% versus 37%) and the lowest in the 15–17 years age group (19% versus 22%); adults: 30% for both groups.
Kiotseridis et al.,[35] 2018 Prospective, observational, noninterventional, open-label study of SQ-T grass pollen SLIT in adults (n=263) and children (n=163) in Denmark and Sweden.	› Overall, 55% of patients completed the 3-year treatment period. › 69% of children completed the 3-year treatment period. › 65% of discontinuations were made shortly after visits 2 and 3 (out of 5 visits). › Significant variation in adherence was observed between the clinics.
Senna et al.,[36] 2010 Analysis of sales figures for pollen and mite SLIT from two large manufacturers in Italy.	› Sales decreased from 100% to 43.7%±8.0% in the first year of treatment, to 27.7%±10.1% in the second year, and to 13.2%±7.5% in the third year. › Across the 20 administrative regions of Italy, sales figures ranged between 60% and 28% in the first year, 47% and 29% in the second year, and 16% and 6% in the third year.
Kiel et al.,[37] 2013 Retrospective analysis of a community pharmacy database containing data from patients starting grass pollen, tree pollen, or house dust mite SLIT (n=3,690) or SCIT (n=2,796) in the Netherlands.	› Overall, only 18% of users reached the minimum required treatment duration of 3 years (SLIT: 7%; SCIT: 23%). › Other independent predictors of premature discontinuation were prescriber (patients of general practitioners demonstrated longer persistence than those of allergologists and other medical specialists), single AIT, lower socioeconomic status, and younger age.
Sieber et al.,[38] 2011 National prescription database study of patients receiving grass pollen AIT during 2005–2007 in Germany (natural extract SLIT, n=112; natural extract SCIT, n=695; allergoid SCIT, n=602).	› In 2006, 71%, 55%, and 59% of patients had at least one renewal prescription of natural extract SLIT, natural extract SCIT, and allergoid SCIT, respectively, with corresponding rates decreasing to 51%, 34%, and 39% in 2007. › Persistence with natural extract SLIT was significantly higher than with natural extract SCIT (p=0.0015 for 2006; p=0.0003 for 2007) and allergoid SCIT (p=0.0152 for 2006; p=0.0111 for 2007). › There were no significant differences between the two SCIT groups.
More and Hagan,[39] 2002 Patients (N=381) enrolled in an AIT programme at a military medical centre in the USA.	› No differences in compliance rates between men and women. › Noncompliant patients were younger than compliant patients (35.4 years versus 42.4 years, respectively; p=0.001). › When patients were stratified by age (<18, 18–45, and >45 years), the youngest and oldest groups were more compliant (p<0.001).
Pajno et al.,[40] 2005 Study in children and adolescents aged 6–15 years with AR, allergic asthma, or both (SLIT, n=1,886; SCIT, n=806) in Italy.	› A significantly greater number of children receiving SLIT prematurely discontinued treatment compared with those receiving SCIT (22% versus 11%; p<0.005).

AIT: allergen immunotherapy; AR: allergic rhinitis; SCIT: subcutaneous immunotherapy; SLIT: sublingual immunotherapy; SQ-T: standardised-quality unit tablet.

CONTINUATION RATES WITH ALLERGEN IMMUNOTHERAPY

Adherence can be divided into the following stages: 1) initiation (also referred to as acceptance), 2) implementation (also referred to as compliance), and 3) persistence.[29] Lack of adherence to treatment is a growing global problem associated with the management of chronic diseases such as AR, contributing to decreased treatment efficacy as well as potential increases in rates of hospitalisation, morbidity, and mortality.[30] In general, the causes of poor adherence may be related to the patient, disease, treatment, or healthcare system.[31]

There is no consensus regarding an acceptable adherence rate to AIT. However, a rate >80% is generally considered to be adequate.[30] Persistence and adherence to AIT is reported to be lower in real-world settings than in clinical studies.[32,33] In clinical trials with a follow-up duration of up to 3 years, average adherence is around 80–90% in both adults and children.[34] In contrast, adherence to AIT in real-world studies is typically poor, although there is a wide variation in reported rates; in studies of SCIT with a duration of follow-up of 3 years, adherence rates are in the ranges of 23–88% in adults and 16–89% in children.[30] In SLIT studies with a similar duration of follow-up, adherence rates are in the range of 7–85% in both adults and children.[30] The wide variation in adherence to AIT is similar to that observed for medication use in other chronic conditions, including rheumatoid arthritis, diabetes, chronic obstructive pulmonary disease, and cardiovascular disease.[30] Possible reasons for this heterogeneity are that studies were performed in different populations and countries, and patients were treated with different allergen vaccines and treatment schedules, using different measures of adherence.[30] Of particular note, research suggests that differences in rates of adherence may exist between the type of treatment regimen (i.e., SLIT versus SCIT) and certain patient groups (i.e., younger versus older patients) in the real-world setting (Table 1).

FACTORS AFFECTING ADHERENCE TO ALLERGEN IMMUNOTHERAPY

Factors known to influence adherence to AIT include the patient's perception of their disease severity, mode of AIT administration or regimen (i.e., sublingual versus injection and the frequency of administration), inconvenience, fear of injections, cost, treatment benefit (or lack thereof, and as perceived by both patients and physicians), side effects, and the patient's level of knowledge about their condition and treatment.[41-43] Broader factors determining adherence include a holistic approach to treatment, the physician–patient relationship, and the quality of treatment delivery.[44]

The first year of therapy is pivotal for AIT adherence, with evidence demonstrating that patients who adhere to their AIT schedule during this time are more likely to complete the rest of their treatment.[45] A retrospective cohort study using prescription renewal rates to compare SLIT-tablet versus SCIT in patients with grass pollen allergy found that despite similar persistence over 3 years, discontinuations in the first year of treatment were more frequent among the SLIT-tablet patients.[31] Those continuing treatment after the first year were less likely to discontinue treatment during the third year, compared with those receiving SCIT.[32] The dose of AIT does not appear to play a notable role in adherence to therapy.[31]

Research has shown that greater perceived disease severity is associated with better adherence to medication.[46] This is of particular relevance for patients with less severe AR, who may not experience a significantly detrimental impact on their QoL prior to starting treatment and who may therefore be less motivated to continue with therapy. Patients' perceptions and expectations of AIT are largely dependent on their knowledge of allergic disease and the treatment options available.[43] It has been suggested that there are numerous misconceptions and a lack of knowledge about AIT among patients.[47] A questionnaire-based study in patients with AR or asthma found that patients often had a negative view of AIT before starting therapy.[48] In a multinational, observational, internet-based survey of patients from five countries, almost two-fifths of early

AIT discontinuers (i.e., those who stopped treatment before the end of the recommended course) reported a perception of poor effectiveness.[41] Although AIT starts to relieve symptoms within a few weeks or months, patients may have unrealistic expectations about the speed of improvement in their symptoms when compared to their experiences with antihistamines and nasal corticosteroids.[41] A questionnaire-based study found that one-fifth of participants with AR receiving AIT did not know when an improvement in symptoms should be expected after starting treatment, and almost one-fifth (18%) believed an improvement would occur within days or weeks.[47] In the same study, around one-third of the study population were aware that AIT may have some potential risk or adverse effects.[47] Moreover, for seasonal allergens (e.g., to plant pollens), AIT is administered perennially or started a few months prior to the pollen season,[49] outside of which, patients may largely be symptom-free. If patients then experience symptoms with the onset of the pollen season (which may itself also be unpredictable and affected by weather patterns and varying daily environmental pollen loads), they may discontinue treatment due to a perceived lack or loss of effectiveness.

Adverse events (AE) are the most common reason for discontinuation of SLIT.[35] AE (mostly local reactions that occur early in treatment) account for at least one-quarter of all dropouts in clinical trials and the rate is likely to be even higher in a real-life setting.[14]

The method of payment is a key determinant of uptake and continuation with AIT. Clear differences are evident when comparing sales of SLIT between reimbursed settings versus the patient paying out of their own pocket.[14] In a study of patients with AR who discontinued SCIT in the USA, 40% reported that this was due to issues of cost, specifically inadequate or nonexistent insurance coverage.[50]

The type of prescriber and frequency of office visits may be predictors of continuation of AIT. A study in the Netherlands found that patients of general practitioners demonstrated longer persistence than those of allergologists and other medical specialists,[37] while evidence has also demonstrated an association between more frequent physician's office visits (such as that required for SCIT administration) and better persistence.[32]

Patient QoL is also thought to influence adherence.[44] A retrospective, noninterventional, cross-sectional study in paediatric patients treated with SLIT showed that QoL scores, assessed using the generic 12-Item Short-Form Health Survey (mental and physical components), were comparable to those of the general population, in contrast to untreated allergic patients who typically report lower QoL, and were correlated with good adherence to SLIT.[44]

However, evidence from placebo-controlled trials of SLIT has demonstrated that adherence depends less on the patient's perception of therapeutic efficacy and more on their motivation to participate in the trial and to meet the researcher's expectations.[33] It was suggested that enrolment of patients into a trial is similar to a concordance process, in which a therapeutic alliance is established between the patient and physician, and this is known to be an important factor in maintaining adherence.[33] Patients also tend to be more adherent when their behaviour is being recorded or observed, a phenomenon known as the Hawthorne effect.[32]

INTERVENTIONS TO IMPROVE ADHERENCE TO ALLERGEN IMMUNOTHERAPY

There is a clear need to improve rates of real-world adherence to AIT[14] and, although research into interventions to improve adherence to AIT is lacking, lessons learned from interventions used in other chronic health conditions may be valuable. The chronic care and patient-centred care models are two well-established models of care that can be used to improve adherence.[51] The latter focusses on the importance of understanding and targeting modifiable barriers to adherence, such as patients' knowledge and health beliefs and healthcare providers' communication skills.[51] Potential barriers to adherence and interventions that may encourage continuation with therapy are summarised in Table 2.

It is important to note that the factors contributing to poor adherence in one setting or healthcare system may not be applicable

to other settings or systems. Ideally, immunotherapy practitioners should audit their own practice to determine the major factors affecting adherence and include these in decision-making processes regarding AIT for individual patients.

Table 2: Barriers to adherence to allergen immunotherapy and interventions to encourage continuation with therapy.[11,18,29,32,35,52,53]

Barrier to adherence	Intervention to encourage continuation
Fear and experience of side effects	› Ongoing monitoring of any side effects. › Effective, standardised management of side effects. › Provision of clear, standardised information, either written or via an app or video. › Educate patients about early local AE and their improvement with continued treatment as a first sign of increasing tolerance. › Nurse education.
Lack of perceived efficacy	› Provision of clear, standardised information either written or via an app or video. › Educate patients about when they should expect to see an improvement in symptoms. › Ongoing monitoring of treatment effect. › Potential use of serum-based biomarkers to demonstrate an immunological response to AIT before improvement in symptoms is evident. › Nurse education.
Perception that upon improvement in allergic symptoms there is no further need to continue therapy	› Provision of clear, standardised information, either written or via an app or video. › Patient-support programmes to foster and maintain patient engagement in treatment. › Nurse education.
Cost	› Point-of-care cost reduction.
Social issues, ability to attend repeat appointments/ long distance to travel from patient's home to physician's office, and lack of convenience	› Reminders to renew prescription sent by telephone (automated or manual), text messages, email, or via social media; use of electronic pillboxes. › Easy-to-administer, convenient formulations. › Greater flexibility of services in offering appointments.
Psychological factors (i.e., patient is in denial about having the disease)	› Patient-support programmes to foster and maintain patient engagement in treatment.
Forgetfulness	› Reminders sent by telephone (automated or manual), text messages, email, or via social media; use of electronic pillboxes.
Poor physician–patient communication and/or poor health literacy	› Provision of clear, standardised information, either written or via an app or video. › Patient mentors. › Collaborative care and raised awareness of AIT. › Nurse education.
Lack of drug availability	› Reminders to renew prescription sent by telephone (automated or manual), text messages, email, or via social media; use of electronic pillboxes. › Individual patient coaching. › Standardisation of logistics and distribution.
Reluctance to use SLIT or SCIT	› SLIT: early detection of local symptoms that could be treated with symptomatic medication; splitting the SLIT tablet prior to administration to take one half after the other, moving to other parts of the vestibulum, and avoiding swallowing the allergen have also been successful in avoiding local symptoms.
Other	› Incentive-based schemes.

AE: adverse event; AIT: allergen immunotherapy; SCIT: subcutaneous immunotherapy; SLIT: sublingual immunotherapy.

Adapted from Demoly et al.[29]

A retrospective analysis conducted in patients with AR with or without asthma found that patient education regarding the treatment course and slow effect of AIT, as well as the need for close follow-up to effectively prevent and treat adverse reactions, are all important in improving adherence to therapy.[54] Improving the information given to patients by prescribers is an important first step in encouraging long-term adherence.[14] An observational, open-label study showed that patients' satisfaction with SLIT in terms of their assessment of effectiveness, tolerability, and convenience is strongly influenced by the information supplied by their healthcare provider.[55] The guidelines on AIT by the German, Austrian, and Swiss allergy societies include a treatment information sheet to inform the patient about practical aspects of AIT, such as expected effects, type and duration of treatment, possible side effects, and alternative treatments.[18] The SLIT information sheet can be found here and the SCIT information sheet here. Regular monitoring is associated with increased adherence and a clear relationship can be seen between number of follow-up visits and adherence to treatment;[56] this is particularly important because SLIT is administered at home without direct supervision.[14] The high levels of adherence found in clinical trials are thought to result partly from regular monitoring of enrolled patients.[41]

Evidence suggests that multifaceted patient-support programmes that encompass communication, educational, and motivational components are associated with improved adherence to AIT.[29] A small study (N=52) reported a higher rate of adherence in patients who underwent educational training versus those who received only instructions about SLIT administration (96% versus 77%, respectively). Patients who did not receive additional education were more likely to discontinue treatment due to minor side effects, such as oral and gastrointestinal local reactions.[57] A study to evaluate an action plan consisting of education, frequent contact, and strictly scheduled visits for patients taking SLIT found that after 1 year, 12% of patients discontinued treatment compared with 35% in the control group ($p<0.001$).[58] In patients with chronic metabolic diseases, empowerment-based self-management interventions have stronger, long-lasting effects than conventional self-management or education.[29]

Forgetfulness is an issue impacting adherence, which can be accentuated by a number of patient health and lifestyle-related factors, such as age, comorbidities, travel, and social activities.[29] eHealth interventions, such as the use of social media, email, and phone services to provide reminders and ongoing monitoring, are considered valuable in improving adherence to SLIT,[59] particularly because these are required more frequently for daily at-home SLIT administration compared with less-frequent administration of SCIT.[29] Incentive-based schemes have been shown to successfully promote medication adherence in selected clinical trials; however, the development of sustainable and cost-effective long-term interventions in the real-world setting may be a challenge.[53]

Until now, the efficacy of AIT has been assessed subjectively based on individual perception. The use of serological biomarkers to evaluate immunological response to AIT, even before the onset of symptom relief, may be a very helpful tool in encouraging adherence. If a lack of response is detected at an early stage, selection of the AIT allergen can then be re-evaluated. Some studies have shown that an elevated ratio of specific IgE to total IgE is a potential positive predictive marker for AIT.[52] The role of serum-based biomarkers in monitoring adherence and compliance with AIT also warrants further exploration. A recent European Academy of Allergy and Clinical Immunology (EAACI) position paper recommended further research into the use of serum IgG4 as a biomarker for compliance, based on the increase in IgG4 levels observed with AIT.[52]

Some interventions to improve adherence have demonstrated limited success or have potential limitations. A systematic review found that <50% of the interventions used in randomised clinical trials were associated with improved adherence, and almost all of those with long-term efficacy were complex and multifactorial. Furthermore, it was not possible to predict which interventions would be successful in a particular setting and over a given timeframe.[60]

CONCLUSION

AIT is the only treatment for AR and asthma that has a disease-modifying effect. However, reported adherence rates with AIT are often low in real-world settings, with high rates of discontinuation during the first year of treatment. Factors known to affect adherence may be related to the patient (e.g., forgetfulness or fear of injections), treatment (e.g., lack of perceived efficacy or lack of knowledge about side effects), or physician (e.g., lack of communication or inadequate counselling), although these may vary according to the healthcare setting or system. Interventions to improve patient adherence should focus on standardised patient education, particularly regarding adverse effects and timing of improvement in symptoms, patient-support programmes, and mentoring, and the use of eHealth reminders. Providing this information via an app or video, if possible, may also promote patient engagement. Effective, standardised management of side effects and regular follow-up visits may also improve patient adherence. The potential role of biomarkers in evaluating early response to AIT and in monitoring treatment adherence in clinical practice also warrants further investigation.

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Interim Results of the Basket of Real-World Randomised Clinical PRISM Trials for M'Sure-S, a Next-Generation Sirolimus-Eluting Stent, Versus Eliminator, an Everolimus-Eluting Stent

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Disclosure: The authors of this paper are part of the clinical research department of Multimedics LLC.

Support: The PRISM randomised control trial study was sponsored by Multimedics LLC.

Received: 24.07.18

Accepted: 08.08.18

Keywords: Definite stent thrombosis (DST), drug-eluting stent (DES), everolimus-eluting stents (EES), outcome, percutaneous coronary intervention (PCI), PRISM, sirolimus-eluting stents (SES), real-world trial.

Citation: EMJ. 2018;3[3]:30-38.

Abstract

Objective: This study compared sirolimus-eluting stents (SES) with everolimus-eluting stents (EES) in coronary artery disease patients.

Methods: A total of 1,174 patients were enrolled in the study; 290 patients (25.28%) were treated with EES and 884 patients (74.72%) were treated with SES. The trial (PRISM) was a randomised (in a 3:1 ratio), multicentre, single-blind, all-comers, single-arm, non-inferiority trial comparing SES and EES-implanted patients with coronary artery disease. The primary endpoint was a composite of safety parameters (including major adverse cardiac events [MACE], cardiac death, and myocardial infarction) and efficacy (parameters concerned to quantitative coronary angiogram). An intention-to-treat analysis was performed at 9 and 18-month follow-ups.

Results: The baseline characteristics were similar for both EES and SES groups. At the 9-month follow-up, MACE occurred in 5.86% and 2.43% of patients in the EES and SES groups, respectively. At the 18-month follow-up, this differential remained almost the same (i.e., 5.17 % of patients treated with the EES versus 2.14% treated with the SES). The rate of definite stent thrombosis at 9-month follow-up was lower in the SES group (11 patients [1.24%]) compared to the EES group (9 patients [3.10%]). At 18-month follow-up, the rate was 2.14% (19 patients) in the SES group and 4.13% (12 patients) in the EES group. When censoring the patients at the time of stent thrombosis, no significant differences between the two stent groups were found.

Conclusion: In this real-world trial, at 9 and 18-month follow-ups, SES (M'Sure-S) exhibited a better safety and efficacy profile when compared to EES in terms of MACE rates and definite stent

thrombosis. However, the difference was not statistically significant and SES was found to be non-inferior to EES.

INTRODUCTION

Drug-eluting stents (DES) used for the treatment of coronary artery disease were an important development in the field of percutaneous coronary intervention (PCI). The use of balloon angioplasty and bare-metal stents resulted in an augmented rate of reocclusions and restenosis. The incidences of restenosis and target vessel revascularisations (TVR) are significantly reduced by DES compared to their antecessors, bare-metal stents.[1,2] The second-generation DES, containing antiproliferative and immunosuppressive agents, are preferred over first-generation DES; this is due to the increased incidences of myocardial infarction (MI) and stent thrombosis observed following the use of first-generation DES.[3] Despite second-generation DES being safer, both generations of DES offer equivalent levels of efficacy. These stents (second-generation stents) contain a cobalt chromium (L605) platform with ultrathin struts covered with a silicon carbide layer.[4] In a study of sirolimus-eluting stents (SES), a first-generation DES exhibited demonstrable angiographic results versus an everolimus-eluting stents (EES).[5] A study conducted by Han et al.[6] examined the material characteristics and limitations of poly-L-lactic acid polymer-based bioresorbable scaffold in PCI. It also compared the strut thickness in bioresorbable scaffolds and metallic DES. The PRISM randomised control trial (RCT) aimed to determine the safety and efficacy of first-generation SES (M'Sure-S, Multimedics LLC, Ahmedabad, India) versus second-generation EES (Eliminator™, Multimedics LLC).

The present study was designed to assess the procedural performance, angiographic result, and long-term clinical outcome obtained by M'Sure-S versus Eliminator. The safety and efficacy outcomes at the different time intervals of the PRISM RCT trial were compared, with a specific focus on long-term clinical performance of the study stents and cardiac events linked with definite stent thrombosis of SES and EES.

MATERIALS AND METHODS

Study Design, Recruitment, Enrollment, and Oversight

The PRISM RCT study was a prospective, randomised (3:1 ratio), open-label, all-comers, single-blind, non-inferiority, multicentre trial, with clinical follow-up at 9 and 18 months. The trial enrolled 1,174 patients; 884 patients were treated with SES and 290 patients were treated with EES. The multicentre study involved European and Indian populations. Patients >18 years old, presenting with symptomatic ischaemic heart disease and/or objective evidence of myocardial ischaemia, and ready for percutaneous transluminal coronary angioplasty, stenting, or emergency coronary artery bypass graft (CABG) were eligible for study participation. The mean age of the patients involved in the trial was 68 years. The research and development department of the PRISM RCT was responsible for data collection and monitoring. All source data were verified by independent monitors on site. All cardiac and non-cardiac adverse events were reviewed and monitored by a safety and data monitoring board. An independent clinical event committee adjudicated all clinical endpoints in a blinded fashion. The investigators involved in the study vouched for the accuracy of the data and analysis. Approval for the trial was granted by each of the institutional ethical committees at the participating sites. Informed consent forms were signed and collected from the participants prior to the study. The trial was registered in a clinical trials registry and conducted as per the ICH/E6/R1 guidelines. Unrestricted access to the data was given to the principal investigator post database lock and the decision was taken to prepare this manuscript for publication.

The most essential inclusion criterion was the presence of a *de novo* target lesion located in the native coronary artery suitable for conventional angioplasty and stenting, and that could be covered by one stent without overlapping. More precisely, the target lesion had to be

present in the native epicardial coronary artery (2.5–4.0 mm in diameter) and had to be able to be covered by a single SES with a maximum length of 40 mm. Lesions with severe calcification, tortuosity, presence of thrombus, bifurcation sites, left main coronary artery involvement, saphenous vein grafts, and those with a left ventricular ejection fraction <30% were excluded from the study.

The main exclusion criteria were pregnancy; known hypersensitivity to or contraindication for sirolimus or any other mTOR; and hypersensitivity to or contraindication for aspirin, clopidogrel, or other thienopyridines. Additionally, hypersensitivity to or contraindication for cobalt, chromium, heparin, or contrast media that are routinely present during stenting procedures were exclusion criteria. Other exclusion criteria included pretreatment of target lesions by stenting methods, previous brachytherapy, presence of significant non-target lesions requiring treatment within 30 days of the index procedure, prior CABG to the target vessel, and acute MI within 48 hours.

Study Procedure

The study subjects were treated by routine angioplasty procedure as per previously published standard protocol with slight modification.[4,7] Briefly, keeping the angiographic inclusion and exclusion criteria in mind, the stents were deployed upon receipt of visual estimation of the vessel diameter and lesion characteristics. At the end of the stent implantation, it was left to the interventional cardiologist's discretion whether to treat the patient further with a post-dilatation balloon catheter. Dual antiplatelet therapy (aspirin concomitantly with clopidogrel) was continued for up to 1 year post-procedure. Procedural success was defined as a successful device implantation with a residual stenosis of <20% of the vessel diameter, event-free sheath removal, and subsequent discharge from the hospital. Angiographic follow-ups were performed at 9 and 18 months. Fractional flow reserve was used in cases of intermediate target vessel stenosis <70% with or without angina or >70% in the absence of angina during follow-up.

Quantitative Coronary Analysis and Clinical Follow-Up

All the coronary angiograms were analysed in an angiographic laboratory by automated software and independent technicians who were unaware of the clinical information and stent allocation pertaining to this study. The quantitative measurements included the in-stent lumen loss, in-segment late lumen loss, lesion length, percentage of diameter stenosis, and minimal luminal diameter (MLD). Follow-up was carried out at 9 and 18 months post-procedure. Follow-up information was collected either by a hospital visit or telephone contact with the patient or the referring physician. Patients were followed-up for up to a total of 18 months.

Study Endpoints

The primary safety endpoint was defined as major adverse cardiac events (MACE) at 30 days, defined as a composite of death, MI (both Q wave and non-Q wave MI), emergent CABG, or clinically driven target-lesion revascularisation (TLR) (repeat PCI or CABG). The primary efficacy endpoint was the in-stent and in-segment late loss at 9 and 18-month follow-up, respectively, determined by off-line quantitative coronary analysis (QCA) at the core laboratory. The secondary efficacy endpoints for the PRISM RCT were angiographic and device success, procedural success, QCA-derived vessel parameters in-stent, and 5 mm proximal and 5 mm distal from the edge of the stent (acute gain, MLD, diameter stenosis, late loss, binary restenosis, in-stent MLD pre, post, and at angiographic follow-up). Binary restenosis was defined as a diameter stenosis ≥50%.

Statistical Analysis

The trial was performed to assess the non-inferiority of SES to EES with respect to the primary endpoint (safety and efficacy) at 9 and 18 months. For superiority for all endpoints, 2-sided 95% confidence intervals (CI) and 2-sided p values were calculated. The primary analysis was performed according to the intention-to-treat principle. To compare the distributions of continuous variables between the study groups, the 2-sample student t test was used, and the chi-square method was used, with a power of 95% and significance

level α=0.005, to measure any difference between the treatment arms. CI functions were computed considering death (and, in a sensitivity analysis, also stent thrombosis) as a competing risk. The MACE per patient was ranked according to the highest category on a scale ranging from 1) death, 2) MI, 3) CABG, to 4) TLR. Gray's test was used for comparing the cumulative incidence functions and Cox regression to determine the cause-specific hazard ratios (HR). Patients treated with the SES were used as the reference group for overall and subgroup analyses for safety and efficacy parameters. HR was calculated for MACE at 18-month follow-up for prespecified patient subgroups (based on baseline demographic and clinical characteristics). A 2-sided p value <0.05 was considered statistically significant. Analyses were conducted using SPSS (SAS institute, Cary, North Carolina, USA).

RESULTS

Baseline and Procedural Characteristics

In this clinical trial, cumulative data comprised results from the 1,174 enrolled patients, 884 of whom were treated with a SES and 290 with an EES. The mean ages of the patients was ≤68 years, and among them male and female patients were bifurcated randomly, in a 3:1 ratio. The statistical analysis of other baseline characteristics like heart rate, previous myocardial infarction, stable angina acute coronary syndrome, diabetes, smoker, hyperlipidaemia, family history of coronary artery disease are represented in Table 1. Both groups achieved 100% of the procedural characteristics.

Quantitative Coronary Analysis

QCA were obtained at four distinct timepoints: pre-stenting, post-stenting, at 9-month follow-up, and at 18-month follow-up. The pre-stenting QCA results showed lesion length (LL), median reference vessel diameter, minimum luminal diameter, and percentage diameter stenosis for SES patients were slightly higher when compared to EES (19.92 mm versus 18.41 mm, p value=0.93; 2.35 mm versus 2.20 mm, p value=0.10; 0.98 mm versus 0.97 mm, p value=0.82; and 79.29% versus 78.45%, p value=0.87, respectively). Here, LL for SES was shown to be non-inferior to EES.

Post-procedural analysis showed that in-stent residual diameter stenosis was slightly higher in the SES group (13.26%) compared to the EES group (12.78%). In-stent acute gain was measured to be 1.34 mm for SES compared to 1.35 mm for EES.

Table 1: Baseline characteristics of both sirolimus and everolimus-eluting stent treatment groups.

Baseline Demographics	Sirolimus group (M'Sure-S stent)	Everolimus group (Eliminator stent)	p value
Number of patients enrolled	884	290	
Mean age (years)	60.00±9.20	61.50±9.50	0.68
Male	716 (80.99%)	225 (77.58%)	1.00
Female	168 (19.00%)	65 (22.41%)	0.87
Hypertension	444 (50.22%)	154 (53.10%)	0.46
Heart rate (bpm)	77.14± 18.14	74.65±19.35	0.85
Previous myocardial infarction	172 (19.45%)	64 (22.06%)	0.56
Stable angina	510 (57.01%)	116 (40.00%)	0.26
Acute coronary syndrome	380 (42.98%)	130 (44.83%)	1.00
Diabetes	220 (24.88%)	64 (22.07%)	0.16
Smoker	412 (46.60%)	143 (49.31%)	0.53
Hyperlipidaemia	106 (11.99%)	36 (12.41%)	0.15
Family history of CAD	221 (25.00%)	81 (27.93%)	0.26

CAD: coronary artery disease.

In-segment analysis of the SES and EES groups revealed a MLD of 2.32 mm and 2.43 mm, respectively, and a residual diameter stenosis of 24.56% and 25.46%, respectively, with an acute gain of 1.34 mm and 1.43 mm, respectively.

At the 9-month timepoint, angiographic follow-up data from 874 and 282 patients were studied; median in-stent MLD for the SES and EES groups were found to be 2.30 mm and 2.20 mm, respectively, with a diameter stenosis of 11.26% and 11.21%, respectively, and a late lumen loss (LLL) of 0.05 mm and 0.04 mm, respectively. Binary restenosis was not found in any patients and this remained at 0.0% at the end of 9 months, thereby indicating the high efficacy of both the stents used in this trial.

There were no significant differences in angiographic measurements of lesions before and after the procedure. Angiographic follow-up at 18 months was performed in 1,119 patients (855 in the SES group and 264 in the EES group). The primary endpoint of the study, mean in-segment LLL, was 0.07 mm in the SES group and 0.10 mm in the EES group; thus, the results of the in-segment LLL met the criteria for non-inferiority of SES versus EES (non-inferiority margin=0.1 mm). The in-stent LLL showed similar findings; the mean in-stent LL was 0.06 mm and 0.08 mm for the SES and EES groups, respectively. Median in-stent MLD was found to be 2.17 mm, with a diameter stenosis of 14.26%; binary restenosis was not found in any patients and this remained at 0% after 18 months, thereby indicating high efficacy. Likewise, median in-segment MLD was 2.35 mm and thus the percentage diameter stenosis was calculated to be 21.24 mm, with no binary restenosis.

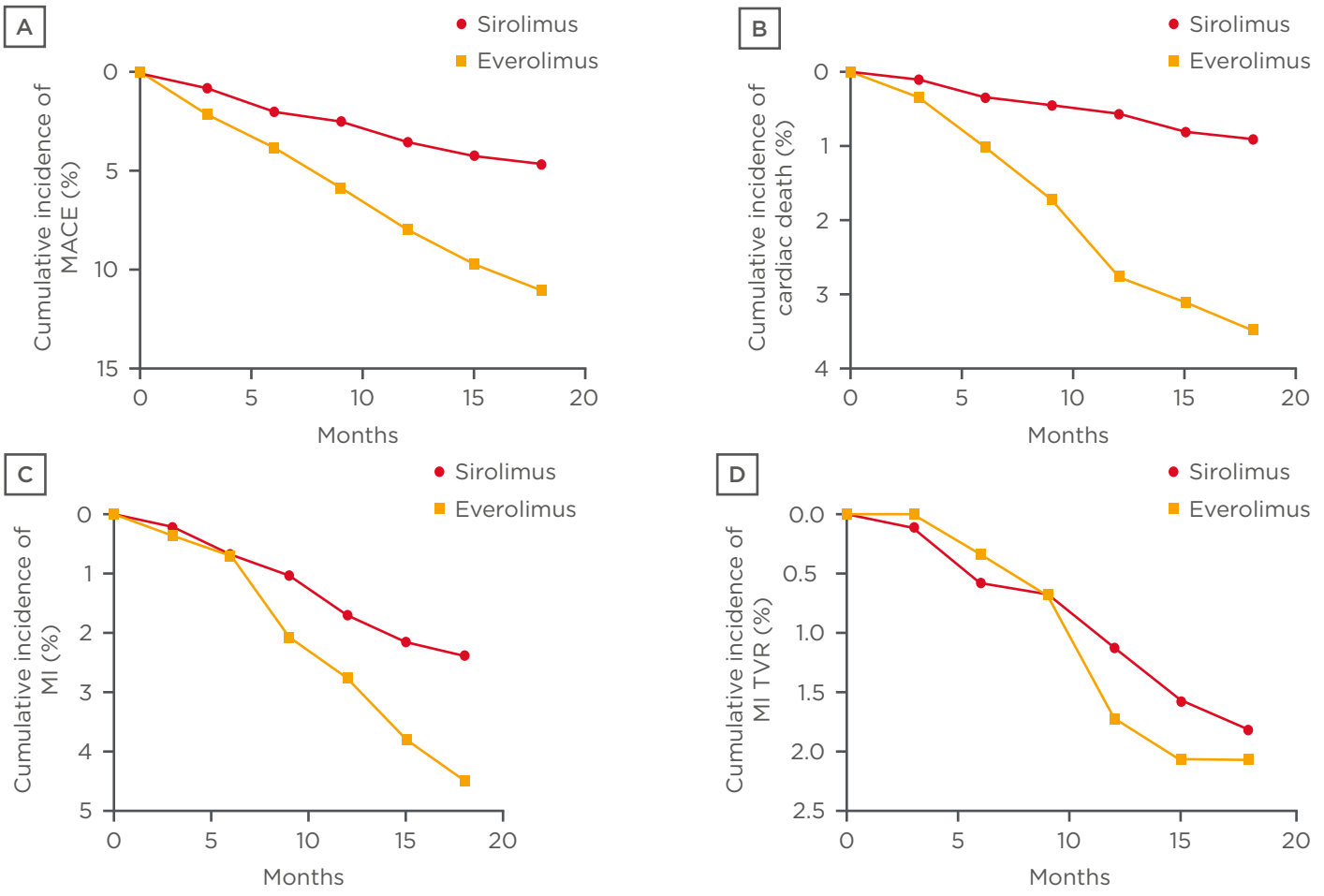

Figure 1: Cumulative event curves up to 18 months are shown for A) MACE, B) cardiac death; C) myocardial infarction; D) myocardial infarction target vessel revascularisation; E) target vessel revascularisation; F) non-target vessel revascularization; G) Non-target vessel revascularisation H) definite stent thrombosis.

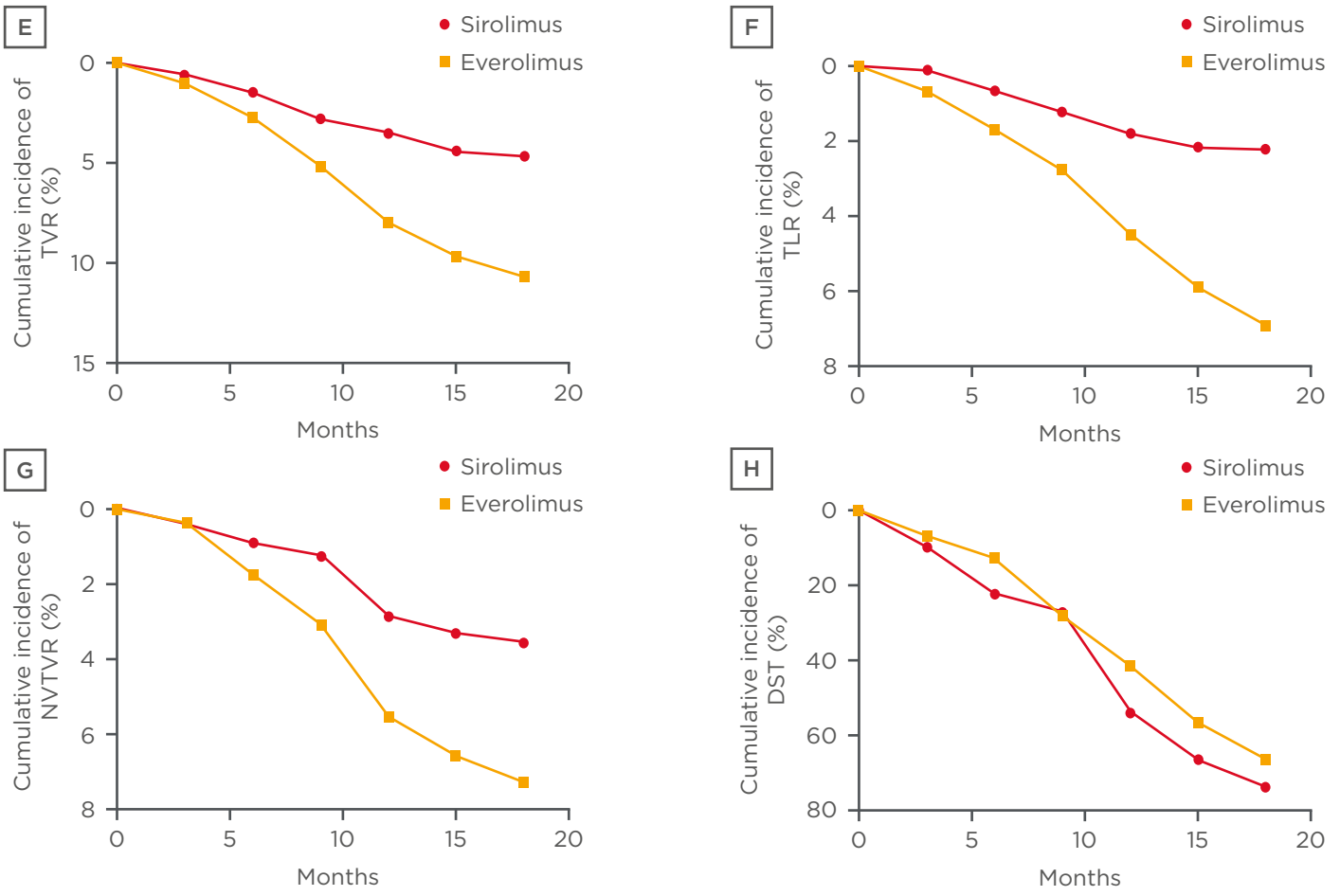

Figure 1 continued.

DST: definite stent thrombosis; MACE: major adverse cardiac events; MI: myocardial infarction; NTVR: non-target vessel revascularisation; TLR: target-lesion revascularisation; TVR: target vessel revascularisation.

Major Adverse Cardiac Events and Stent Thrombosis

All patients in the trial achieved the primary endpoint and were followed for 9 months. At 9-month follow-up, MACE occurred in 22 patients (2.48%) who received SES and in 17 (5.86%) patients who received EES (HR: 0.98; 95% CI: 1.00–1.06; p=0.28). However, at 18-month follow-up, the occurrence of MACE was reduced to 19 (2.14%) and 15 (5.17%) in the SES and EES patients, respectively (Figure 1A).

Initially, from 0–9-month follow-up, 4 patients (0.45%) and 5 patients (1.72%) died due to cardiac death in the SES and EES-treated groups (HR: 0.96; 95% CI: 0.97–1.07; p=0.47 [Figure 1B]), respectively; furthermore, the same percentage of deaths was observed at 9–18-months follow-up in both groups. From the data it was observed that death rates in the SES group were lower compared to the EES group, however, the difference was non-significant.

MI was observed in 21 patients who received SES and 13 patients who received EES. At 9-month follow-up, 9 and 6 patients were found with MI who had received SES and EES, respectively (HR: 0.89; 95% CI: 0.99–1.02; p=0.25). The incidence of MI increased to 12 and 7 at 18-month follow-up, respectively (HR: 0.95; 95% CI: 0.99–1.03; p=0.29 [Figure 1C]). However, SES-implanted patents had a lower incidence of MI compared to EES patients.

MI TVR was performed in 16 and 6 patients who received SES and EES, respectively (HR: 0.89; 95% CI: 0.98–1.03; p=0.36 [Figure 1D]). At 9-month follow-up 6 and 2 patients were found to have MI TVR who had received SES

and EES; but the incidence was raised to 10 and 4 at 18-month follow-up, respectively. There was a need for TVR in 47 and 31 patients in the SES and EES patient groups, respectively (HR: 0.92; 95% CI: 1.01–1.10; p=0.89 [Figure 1E]). There was also a need of TLR in 20 patients with SES and in 18 patients with EES (HR: 0.98; 95% CI: 0.73–1.20; p=0.65 [Figure 1F]). Non-TVR was also required in 31 patients with SES and in 21 patients with EES (HR: 0.97; 95% CI: 0.99–1.07; p=0.29 [Figure 1G]). The total number of definite stent thromboses was lower in SES patients (30 [3.40%]) compared to EES patients (21 [7.23%]) (HR: 1.17; 95% CI: 0.99–1.02; p=0.24). The incidence of definite stent thrombosis (DST) did not differ significantly at 0–9-month follow-up (11 [1.24%] with SES versus 9 [3.10%] with EES; HR: 0.97; 95% CI: 0.99–1.02; p=0.10). However, at 18-month follow-up, DST was not significantly lower in the SES group than the EES group (19 [2.14%] versus 12 [4.13%] patients, respectively; HR: 1.03; 95% CI: 0.99–1.04; p=0.30 [Figure 1H]). Results of the corresponding test for interaction were non-significant, which was deduced based on Cox regression analysis. Graphical representation of cumulative incidences of all the primary events of SES and EES implantation are shown in Figure 1.

DISCUSSION

Over recent decades, stent technology has gained momentum in PCI.[8] The surface of the metal and the chemical properties of the materials play a pivotal role in designing an ideal, safe, and efficacious stent. In recent years, there has been a revolutionary change in stent material and design. The bare-metal stents prevent negative arterial remodelling in percutaneous transluminal coronary angioplasty, acute recoil, and closure of vessels.[9] However, the use of bare-metal stents is limited by augmented instances of restenosis and repeat revascularisation and stent thrombosis rates.[10-15] Thus, newer generation DES were developed that inhibit neointimal proliferations, reducing repeat revascularisation.

In this arbitrary, prospective comparison of SES and EES, the efficacy of SES in suppressing neointimal growth (expressed as LL) was non-inferior to the EES. Both stents showed exceptional LL profiles at 9 and 18-month angiographic follow-up. Clinical outcomes, including MI, cardiac death, and TLR, were typically similar between the two stent types, although this study was underpowered to demonstrate the variation in clinical outcome between the two stents. Moreover, the distinct and apparent DST rates were not statistically different between the two types of stents.

The outcome of this trial was demonstrating the non-inferiority of M'Sure-S versus Eliminator. The trial outcome of both EES and SES included low rates of DST and TLR. Previously developed DES are known to cause late stent thrombosis and have thick struts, which act as a barrier for early endothelialisation. SES has low strut thickness (59 µm), which promises early endothelialisation and reduces the risk of stent thrombosis. SES has drug-elution kinetics of 28–30 days and a polymer degradation that is short and well documented.[16-19] SES was found to be safe and efficacious in preclinical models and in the primary safety and efficacy study.[20]

In terms of efficacy parameters, at 9-month angiographic follow-up, 0.05 mm of in-stent medial LL were observed and no binary restenosis was recorded. Analysis of data demonstrated safety and efficacy of SES in line with other published randomised trials.[21]

This study demonstrated a high safety profile of DES, with 13%, 10%, and 0% of patients exhibiting MACE, stent thrombosis, and binary restenosis, respectively. Data from other similar studies demonstrated that, in comparison to EES, SES had a better enduring safety and efficacy report.[10,22,23] These results also revealed a 14% reduction in MACE rate in SES patients, which was largely due to a lower risk of very late DST. During 0–9 months, MACE rate did not differ significantly, which is the main prevalent timepoint considered for determining the primary endpoint in head-to-head contrasts of DES. However, from 9–18 months the MACE rate was non-inferior with SES versus EES.

Throughout the 18 months, the liability of DST was sporadic and alike in both groups. In a comparison of first-generation DES (SES and EES), the initial pre-eminence of the EES was lost at 18-month follow-up. DST occurred with an annual rate of 1.24–3.10% for both stent types.[24-26] In this trial (SES versus EES), DST was

more recurrent after implantation of EES than after SES, although contradictory results were recorded between 9 and 18-month follow-up. The present results from the PRISM RCT with follow-up to 18 months indicated that the composite endpoint safety and efficacy factors of SES versus EES were not significant. The rates of DST also presented similar results, with both being equally efficacious, as per studies.[27] In this PRISM RCT trial, SES patients exhibited better safety and efficacy profiles in MACE rates and DST when compared to EES patients. However, the difference found was not statistically significant. Like most stent trials, the PRISM RCT trial was designed as a single-blinded study, and the authors believe that the scarcity of double-blindness would not influence the results, as all endpoints were objective and determined by an event committee that was blinded to treatment group assignment during the adjudication process. This registry-based randomised clinical trial design has been used in all PRISM RCT trials and has established significant attention as a way of experimenting comprehensive, self-regulating clinical trials. Advantages of this approach include a substantial reduction in the expense associated with a randomised trial because an established registry infrastructure was able to be used. Moreover, the study design provides data that are more comparable to real-life situations because of the absence of study-related interventions.

CONCLUSION

At 9 and 18-month follow-up, SES established a better safety and efficacy profiles, and the difference in MACE rate between SES and EES-treated patients was non-significant. This effect was largely due to a lower risk of DST.

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Alcohol or Not: A Review Comparing Initial Mechanisms, Contributing Factors, and Liver Transplantation Outcomes Between Alcoholic and Nonalcoholic Steatohepatitis

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Disclosure: The authors have declared no conflicts of interest.

Acknowledgements: The authors thank Dr Hui-Ling Chen for providing critical comments.

Received: 22.01.18

Accepted: 14.06.18

Keywords: Alcoholic steatohepatitis (ASH), fatty liver disease, liver transplantation, nonalcoholic steatohepatitis (NASH), two-hit hypothesis.

Citation: EMJ. 2018;3[3]:40-48.

Abstract

Chronic liver diseases take many forms; alcohol-related liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD) are two common illnesses that potentially lead to cirrhosis, liver failure, and liver cancer. It is estimated that a quarter of heavy drinkers develop ALD and the same portion of people without heavy drinking habits have NAFLD. Alcohol intake is regularly used to differentiate NAFLD from ALD; however, diagnosis based on the discrimination threshold may be suboptimal when facing an obese patient with a high level of alcohol exposure. Therefore, understanding the common and/or different mechanism(s) driving each disease is extremely important. The 'two-hit' or 'multi-hit' hypothesis is used to explain the pathogenesis of both diseases. The 'first hit' refers to developing steatosis, the accumulation of fat components in the liver, and the 'second hits' are factors leading to oxidative stress, inflammation, and fibrosis, such as metabolic syndromes (e.g., morbid obesity, hyperglycaemia, hyperlipidaemia, disturbed circadian cycles, and altered intestinal microbiota) and environmental toxins (e.g., cigarette smoke and pollutants). Heritable factors also affect the probability and disease progression of both ALD and NAFLD. Whereas *PNPLA3* and *TM6SF2* variants are influential genetic risk factors for the diseases, epigenetic factors, such as DNA methylation, post-translational histone modifications, and small non-coding RNA, are of paramount importance. Moreover, considering that both ALD and NAFLD patients may eventually develop end-stage liver disease and require liver transplantation, the authors extensively investigated the worldwide outcomes from original literature for these two aetiologies, and the results showed no obvious differences in post-transplantation survival between them. Precise percentage determination of these two aetiologies contributing to steatohepatitis and its secondary injuries in the future would allow for better strategies for therapeutic and preventive intervention.

INTRODUCTION

Alcohol-related liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD) are two very common types of chronic liver disease worldwide. ALD and NAFLD are multifactorial diseases with broad spectrums, including isolated steatosis (defined as hepatic triglyceride content [HTGC] >5%), steatohepatitis, fibrosis, and cirrhosis, and both can potentially lead to end-stage liver disease or develop into hepatocellular carcinoma (HCC),[1] eventually requiring liver transplantation as a curative treatment. While ALD has a strong connection with heavy drinking, NAFLD can be found in >25% of adults that do not excessively consume alcohol in the USA.[2] Generally speaking, NAFLD can be differentiated from ALD by using information about the alcohol consumption of the patient (<30 g per day for men and <20 g per day for women). However, the condition can be complicated when a patient has strong risk factors for NAFLD, such as Type 2 diabetes mellitus or obesity, while consuming excessive alcohol at the same time. Therefore, understanding the common and different mechanisms driving each disease is extremely important. In this literature review, the authors consider the different aetiologies in ALD and NAFLD to understand whether there are differences in post-transplant outcomes. The aim of this review is to compare the initial mechanisms, contributing factors, and liver transplantation outcomes between alcoholic steatohepatitis (ASH) and nonalcoholic steatohepatitis (NASH).

EPIDEMIOLOGIC FEATURES

Alcoholic Liver Disease and Nonalcoholic Liver Disease are Increasingly More Prevalent

Currently in the USA, chronic hepatitis C virus (HCV) is the top cause of liver decompensation leading to liver transplantation, followed by ALD. NASH is an advanced form of NAFLD and is more commonly associated with liver fibrosis compared with NAFLD outcomes. NASH is the third most common disease requiring liver transplantation.[3] As one of the leading causes of hepatic decompensation, ALD is the second most common indication for liver transplantation and accounts for 40% of deaths from cirrhosis in Western countries.[4] Studies show that 25% of heavy drinkers develop ASH, which is an advanced form of ALD, and, among them, about 40% will develop cirrhosis.[5]

On the other hand, the proportions of adults in the USA with nonalcoholic steatosis, NASH, and NASH-related cirrhosis or HCC are estimated to be 25%, 5–6%, and 1–2%, respectively.[6,7] Such data suggest that in spite of the high prevalence of NAFLD, only a small portion of patients with nonalcoholic steatosis will eventually progress to end-stage liver disease. Nevertheless, considering the growing number of morbid obesity and metabolic syndromes, as well as effective treatment options for HCV eradication (direct-acting antiviral drugs), NASH will probably be the most common indication for liver transplantation in the near future.[8] It is estimated that by 2025, 25 million Americans will have NASH, with 5 million of them developing cirrhosis or HCC.[9]

PATHOGENESIS

The 'Two-Hit' Hypothesis Can Apply to Both Alcoholic Steatohepatitis and Nonalcoholic Steatohepatitis

Isolated hepatic steatosis is 3–4-times as prevalent as NASH,[10] and the key features differentiating NASH from isolated steatosis are the presence of cell injury and death.[11] Much work has been done to identify definite mechanisms driving ALD and NAFLD disease spectrums. Obesity and extensive alcohol use are widely known risk factors related to fatty liver disease; however, only a minority of isolated steatosis cases will progress to steatohepatitis, cirrhosis, and HCC. Thus, the phenomenon is more likely a result of multifactor interaction. The 'two-hit' hypothesis, which was proposed by Day and James 20 years ago, is currently the leading hypothesis describing the pathogenesis of NASH as well as ASH.[12] The 'first hit' refers to steatosis, the excessive accumulation of free fatty acids and triglycerides in hepatocytes. Earlier research has proven that many kinds of lipid-related intermediates are cytotoxic (lipotoxic) alone or together with the presence of other factors ('second hits')

and cause damage to hepatocytes through lysosomal dysregulation and elevation of endoplasmic reticulum stress.[13,14] An example of lipotoxicity demonstrated both *in vivo* and *in vitro* was that deactivation of stearoyl-CoA desaturase-1, the enzyme that converts saturated fatty acids to monounsaturated fatty acids, sensitised hepatocytes to monounsaturated fatty acid-induced caspase activation and apoptosis (Table 1).[15]

The Mechanisms of Steatosis are Closely Associated with Insulin Resistance

The livers of patients with NAFLD usually contain mixed large (macrovesicular) and small (microvesicular) droplets or predominantly large droplets within hepatocytes, while the livers of patients with ALD are more predominantly microvesicular (known as alcoholic foamy degeneration).[16] Such pathologies may be due to different fat accumulation processes in ALD and NAFLD and could be helpful in determining the percentage of contribution of ALD and NAFLD. In the case of NAFLD, insulin resistance and consequent hyperinsulinaemia, which causes lipolysis of peripheral fat and increased fat absorption of the liver, are thought to be the triggers of steatosis. However, in the case of ALD, the definite mechanisms of steatosis are more complicated, given the fact that ethanol has dose-dependent effects on insulin signalling: high-dose ethanol exposure weakens insulin signalling in hepatocytes through elevation of tribbles-related protein 3, leading to decreased sterol regulatory element binding protein-1. In contrast, low-dose ethanol consumption enhances hepatic insulin signalling associated with reduced p55γ (a phosphatidylinositol 3-kinase regulatory subunit isoform) to increase nuclear sterol regulatory element binding protein-1.[17] Even so, the protective effect of low-dose alcohol exposure is controversial. Bellentani et al.[18] claimed that patients with NAFLD should not seek a preventive effect via drinking a small amount of alcohol on a daily basis because "the alcohol and the metabolic risk factors for progression of liver disease should be considered always together", implying that, in many cases, the steatohepatitis may not be purely alcoholic or nonalcoholic but rather a mixed coexistance of both ALD and NAFLD.

Metabolic Syndromes and Environmental Toxins Act as Second Hits Toward Steatohepatitis

The second hits, including obesity, hyperglycaemia, hyperlipidaemia, and other metabolic syndromes, as well as environmental toxins, such as cigarette smoke and pollutants, lead to oxidative stress, inflammation, and fibrosis, which are important drivers of the pathogenesis and progression of steatohepatitis.[19,20]

Table 1: Brief comparison of pathogenic causes of nonalcoholic steatohepatitis and alcoholic steatohepatitis.

	NASH	ASH
Histological changes	Mixed large and small droplets or large droplets predominant	Small droplets predominant
Genetics	*PNPLA3, TM6SF2* *ENPP1* *IRS 1*	*PNPLA3, TM6SF2* Sex (female) *ADH, ALDH*
Metabolic syndromes	Obesity, hyperglycaemia, hyperlipidaemia, and others	
Environmental factors	Cigarettes and pollutants	
Changes in the gut microbiota	Disrupted circadian cycles, feeding content, low-grade inflammation status of host	Alcohol consumption

ADH: alcohol dehydrogenase; ALDH: aldehyde dehydrogenase; ASH: alcoholic steatohepatitis; ENPP1: ectonucleotide pyrophosphate/phosphodiesterase 1; IRS 1: insulin receptor substrate 1; NASH: nonalcoholic steatohepatitis; PNPLA3: patatin-like phospholipase domain-containing 3; TM6SF2: transmembrane 6 superfamily, member 2.

Oxidative stress could increase the generation of cytokines such as TNF-α, transforming growth factor beta, and Fas ligand, participating in the progression of steatohepatitis, such as cell death, further inflammation, and fibrosis.[21-23] To protect themselves from further damage, injured hepatocytes undergo adaptations known as stress responses, which rebalance signalling cascades and other aspects of cell physiology. As a result of the unstable microenvironment, the hepatocytes are more vulnerable to other stimulants and stressors, which may also cause apoptosis. For example, in mice models, >50% loss of glucose-regulated protein 78, which is a master regulator of endoplasmic reticulum homeostasis, causes compartment dilation in endoplasmic reticulum, as well as other stress-induced responses including fat accumulation, insulin resistance, increased susceptibility to alcohol, overfeeding, and drug and toxin-related injury, leading to cell apoptosis.[24] Dying cells then produce mediators that promote the regeneration process to replace themselves. In a liver undergoing active dying and regenerating processes, regenerative cell types, including immune cells, myofibroblasts, and hepatic progenitor cells, will be present in a larger amount than those in a normal, healthy liver.[25,26] The first and second hits can interact with each other, causing hepatocyte injury, and the dying and regenerating processes finally become futile, resulting in progressive scarring, which leads to steatohepatitis, fibrosis, cirrhosis, and liver cancer, resulting in hepatic decompensation.

Circadian Cycles and Intestinal Microbiota are Involved in Steatohepatitis Pathogenesis via the Gut–Liver Axis

Until recently, the link between stressful daily routines and liver diseases was unclear, but new experimental and clinical evidence has revealed the interaction between circadian cycles, gut microbiota, intestinal barrier integrity, and NAFLD.[27] Normally, intestinal microbes and the host share a mutually symbiotic relationship, forming a complex and diverse ecosystem. When the host experiences disrupted circadian cycles, which are often seen in patients with shift work and frequent long distance travel, the normal intestinal flora will be influenced rapidly.[27] Feeding content and frequency, as well as chronic, low-grade inflammation status of the host, accompanied by altered inflammatory cytokines, such as elevated TNF-α and IL-6, impact intestinal microbiota as well.[28] Altered intestinal microbiota lose the normal function of intestinal wall barriers, leading to increased intestinal permeability and further production of fatty acids in the small bowel, causing additional fatty acid absorption, thereby strengthening the first hit and resulting in obesity and obesity-related steatohepatitis.[29] Besides this, altered intestinal microbiota dysregulate hepatic inflammation via supplement of bacterial endotoxins and other toll-like receptor ligands, which induce hepatocytes to produce inflammatory mediators, including TNF-α, IL-1, and plasminogen activator inhibitor-1.[23] In short, host factors (diet content, feeding frequency, biological clocks, and chronic inflammation) influence normal gut flora, and, conversely, abnormal gut flora affect the host, leading to pathological inflammation and NASH. Such cross-talk between the liver and the gut, known as the gut–liver axis, are bidirectional actions that play a critical role in NASH pathogenesis.

In the case of ASH, recent animal studies by Lowe et al.[30] showed a link between alcohol, gut microbiota, hepatic neutrophil infiltration, and hepatitis. In their study, changes in gut microbiota were found in alcohol-fed mice and were related to hepatic neutrophil infiltration, inflammation signalling, and steatotic changes. Elevated serum alanine and aspartate aminotransferase, and activation of fat metabolic signalling pathways, were also discovered. Suppression of gut bacterial load by antibiotics reduced alcohol-related liver steatosis but not serum transaminases. In human cohorts, Dubinkina et al.[31] demonstrated an association between alcohol consumption, gut microbiota, and liver dysfunction. Increased gut permeability, impaired ability to transform toxic ethanol metabolites, such as acetaldehyde, and dysregulation of bile volume and composition are all possible mechanisms linking alcohol consumption and liver disease.

Role of Genetics: Vulnerable Background for 'Hits'

Heritable components play roles in determining the risks of both ALD and NAFLD. Sex is a well-

known risk for developing ALD: among people who have similar levels of ethanol exposure, women are more susceptible to ALD than men.[32] Enomoto et al.[33] demonstrated in a rat model that oestrogen promotes the production of TNF-α via increasing the permeability of the intestinal wall to endotoxins and upregulating endotoxin receptors on Kupffer cells. Moreover, twin studies have shown that ALD was three-times higher in monozygotic twins than in dizygotic counterparts.[34] On the other hand, familial aggregation studies indicated that genetic factors play roles in developing NAFLD.[35,36] Much work had been done in recognising the associated genetic and epigenetic factors regarding the risk of NAFLD.

PNPLA3, *TM6SF2*, and Other Genetic Variants are Risk Factors for Alcoholic Liver Disease and Nonalcoholic Fatty Liver Disease

Many gene polymorphisms have been identified as strongly relevant to either ALD or NAFLD, including *PNPLA3* variants and *TM6SF2* variants, that promote the development of steatohepatitis and further hepatic injury.[5,37] A statistically significant index single-nucleotide polymorphism (SNP) in *PNPLA3* (rs738409, I148M), which is more prevalent in Asian and Native American populations, was identified as being associated with higher hepatic susceptibility to both alcoholic and nonalcoholic steatosis, steatohepatitis, cirrhosis, and liver cancer.[38,39] Combined with another SNP (rs6006460, p.S453I) in the same gene, these variants lead to even further increases in HTGC, increasing the severity of ALD and NAFLD disease spectrums.[39] *TM6SF2*, located within the 19p13.11 locus, is responsible for encoding a protein that regulates liver fat metabolism, influencing triglyceride secretion and hepatic lipid droplet content. A *TM6SF2* variant (rs58542926, p.E167K), which downregulates hepatocyte very-low-density lipoprotein secretion, is associated with a higher risk of increased HTGC, leading to steatohepatitis, fibrosis, and further liver damage.[40] However, neither *PNPLA3* nor *TM6SF2* variants are necessary for development and progression of steatohepatitis.[19,37] In addition, other aetiology-specific genetic polymorphisms are only related to either ALD or NAFLD independently. For example, variants in genes encoding class I alcohol dehydrogenase and aldehyde dehydrogenase alter the activity of the enzymes, thus potentially determining ethanol tolerant ability, risk of addiction to alcohol, and risk of ALD,[41,42] while variants in genes encoding ectonucleotide pyrophosphate/phosphodiesterase 1 and insulin receptor substrate 1 impair insulin signalling, thus elevating the risk of nonalcoholic steatosis and NASH.[43]

Epigenetic Factors Affect the Probability of Developing Steatohepatitis, Directly or Indirectly

DNA methylation, post-translational histone modifications, and RNA-based mechanisms (e.g., small non-coding RNA [miRNA]) are important epigenetic factors that regulate gene expression without altering the primary DNA sequences. Some studies suggested that inactivation of some tumour-suppressor genes, due to promoter DNA hypermethylation, is responsible for liver fibrosis and cancer. For example, phosphatase and tensin homologue, a tumour suppressor gene frequently inactivated on chromosome 10q23, inhibits extracellular signal regulated kinase and protein kinase B pathway and downregulates cell cycles and proliferation.[44,45] miRNA, which are small, non-coding and single-strand RNA comprising 19–22 nucleotides, are involved in the regulation of cell development, proliferation, differentiation, and apoptosis through a variety of translation regulatory functions. Silencing of certain miRNA by promoter hypermethylation is related to the regulatory pathways involved in inducing liver fibrosis (e.g., MeCP2, a member of methylated DNA-binding domain proteins, and DNA methyltransferases family-related pathways).[44,46] Moreover, correlation between the risk of obesity and metabolic syndromes in children and mothers experiencing starvation was noted through epigenetic modification, potentially leading to the formation of NAFLD.[47] Such epigenetic factors as well as genetic components directly or indirectly influence the probability of steatohepatitis and further liver damage.

Table 2: Outcome of liver transplantation for nonalcoholic steatohepatitis.

Study	Time period	Number of patients with NAFLD	Post LT 1-year survival	Post LT 3-year survival	Post LT 5-year survival
Charlton et al.,[8] 2011	2001–2009	1,959	84%	78%	N/A
Bhagat et al.,[48] 2009	1997–2007	71	82%	79%	75%
Singal et al.,[49] 2013	1994–2009	1,368	89%	85%	84%
Afzali et al.,[50] 2012	1997–2010	1,810	88%	82%	77%
Agopian et al.,[51] 2012	1993–2001	144	N/A	70%	N/A
Kennedy et al.,[52] 2012	1999–2009	129	90%	88%	85%
Barritt et al.,[53] 2011	2004–2007	21	76%	76%	N/A
Malik et al.,[54] 2009	2004–2007	98	79%	75%	72%

LT: liver transplant; N/A: not applicable; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis.

OUTCOMES OF LIVER TRANSPLANTATION AS THE ULTIMATE THERAPEUTIC TREATMENT: CURRENT STATUS AND A LOOK TOWARDS THE FUTURE

Liver transplantation is the ultimate treatment choice for decompensated livers. As mentioned above, ASH and NASH are the second and third most common indications for liver transplantation, respectively, following HCV.[3,8] Nowadays, effective treatment for liver chronic viral infection has been well developed; therefore, in the near future, ASH and NASH are set to become the two most common indications for liver transplantation. With this expectation in mind, there are two important questions the medical community needs to address: are the transplantation outcomes of ASH and NASH patients similar? Secondly, are the disease recurrence rates between ASH and NASH similar?

Bhagat et al.[48] compared the 1-year, 3-year, 5-year, and 9-year post-transplant survival between ASH and NASH patients and reported no significant difference (p=0.17). In their study, sepsis with or without multiorgan failure was the leading cause of mortality in both ASH and NASH groups, followed by malignancies in the ASH group and cardiovascular causes, including myocardial infarction and stroke, in the NASH group.[48] In order to have a broader view, the authors of this paper extensively reviewed original literature regarding liver transplantation outcomes of either ASH or NASH as the indication (Table 2 and Table 3)[48-63] from 1982–2010. Studies that did not distinguish ASH and NASH from other liver diseases, such as HCV or autoimmune hepatitis, were excluded.

Post-Transplantation Survival Between Alcoholic Steatohepatitis and Nonalcoholic Steatohepatitis Patients are Similar Worldwide

Although the comparison may not be totally valid because the patients were observed and followed-up in different centres and during different eras, the results of this review still provide a complete picture, as different studies showed similar trends. Survival at 1, 3, and 5 years after liver transplantation for NASH was 76–90%, 75–88%, and 72–85%,[8,48-54] compared with 74–92%, 73–90%, and 72–88% for ALD,[48,55-63] respectively. It could be summarised that outcomes for patients receiving liver transplantation for ASH and NASH are similar. However, Bhagat et al.[48] reported a significantly higher post-transplant recurrence rate of steatohepatitis in livers with NASH than with ASH (33% versus 0%; p<0.0001) but no significant difference in retransplantation rates. Such outcomes may be a result of the different initial mechanisms of steatohepatitis. In addition, active alcohol abuse is an absolute contraindication for liver transplantation whereas metabolic syndrome is not; in other words, the underlying conditions leading to NAFLD are not eliminated when a patient with NASH receives liver transplantation.

Table 3: Outcome of liver transplantation for alcoholic steatohepatitis.

Study	Time period	Number of patients with ALD	Post LT 1-year survival	Post LT 3-year survival	Post LT 5-year survival
Bhagat et al.,[48] 2009	1997–2007	83	92%	86%	86%
Burra et al.,[55] 2010	1988–2005	9,880	84%	78%	73%
Gedaly et al.,[56] 2008	1995–2007	147	96%	90%	84%
Pfitzmann et al.,[57] 2007	1989–2002	300	96%	N/A	88%
Lim et al.,[58] 2004	1988–1997	3,063	82%	74%	68%
Bellamy et al.,[59] 2001	1991–1992	123	84%	N/A	72%
Mackie et al.,[60] 2001	1996–1999	64	82%	82%*	N/A
Gerhardt et al.,[61] 1996	1985–1991	67	90%	84%†	82%
Lucey et al.,[62] 1992	1985–1989	45	78%	73%*	N/A
Kumar et al.,[63] 1990	1982–1988	73	74%	N/A	N/A

*2–year survival; †2–4-year survival

ASH: alcoholic steatohepatitis; ALD: alcoholic liver disease; LT: liver transplantation; N/A: not applicable.

However, given the fact that alcohol consumption is contraindicated, many ASH patients experience 'alcohol relapse', with a relapse rate of 10–50%.[64] Further study is needed to clarify factors that determine the prognosis. Both ASH and NASH patients could benefit from liver transplantation with no significant survival difference and, considering underlying 'abnormal metabolic' status, there may be a role for aggressive control of metabolic syndrome in post-transplant NASH patients. As for ASH patients, cardiovascular disease and *de novo* malignancies seem to have great impacts on patients' quality of life and survivability, and thus they should be taken into consideration by the transplant team.[64]

CONCLUSION

ALD and NAFLD are increasingly important chronic liver diseases that lead to advanced liver damage, such as cirrhosis and liver cancer. While the alcohol volume threshold is widely used to distinguish NAFLD from ALD, there may be a combination of causes in many cases, considering that many people eat and drink a lot at the same time. ALD and NAFLD share many common pathological pathways as well as similar presentation. The two-hit hypothesis could apply to the pathogenesis of both ALD and NAFLD, with the first hit being steatosis, the excessive accumulation of fat components in hepatocytes, and the second hits being the stressors that cause the progression of steatohepatitis. Altered intestinal microbiota is a newly recognised risk factor leading to NASH and ASH via the cross-talk between the liver and the gut. Additionally, a number of genetic polymorphisms, including *PNPLA3* and *TM6SF2* variants, as well as epigenetic factors such as DNA methylation, post-translational histone modifications, and RNA-based mechanisms, increase the risk of ALD and NAFLD and affect the severity and progression. Although the complexity of steatohepatitis aetiologies results in great challenges in prevention and treatment, new drugs targeting inhibition of the inflammatory process and the improvement of insulin signalling are being tested in patients with NASH.[65] With the increasing number of patients requiring liver transplantation with ASH and NASH as aetiologies, there seems to be no significant difference in outcomes for these patients, and such results are consistent with previously published data.[48]

Currently, there are no clinical examination techniques that are able to definitely distinguish NASH from ASH or determine the precise percentage of contribution in each patient. The authors believe that since steatohepatitis is a multifactorial condition, people with

steatohepatitis probably have traits of both ASH and NASH. In other words, both diseases may coexist within one liver but consist of different spectrums. A precise diagnostic tool could allow physicians to formulate better therapeutic or preventive strategies. For example, for an ASH-dominant liver, quitting drinking is the best solution, whereas for a NASH-dominant liver, controlling body weight and blood sugar may be more efficient. Diagnosis based on drinking volume alone is not sufficient. Combining a specific set of biomarkers, genetic information, dynamic liver testing, and advanced imaging techniques may be promising new solutions for the diagnosis of steatohepatitis in the future.[66]

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Recent Updates on Corticosteroid Resistance in Asthma

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Disclosure: The authors have declared no conflicts of interest.

Acknowledgements: This work was supported by the projects BSC 0116, Council of Scientific & Industrial Research (CSIR), and GAP 0118 (Department of Biotechnology) at the Institute of Genomics & Integrative Biology, Council of Scientific and Industrial Research, Delhi, India.

Received: 12.01.18

Accepted: 26.06.18

Keywords: Asthma, glucocorticoids (GC), glucocorticoid receptor (GR), steroid resistance.

Citation: EMJ. 2018;3[3]:49-57.

Abstract

Corticosteroids are one of the most effective medications available for a wide variety of inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, autoimmune diseases, and chronic lung diseases such as asthma; however, 5–10% of asthma patients respond poorly to corticosteroids and require high doses, secondary immunosuppressants, such as calcineurin inhibitors and methotrexate, or disease-modifying biologics that can be toxic and/or expensive. Though steroid-resistant asthma affects a small percentage of patients, it consumes significant health resources and contributes to substantial morbidity and mortality. In addition, the side effects caused by excessive use of steroids dramatically impact patients' quality of life. Recognition of patients who respond poorly to steroid therapy is important due to the persistent and considerable problems they face in managing their conditions, which bears a significant socioeconomic burden. Along with the recognition of such patients, elucidation of the molecular mechanisms of steroid resistance is equally important, so that administration of a high dosage of steroids, and the consequent adverse effects, can be avoided. This review provides an update on the mechanisms of steroid function and the possible new therapeutic modalities to treat steroid-resistant asthma.

INTRODUCTION

Glucocorticoids (GC), also called glucocorticosteroids or steroids, are the mainstay therapy for numerous inflammatory diseases, such as asthma, dermatitis, rheumatoid arthritis; prevention of graft rejection; and autoimmune diseases. GC are steroid hormones that are synthesised and released by the adrenal cortex. GC have profound effects on various cell processes and organ-specific biological functions.[1] The regulation of downstream genes by GC involves either gene repression or activation. While gene activation leads to beneficiary actions of GC, repression of gene signalling is hypothesised to cause adverse effects due to prolonged use of steroids.[2] Although the majority of patients with inflammatory diseases and immune disorders respond to oral GC, 10–20% of patients do not respond even to very high doses of GC.

Resistance to steroids has also been reported in rheumatoid arthritis and inflammatory bowel disease and has been extensively studied in asthma. Despite the fact that only 5–10% of asthmatic patients do not respond to steroids, this leads to a significant socioeconomic burden and affects their quality of life as a result of the side effects associated with the prolonged usage of high doses of steroids.[3] Therefore, it is important to identify the patients with poor responses to steroids and to elucidate the molecular mechanism of steroid resistance.

This review focusses on the features, possible resistance mechanisms known so far, and recent developments in the treatment regimen of steroid-resistant asthma. To understand steroid-resistant asthma, the authors also bring the normal function of the steroids in a cell to the attention of the readers.

THE DIVERSE PHENOTYPES OF ASTHMA

Asthma is characterised by reversible expiratory airflow obstruction or airway hyper-responsiveness and airway inflammation. Approximately 235 million people have asthma worldwide.[4] In contrast to the previous Th2 dominant hypothesis, the phenotypes of asthma have been classified into high Th2 (eosinophilic inflammation) and low Th2 types (neutrophilic inflammation with Th1 and Th17 involvement).[5,6] Asthmatics with high Th2 phenotypes are responsive to corticosteroids, whereas non-Th2 asthmatics are much less so. The current treatment includes increasing the dosage of corticosteroids or using secondary immunosuppressants that are more toxic. Biologics, such as monoclonal antibodies against proinflammatory cytokines like IL-4, IL-5, IL-13, and IgE, have also been tried.

CLINICAL PRESENTATION OF GLUCOCORTICOID-RESISTANT ASTHMA

The response to steroids can be visualised as a spectrum, with steroid resistance placed at one end. Complete resistance to steroids is a rare case; usually poor response to steroids is observed, so that a high dose of steroids is required to control asthma, a condition termed steroid-dependent asthma. In 1968, Schwartz et al.[7] described GC resistance for the first time in six patients with low eosinopenic response, who did not respond clinically to high doses of systemic GC, although they showed reversibility to inhaled β-agonists. Later, a number of studies reported such insensitivity to steroids in asthmatic patients.[3] The detailed analysis of these patients revealed that the unresponsiveness was due to the inefficiency in the anti-inflammatory effects of steroids rather than their metabolic or endocrine functions. The thickness of the airway epithelium and basement membrane in steroid-resistant patients was larger than the patients with steroid sensitivity, in spite of having similar epithelial damage.[8] GC-resistant asthma is defined by a failure to improve forced expiratory volume in 1 second (FEV_1) by >15% even after an adequate dose of prednisolone (40 mg) for 2 weeks. These patients present bronchodilation with inhaled β2 agonists and typical diurnal variation of peak expiratory flow.[9] The diagnosis of steroid-resistant asthma is entirely based on the clinical history, symptoms, and lung function in the context of GC use. For a clear diagnosis, one has to rule out other diseases, such as cystic fibrosis and bronchiectasis.[10] Currently, there is no available clinical marker or established immune adherence test for the diagnosis of steroid-resistant asthma.

To understand the mechanisms behind steroid resistance in asthma, one should first understand the functioning and the mechanism of steroid receptor action. This will enable the identification of more cues for the failure of its function, leading to steroid resistance.

THE GLUCOCORTICOID RECEPTOR AND ITS FUNCTION

The GC receptors (GR) belong to the nuclear receptor Type I family. The human GR (*hGR*) gene is located on the chromosome 5q11-q13.[11] The *hGR* gene consists of nine exons, wherein the protein coding region is present between exon 2 and exon 9 (Figure 1). *hGR* is known to have three alternative promoters: 1A, 1B, and 1C. Although *hGR* does not have prominent TATA or CCAAT boxes, it contains multiple GC boxes.[12]

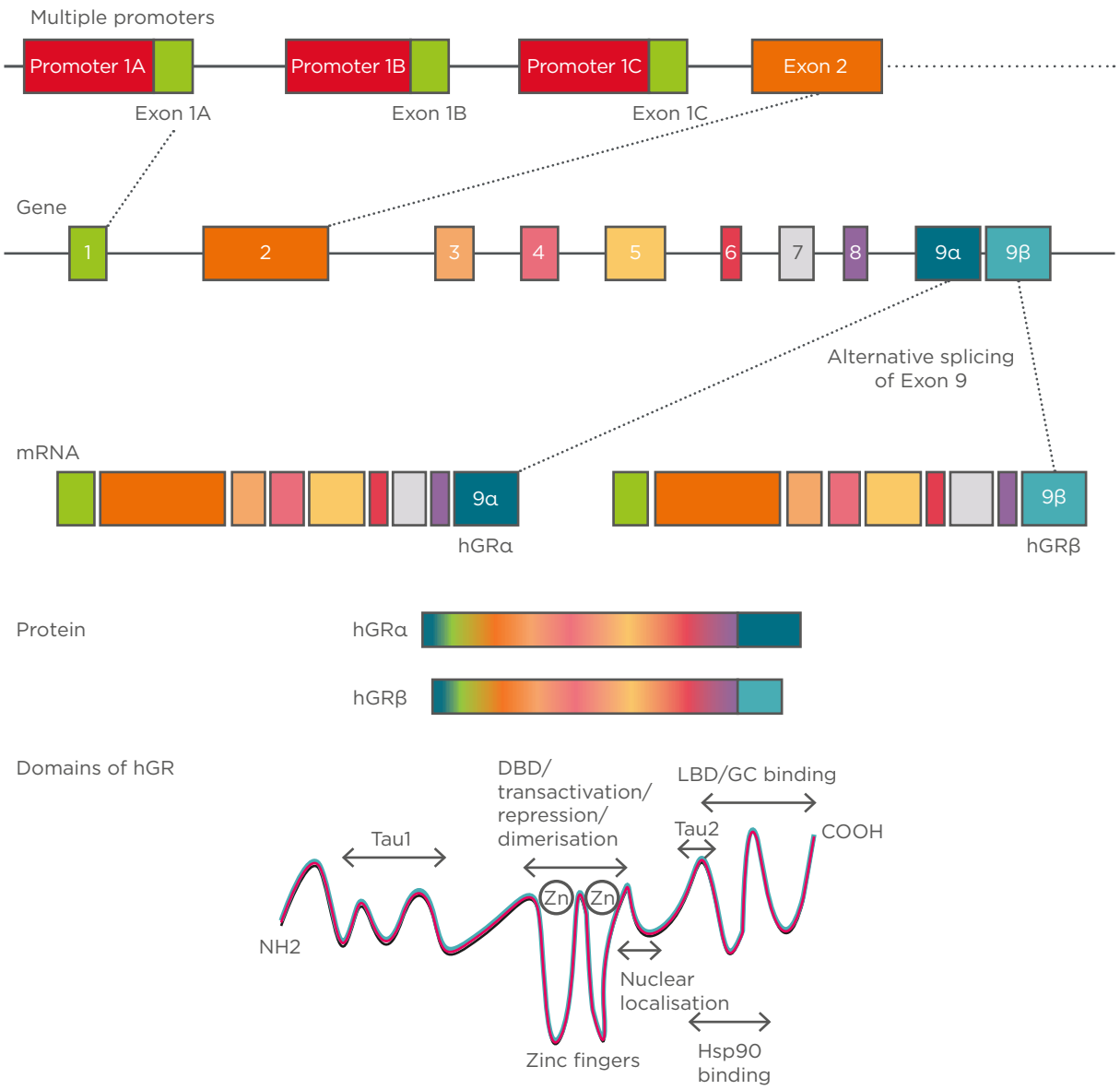
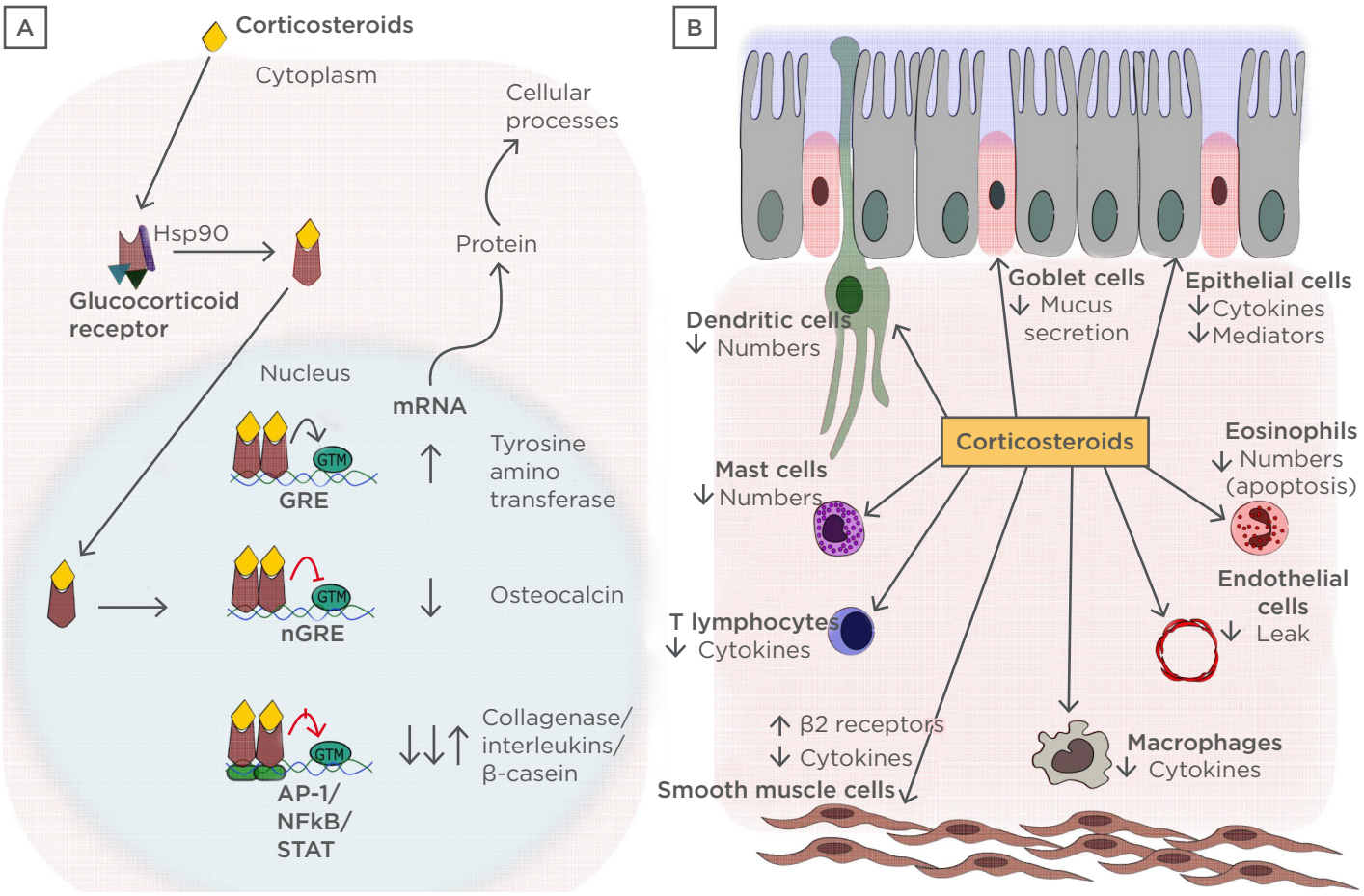


Figure 1: *hGR* gene structure and its isoforms.

The *hGR* gene has nine exons and three variable promoters giving rise to several glucocorticoid receptor isoforms that differ in the 5'-untranslated region. Alternative splicing of exon 9 at the 5' end gives rise to α and β isoforms. Domains of hGR show the LBD to be present at the C-terminal, which differentiates between hGRα and hGRβ in their functionality.

DBD: DNA binding domain; GC: glucocorticoid; hGR: human glucocorticoid receptor; Hsp90: heat shock protein 90; LBD: ligand binding domain.

Alternative splicing of GR pre-mRNA gives rise to two dominant isoforms: GRα and GRβ. GRα is located in the cytoplasm whereas GRβ remains in the nucleus and acts as a dominant negative or decoy receptor for GC[13] (Figure 1). hGRβ was identified as having a potential contribution to GC resistance in several diseases. It has been shown that while proinflammatory cytokines such as TNF-α and IL-1 increase the expression of GRβ, the formation of the GRβ/α heterodimer attenuates the function of GRα[14] (Figure 1). The GR protein has a modular structure similar to other members of its nuclear receptor family. It has three major domains: a) variable N-terminal domain (421 amino acids), b) central DNA binding domain (65 amino acids), and c) C-terminal domain (250 amino acids). Furthermore, the motif containing the nuclear localisation signals is present in both the DNA binding domain and ligand binding domain.[15]



Basic mechanism of steroid action

Cellular effects of corticosteroids

Figure 2: A) Basic mechanisms of steroid action and B) cellular effects of corticosteroids: pleiotropy in various cells.

A) The descriptive figure illustrates how glucocorticoids induce their effects in the cells. B) The cellular effects that are brought about in different types of cells due to the various gene regulations carried by corticosteroids.

AP-1: activator protein-1; GRE: glucocorticoid receptor element; GTM: general transcription material; Hsp90: heat shock protein 90.

Mechanism of Glucocorticoid Receptor Action

Endogenous GC participate in various physiological processes in a variety of cells, including hepatocytes, epithelial cells, neurons, immune cells, and cardiomyocytes (Figure 2). GC regulate multiple pathways, including carbohydrate metabolism, programmed cell death, amino acid metabolism, and inflammation.[1] They have three basic modes of action: first, the binding of heterodimerised receptors to the GR element (GRE) in the target genes to activate the transcription; second, inhibition of target gene expression by binding of GR heterodimer onto the negative GRE (nGRE); third, transactivation or transrepression by physical interaction with other transcription factors.[16] The GR bind with ligands and translocate into the nucleus. The GRα usually resides in the cytoplasm as part of a large multiprotein complex composed of chaperones such as Hsp90, Hsp70, p23, and immunophilin p59. These proteins bind to the unliganded GR and maintain its conformation in the cytoplasm without compromising the ligand binding efficiency.[17] When the ligand binds to the receptor, the protein complex dissociates, causing a conformational change in the receptors and exposing the nuclear localisation signals. This leads to the translocation of the GR into the nucleus through nuclear pores.[16]

Once GR enters the nucleus, the activated hormone-bound receptor dimerises and binds to the GRE via a zinc finger motif containing a DNA binding domain. Binding of GR to GRE leads to conformational changes in the receptor, which allow it to interact with several coactivators, such as cyclic adenosine monophosphate response element binding protein, p300, steroid receptor coactivator-1, p/CIF, SWI/SNF, and NcoA-1, which are critical in modulating the chromatin structure.[2] These coactivators activate gene transcription by unwinding the chromatin structure. Corticosteroids suppress inflammatory responses by activating the expression of anti-inflammatory proteins such as annexin-1, secretory leukoprotease inhibitor, IL-10, and IκBα. To generate this response, a large steroid concentration is required, but in a clinical scenario, a very small dose of corticosteroids is enough to generate the anti-inflammatory response.[18] Therefore, it is a controversial suggestion that the activation of genes would lead to the fruitful functioning of steroids. Hence, it is thought that an increase in transcription might generate systemic side effects, such as osteoporosis, growth retardation in children, skin fragility due to increased apoptosis, and metabolic effects observed due to overuse of steroids. To support this hypothesis, it was shown that mutant GR, which was unable to bind to GRE, did not show any loss of anti-inflammatory properties of steroids and was capable of suppressing NFκB-activated genes. This could also mean that GR has a different pathway of inhibiting NFκB-mediated inflammation[19] (Figure 2).

At the promoter level, GR can also inhibit the expression of its target gene through nGRE.[20] The inhibition in the transcription of genes takes place typically through two mechanisms; first, GR competes for its binding site on nGRE and hinders the binding of the other transcription factors, inhibiting the transcription of the downstream genes such as osteocalcin.[21] The second mechanism is via composite GRE, wherein the GR dimer bound with nGRE interacts with adjacent transcription factors and results in either gene repression or activation, which is dependent on the subunit of the composition. nGRE have been identified in the promoters of genes encoding for glutathione S-transferase, insulin, and vasoactive intestinal polypeptide receptor 1.

The GR can also regulate the expression of genes independent of direct binding to GRE. The GR exerts its anti-inflammatory role mainly through interacting with other transcription factors, such as NFκB and AP-1, binding to each other via the leucine zipper interaction.[23] NFκB is the master regulator of many proinflammatory cytokines and immune genes, and it seems intuitive that GR might reduce inflammation by inhibiting NFκB.[24] Another indirect way that GR inhibits protein synthesis is by reducing the stability of RNA encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) and cyclo-oxygenases. The GR receptor also represses proteins of the MAPK family by inhibiting the phosphorylation essential for their activation.

The capability of GC to produce anti-inflammatory actions also stems from their ability to induce apoptosis in inflammatory cells, such as thymocytes, eosinophils, and monocytes, and provide protection against apoptotic stimuli in cells with non-lymphoid origin.[25] GC have also been recently reported to inhibit mucus secretion in airways by repressing the expression of mucin genes such as *MUC2* and *MUC5AC*.[26] In addition, GC are efficient at reducing neurogenic inflammation by inhibiting the synthesis of tachykinins and tachykinin receptors, which amplify the inflammatory responses.[27]

MOLECULAR MECHANISMS OF STEROID RESISTANCE

With more than a decade of studies attempting to identify the molecular mechanisms of steroid resistance, a lot of pathways and mechanisms have been explored; however, there are four overarching steps that are essential in GC dysfunction: a) reduced GR expression, b) defective binding of steroids to the receptor, c) reduced ability of the receptor to bind to the DNA, either due to competition or reduced nuclear translocation, and d) increased expression and antagonism from proinflammatory transcription factors (AP-1 and NFκB).[3] In addition, a number of other mechanisms, like increased GRβ expression, defective histone acetylation, and involvement of immune cells have also been demonstrated. Here, a few key mechanisms of steroid resistance are described.

Defective Glucocorticoid Receptor Binding and Nuclear Translocation

Reports indicate that cytokines such as IL-2, IL-4, and IL-13 are overexpressed in the airways of steroid-resistant asthma patients.[3] In T cells and inflammatory cells, these cytokines are presumed to reduce GR affinity and elicit a local resistance to the anti-inflammatory action of GC.[28] These cytokines reduce steroid functioning by preventing phosphorylation of the GR. Impairment in GR phosphorylation and subsequent impaired translocation of the receptor into the nucleus and reduced binding to GRE have been reported in large proportions of patients with steroid unresponsiveness. p38 mitogen activated protein kinase (p38MAPK) is thought to participate in the phosphorylation of GR, which does not allow for nuclear translocations, and this effect is blocked upon addition of a p38MAPK inhibitor.[29,30] IL-2, IL-4, IL-5, and IL-13 are known to induce the phosphorylation of serine 226 on GR, which is inhibited by p38MAPK inhibitor. Selective inhibition of p38MAPK isoforms alpha, beta, and gamma increases the responsiveness to steroids in alveolar macrophages and peripheral blood mononuclear cells (PBMC) of severe asthma patients in response to IL-2 plus IL-4.[31]

TNF-α-induced p38MAPK, c-Jun N-terminal kinase (JNK), also phosphorylates GR at serine 226, inhibiting its binding with GRE in PBMC isolated from severe asthma patients.[32] To combat the phosphorylation-induced inefficiency of GR, corticosteroids and long-acting β2 agonists (LABA), like formoterol, activate MKP-1 and protein phosphatase 2A (PP2A), endogenous inhibitors of the JNK and p38MAPK pathways.[33] In alveolar macrophages of patients with a poor response to steroids, it was observed that MKP-1 expression was significantly reduced and was negatively corelated with p38MAPK activity. PP2A expression and activity was found to be reduced in PBMC of steroid-resistant asthma patients and knockdown of PP2A or inhibition reduced steroid responsiveness by inhibiting nuclear translocation and increasing JNK1 phosphorylation.

Inducible nitric oxide synthase (iNOS) has been reported to be increased in severe asthmatic patients, and high levels of nitric oxide have been reported to modify the GR ligand binding site by nitrosylating the GR at the Hsp90 interaction site, thereby inhibiting the translocation of GR into the nucleus. Whether this is relevant to steroid-resistant patients or not remains to be studied.[28,34] Intriguingly, iNOS is increased by smoking,[35] and steroid insensitivity in smokers with asthma may be attributed to this mechanism.

Increased Glucocorticoid Receptor-β Expression

The dominant negative isoform of GR, GRβ, has been described to be increased in the lymphocytes, neutrophils, and PBMC of steroid-resistant patients.[36,37] As previously explained, GRβ is induced by proinflammatory cytokines and competes with GRα for the GRE, and hence it acts as a dominant negative inhibitor. GRβ also acts as a decoy receptor as it does not have any ligand binding sites.[14] Microbial super antigens, such as Staphylococcal enterotoxins, also increase the expression of GRβ and this leads to steroid resistance in nasal explant models.[37] Another possible mechanism was discovered in alveolar macrophages of steroid-resistant asthmatic patients, where knockdown of GRβ with siRNA resulted in GRα nuclear translocation and increased steroid responsiveness.[37]

Defective Histone Acetylation

In a different group of steroid-resistant asthmatic patients, compromised anti-inflammatory action of steroids was observed along with reduced side effects. This was attributed to the inability of GR to acetylate lysine residue (K5) and hence transactivation of genes, which is required both for anti-inflammatory action and side effects. Repression of gene expression is also mediated by recruitment of histone deacetylase 2 (HDAC2) to deacetylate the chromatin and cause structural changes. HDAC2 expression has been reported to be reduced in alveolar macrophages, airways, and peripheral lungs in patients with severe asthma and steroid resistance.[38-40] The mechanism for this has been elucidated: oxidative and nitrative stress led to the formation of peroxynitrite, which nitrates tyrosine residues on HDAC2, resulting in ubiquitination, degradation, and inactivation of HDAC2. Oxidative stress also phosphorylates PI3Kδ, which further leads to phosphorylation and inactivation of HDAC2.[40-42]

Table 1: List of possible drugs and novel therapeutic strategies for the treatment of steroid-resistant/severe asthma by interfering with the pathways that cause it.

Study	Drugs	Target molecule	Mechanism of action
Mercado et al.,[31] 2011	p38 MAPK inhibitors	p38 MAPK	Inhibits phosphorylation of serine 226 on the GR.
Mercado et al.,[47] 2012	LABA formoterol	PP2A	Increases PP2A, hence dephosphorylating p38 MAPK-γ and reducing phosphorylation of GR on serine 226.
Chong et al.,[46] 2011	Roflumilast	PDE4	PDE4 inhibitor.
Cosio et al.,[50] 2004	Theophylline	PI3Kδ	Inhibits PI3Kδ and restores HDAC2 activity in macrophages from patients with COPD.
Mercado et al.,[51] 2011	Nortriptyline	PI3Kδ	Inhibits PI3Kδ and restores HDAC2 activity in macrophages from patients with COPD.
Kobayashi et al.,[45] 2013	Macrolides	PI3Kδ pathway	Targets the PI3K pathway and restores HDAC2 activity.
To et al.,[52] 2010	IC87114	PI3Kδ inhibitor	Targets the PI3K pathway and restores HDAC2 activity.

COPD: chronic obstructive pulmonary disease; HDAC2: histone deacetylase 2; PDE4: phosphodiesterase type 4; PP2A: protein phosphatase 2A; GR: glucocorticoid receptor; p38 MAPK: p38 mitogen activated protein kinases.

Immune Mechanisms

Th17 cells have been shown to be the hallmark cell type as they not only increase in number but also induce neutrophilic inflammation in steroid-resistant patients. In mice, adoptive transfer of Th17 cells leads to steroid resistance. In addition to this, IL-17 increases the expression of GRβ in airway epithelial cells more than in other cell types and is not suppressed by corticosteroids *in vitro*. When Th17 is increased, Treg cell response is reduced as they fail to secrete IL-10 in response to steroids; however, it was recently shown that oral administration of vitamin D3 enhanced *ex vivo* Treg response to steroids.[43] This suggests that vitamin D3 could be used as a potential therapeutic drug along with steroids.

Possible Therapeutic Strategies to Control Steroid-Resistant Asthma

With the present studies and insight into the mechanism of action of steroids, many potential strategies have been identified to control steroid-resistant asthma. One of the most common strategies is the use of broad-spectrum antibiotics.[44] This is due to the fact that most cases of neutrophilic asthma that are resistant to corticosteroids are because of bacterial infections.[44] Interestingly, solithromycin, a novel macrolide, improved oxidative stress-mediated steroid insensitivity, and, when it was given along with steroids, also inhibited neutrophilia.[45] In this respect, phosphodiesterase 4 inhibitors and oral roflumilast are in clinical development as an anti-inflammatory regimen; however, these oral uses are limited by their side effects, such as nausea, diarrhoea, and headaches, and inhaled drugs have proven ineffective so far.[46] p38MAPK inhibitors look promising based on the theoretical and experimental studies in steroid-resistant asthma.[31,47] However, in rheumatoid arthritis, prolonged administration of p38MAPK inhibitors led to the development of tolerance for the drug, suggesting that this might not be the only essential pathway.[48] HDAC2 restoration with plasmid vectors has been reported to improve steroid responsiveness in macrophages of severe asthma patients, but administration of plasmids to patients does not seem clinically viable.[49] In such a case, selective activation of HDAC2 could prove beneficial; drugs like theophylline and nortriptyline are known to increase HDAC2 expression and increase HDAC2 activity by inhibiting PI3Kδ.[50-52] Similarly, sulforaphane and curcumin have been found

to increase the expression of HDAC2 and reduce oxidative stress. Inhaled LABA are usually prescribed for asthmatics, and it is now well established that, when given alongside steroids, they improve the action of steroids by increasing the nuclear translocation of GR necessary for its anti-inflammatory actions. LABA achieves this by inhibiting the phosphorylation of GR at serine 226, inhibiting JNK1 and p38MAPKγ.[53-55] This benefit is mutually shared as GR increases the expression of β2 agonist receptors, increasing receptor turnover and preventing receptor blunting[3,30] (Table 1).

CONCLUSION

Although quite a few therapeutic strategies have been identified, a more concrete approach is required that ensures the side effects are reduced. For instance, blocking pathways and proteins that might also play a role in basic cell homeostasis could prove to be futile and even dangerous. With this premise, one can think of therapeutic agents, such as vitamin D3, which have been shown to be beneficial. However, recent studies raise the concern that the function of vitamin D3 is ambiguous as it did not show any protective effects against upper respiratory tract infections in adults with asthma along with stable doses of corticosteroids.[56,57] This indicates that any further complications in asthma, such as viral load, could alter the expected effect of vitamin D3. Not only vitamins but dietary supplements, such as omega-6 fatty acid, are also known to be correlated with asthma prevalence. Our group has shown that one such omega-6 fatty acid metabolite, 13-S-HODE, leads to severe asthmatic symptoms in mice and is resistant to steroid therapy.[58] This study is also relevant as it has been shown that 13-hydroxyoctadecadienoic acid increases in the sera of asthmatics compared to controls. This indicates that 13-hydroxyoctadecadienoic acid is an intrinsic parameter that may lead to steroid resistance in asthmatics if left unchecked. Thus, dietary supplementation may also prove to be important in genesis of the steroid-resistant features in asthmatics. Keeping this in mind, it is essential to identify and study more pathways to give rise to a more promising therapeutic with minimal side effects. This can be done by developing efficient mice models and studying molecular mechanisms from a new point of view.

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Diagnostic Accuracy of Magnetic Resonance Cholangiopancreatography Versus Endoscopic Retrograde Cholangiopancreatography Findings in the Postorthotopic Liver Transplant Population

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Disclosure: The authors have declared no conflicts of interest.

Received: 11.07.17

Accepted: 05.03.18

Keywords: Anastomotic stricture, ductal dilation, endoscopic retrograde cholangiopancreatography (ERCP), liver transplant, magnetic resonance cholangiopancreatography (MRCP).

Citation: EMJ. 2018;3[3]:58-64.

Abstract

Introduction: Magnetic resonance cholangiopancreatography (MRCP) is an important diagnostic tool in evaluating patients with biliary laboratory abnormalities after orthotopic liver transplant (OLT) to determine the need for more invasive procedures, such as endoscopic retrograde cholangiopancreatography (ERCP), which can deliver therapeutic interventions. The aim of this study was to determine the diagnostic accuracy of MRCP findings using ERCP as the gold standard in a group of post-OLT patients.

Methods: A retrospective review of 273 patients who underwent OLT at the University of South Florida and Tampa General Hospital, Tampa, Florida, USA, from January 2012–April 2015 was performed. A total of 52 patients who had a MRCP and underwent a subsequent ERCP were studied. Presence of anastomotic stricture, common bile duct dilation >0.7 mm, bile leak, stone, intrahepatic stricture, or extrahepatic stricture on either modality was recorded. SPSS statistical analysis software (version 22 for Windows, SPSS Inc., Chicago, Illinois, USA) was used to calculate diagnostic accuracy.

Results: The mean age of the population examined was 54.5±10.5 years; 73% of the patients were male (38 of 52). Overall agreement between the two procedures ranged from 71–96%.

The sensitivity, specificity, and positive and negative predictive values of MRCP for anastomotic strictures were 77%, 59%, 79%, and 56%, respectively. The sensitivity, specificity, and positive and negative predictive values of MRCP for common bile duct dilation of >0.7 mm were 64%, 95%, 82%, and 88%, respectively.

Conclusion: Despite significant improvement in the technology to better visualise the biliary system on MRCP, this study found that MRCP does not appear to be sensitive or specific in this chosen population. ERCP should be considered to confirm all positive MRCP findings, and in normal MRCP cases if there are other clinical data suggesting biliary abnormalities.

INTRODUCTION

Magnetic resonance cholangiopancreatography (MRCP) is quickly becoming the gold standard of care in the evaluation of patients who present with laboratory findings that are consistent with cholestasis. Several studies have found MRCP to be both sensitive (>90%) and specific (>90%) for the diagnosis of stones, tumours, and duct injuries.[1-5] It has even been suggested that MRCP could replace more invasive procedures, such as endoscopic retrograde cholangiopancreatography (ERCP), for diagnostic purposes in the general population.[4]

As the number of individuals receiving liver transplants increases, it is important to understand that these previous studies may not apply to patients presenting with cholestasis. There are currently >13,000 patients on the liver transplant waiting list in the USA as of 4th April 2018 according to the United Network for Organ Sharing (UNOS).[6] Despite advances in surgical techniques, organ selection, and immunosuppression therapy, the biliary tract continues to be the most common site for postoperative complications.[7] Complications such as stricture at the site of anastomosis, stones, and leaks can require long-term therapy and have a significant impact on graft survival and quality of life for orthotopic liver transplant (OLT) patients.[7]

Patients with biliary complications typically present with cholestatic laboratory abnormalities (elevations in total bilirubin, alkaline phosphatase, and gamma-glutamyltransferase [GGT], with or without elevation in aspartate aminotransferase [AST] and alanine aminotransferase [ALT]). A high index of clinical suspicion must be maintained as these patients are often asymptomatic, without fever or pain due to immunosuppression and hepatic denervation after transplant.[8] MRCP is usually completed in these situations to determine the need for more invasive procedures such as ERCP, which can be used diagnostically and therapeutically. The goal of this study was to determine the diagnostic accuracy of MRCP findings in the post-OLT population using ERCP as the gold standard.

MATERIALS AND METHODS

Approval for this study was obtained from the Institutional Review Boards of the University of South Florida and Tampa General Hospital. A retrospective analysis of all patients that underwent an OLT at the University of South Florida and Tampa General Hospital, between January 2012 and April 2015, was performed.

All patients >18 years old who underwent a MRCP and a subsequent ERCP were included in this study. Demographic data were collected, including the age, sex, and BMI of the patients. Presence of anastomotic stricture, common bile duct (CBD) dilation >0.7 mm, bile leak, stone, intrahepatic stricture, or extrahepatic stricture on either modality were recorded as primary outcomes.

In a separate analysis, all post-OLT patients >18 years old who were found to have an anastomotic stricture on ERCP were studied to determine the predictive value of initial laboratory tests for anastomotic stricture. In addition to demographic data (age, sex, and BMI), the presence and severity of any abnormality in temperature, AST, ALT, total bilirubin, alkaline phosphatase, and GGT were recorded.

Table 1: Number of patients identified with common disorders following magnetic resonance cholangiopancreatography versus endoscopic retrograde cholangiopancreatography.

Anastomotic stricture

		Endoscopic retrograde cholangiopancreatography	
		Yes	No
Magnetic resonance cholangiopancreatography	Yes	27	7
	No	8	10

Common bile duct dilation

		Endoscopic retrograde cholangiopancreatography	
		Yes	No
Magnetic resonance cholangiopancreatography	Yes	9	2
	No	5	36

Bile leak

		Endoscopic retrograde cholangiopancreatography	
		Yes	No
Magnetic resonance cholangiopancreatography	Yes	0	2
	No	4	46

Stone

		Endoscopic retrograde cholangiopancreatography	
		Yes	No
Magnetic resonance cholangiopancreatography	Yes	2	0
	No	2	48

Extrahepatic stricture

		Endoscopic retrograde cholangiopancreatography	
		Yes	No
Magnetic resonance cholangiopancreatography	Yes	1	1
	No	2	48

Intrahepatic duct stricture

		Endoscopic retrograde cholangiopancreatography	
		Yes	No
Magnetic resonance cholangiopancreatography	Yes	0	5
	No	0	47

No disorders identified

		Endoscopic retrograde cholangiopancreatography	
		Yes	No
Magnetic resonance cholangiopancreatography	Yes	5	1
	No	4	24

Endoscopic Retrograde Cholangiopancreatography and Magnetic Resonance Cholangiopancreatography

MRCP was performed using a General Electric 3 Tesla magnetic resonance imaging (MRI) scanner to generate multiplane and multisequence images obtained pre and post-intravenous Multihance® contrast (Bracco SpA, Milan, Italy). ERCP was performed with an Olympus V-Scope™ TJF-160VF side-viewing duodenoscope (Olympus, Tokyo, Japan). Omnipaque™ contrast (GE Healthcare, Little Chalfont, UK) was used for fluoroscopic views of the biliary and pancreatic systems.

Statistical Analysis

Patient characteristics and findings from diagnostic tests were summarised using

descriptive statistics and reported as mean and standard deviations for continuous variables, frequency, and percentages for categorical variables. Mann–Whitney U and chi-squared tests were used to compare continuous and categorical variables. The association between categorical variables was summarised as odds ratio (OR), 95% confidence intervals (CI), and mean difference. The Bonferroni correction was applied to adjust for multiple comparisons. The statistical significance for all comparisons was set at 5%. Diagnostic accuracy, measures of sensitivity, specificity, and positive and negative predictive values, along with 95% CI, were also assessed for the compared tests. The agreement between findings on diagnostic tests was also assessed using the Kappa statistic: agreement was considered slight if the κ value was 0.00–0.20, fair if κ was 0.21–40.00, moderate if κ was 0.41–0.60, substantial if κ was 0.61–0.80, and almost perfect if κ was 0.81–1.00. All statistical analyses were performed using SPSS statistical analysis software (Version 22 for Windows, SPSS Inc., Chicago, Illinois, USA).

RESULTS

A retrospective review of 273 patients who underwent OLT at the University of South Florida and Tampa General Hospital between January 2012 and April 2015 was performed. From that group, 52 patients were selected who had previously received an ERCP following MRCP. The mean age of the population was 54.5±10.5 years. The population was predominantly male (73.1%). Average BMI was 27±4.9. The most common primary biliary duct anastomosis type during liver transplant was duct-to-duct (n=50, 96.2%), followed by Roux-en-Y (n=2, 3.8%). The patient population exhibited a number of comorbidities, including hypertension (55.8%), diabetes (32.7%), dyslipidaemia (9.6%), coronary artery disease (5.8%), and chronic obstructive pulmonary disease (3.8%), as well as having a history of alcohol use (44.2%) and tobacco use (65.4%). There were a number of reasons for transplant, including alcoholic cirrhosis (25.0%), hepatitis C virus-induced cirrhosis (40.4%), hepatocellular carcinoma (3.8%), primary biliary cholangitis (3.8%), autoimmune hepatitis (9.6%), nonalcoholic steatohepatitis (7.7%), and other (9.6%).

Table 2: Diagnostic accuracy of magnetic resonance cholangiopancreatography.

Findings	Found	MRCP (n=52)	ERCP (n=52)	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)	Kappa index (95% CI)	Agreement
Anastomotic stricture	No Yes	18 34	17 35	77.14% (59.86–81.58%)	58.82% (32.92–81.56%)	79.41% (62.10–91.30%)	55.56% (30.76–78.47%)	0.35 (0.09–0.62)	71.15%
CBD dilation (>0.7 mm)	No Yes	41 11	38 14	64.29% (35.14–87.24%)	94.74 (82.25–99.36%)	81.82% (48.22–97.72%)	87.80% (73.80–95.92%)	0.63 (0.39–0.88)	86.54%
Bile leak	No Yes	50 2	48 4	0.00%	95.83% (85.75–99.49%)	0.00%	92.00% (80.77–97.78%)	-0.05 (-0.11–0.00)	88.46%
Stone	No Yes	50 2	48 4	50.00% (6.76–93.24%)	100.00% (92.60–100.00%)	100.00% (15.81–100.00%)	96.00% (86.29–99.51%)	0.65 (0.20–1.00)	96.15%
Intrahepatic duct stricture	No Yes	47 5	52 0	0.00%	90.38% (78.97–96.80%)	0.00%	100.00% (92.45–100.00%)	0.00	90.38%
Extrahepatic stricture	No Yes	50 2	49 3	33.33% (0.84–90.57%)	97.96% (89.15–99.95%)	50.00% (1.26–98.74%)	96.00% (86.29–99.51%)	0.37 (-0.18–0.93)	94.23%
No disorder	No Yes	46 6	43 9	55.56% (21.20–86.30%)	97.67% (87.71–99.94%)	83.33% (35.88–99.58%)	91.30% (79.21–97.58%)	0.61 (0.31–0.92)	90.38%

CBD: common bile duct; CI: confidence interval; ERCP: endoscopic retrograde cholangiopancreatography; MRCP: magnetic resonance cholangiopancreatography.

Table 3: Laboratory findings in patients with normal and abnormal endoscopic retrograde cholangiopancreatography.

	Normal number (%), N=19	Anastomotic stricture number (%), N=37	p value	Odds ratio	Confidence interval
Febrile	0 (0.0)	2 (5.4)	0.786	-	0.53–0.79
Tmax 100.4–101.4 (°F)	0 (0.0)	1 (2.7)	1.000	-	0.54–0.79
Tmax ≥101.5 (°F)	0 (0.0)	1 (2.7)	1.000	-	0.54–0.79
AST abnormal	16 (84.2)	33 (89.2)	0.915	1.55	0.31–7.75
AST 35–70 (U/L)	4 (21.1)	11 (29.7)	0.707	1.59	0.43–5.87
AST ≥71 (U/L)	12 (63.2)	22 (59.5)	1.000	0.86	0.27–2.68
ALT abnormal	16 (84.2)	32 (86.5)	1.000	1.20	0.25–5.66
ALT 56–110 (U/L)	5 (26.3)	7 (18.9)	0.768	0.65	0.18–2.43
ALT ≥111 (U/L)	11 (57.9)	25 (67.6)	0.674	1.52	0.49–4.75
Total bilirubin abnormal	15 (78.9)	32 (86.5)	0.732	1.71	0.40–7.28
Total bilirubin 1.2–4.0 (mg/dL)	9 (47.4)	16 (43.2)	0.992	0.85	0.28–2.57
Total bilirubin ≥4.1 (mg/dL)	6 (31.6)	18 (48.6)	0.349	2.05	0.64–6.56
Alkaline phosphatase abnormal	14 (73.7)	29 (78.4)	0.952	1.30	0.36–4.69
Alkaline phosphatase 151–300 (U/L)	11 (57.9)	12 (32.4)	0.122	0.35	0.11–1.01
Alkaline phosphatase ≥301 (U/L)	3 (15.8)	17 (45.9)	0.050	4.53	1.13–18.24
GGT abnormal	16 (88.9)	34 (97.1)	0.550	4.25	0.36-50.39
GGT 65–128 (U/L)	3 (16.7)	3 (8.6)	0.672	0.469	0.08-2.60
GGT ≥129 (U/L)	13 (72.2)	31 (88.6)	0.265	2.98	0.69-12.91

ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyltransferase; Tmax: maximum temperature.

The most common finding on MRCP was anastomotic stricture in 34 patients followed by CBD dilation in 11 patients. Anastomotic stricture was also the most common finding following ERCP, diagnosed in 35 patients, followed by CBD dilation in 14 patients. Very few patients were found to have bile leaks, stones, or intrahepatic or extrahepatic strictures on either of the two modalities, as summarised in Table 1.

The sensitivity and specificity for anastomotic strictures (n=35 on ERCP) for MRCP were 77.1% and 58.8%, respectively, while the positive and negative predictive values were 79.4% and 55.6%, respectively. Agreement between MRCP and ERCP for anastomotic stricture was 71.2%. For CBD dilation (n=14 on ERCP), the sensitivity and specificity of MRCP were 64.3% and 94.7%, respectively, while positive and negative predictive values were 81.8% and 87.8%, respectively. Agreement between the two modalities was 86.5% for CBD dilation (Table 2). With so few cases of bile leaks (n=4 on ERCP), stones (n=4 on ERCP), and intrahepatic or extrahepatic strictures (n=0 and n=3 on ERCP, respectively), we were unable to run diagnostic accuracy calculations between the two modalities.

A subgroup analysis was completed in 56 patients after selecting the individuals who underwent an ERCP that was either normal or had an anastomotic stricture, in an attempt to determine if the presence of, or the severity of, laboratory abnormalities, such as temperature, AST, ALT, total bilirubin, alkaline phosphatase, and GGT, could help predict anastomotic strictures. A total of 19 patients had normal findings on ERCP (normal group) and 37 patients had an anastomotic stricture (stricture group). Mean alkaline phosphatase levels were higher in the stricture group versus the normal

group (mean difference: 253.6 U/L; 95% CI: 2.45–504.81; p=0.048). Severe elevation of alkaline phosphatase (>301 U/L) had an OR of 4.53 (95% CI: 1.13–18.24; p=0.05) for anastomotic stricture. Severe elevation in total bilirubin concentration (>4.1 mg/dL) and any abnormal GGT value had a greater OR for anastomotic stricture; however, this was not statistically significant, with an OR of 2.05 (95% CI: 0.64–6.56; p=0.35) and 4.25 (95% CI: 0.36–50.39; p=0.55), respectively (Tables 3 and 4). Complications within 14 days of ERCP included pancreatitis (n=1), stent migration up (n=1), and stent migration out (n=1).

DISCUSSION

MRCP has substantially improved the ability to view the biliary system with radiographic imaging. New technology even allows for three-dimensional (3D) imaging that can be reformatted to produce a rotating display to analyse the biliary tract from any angle.[9] This study found that MRCP was not sensitive or specific at evaluating the biliary system in the post-transplant population. Furthermore, it was discovered that a negative MRCP was essentially no better than chance at diagnosing an anastomotic stricture, with a negative predictive value of 56%. There were eight cases when an anastomotic stricture, defined as focal narrowing at the site of anastomosis with contrast injection as observed by the endoscopist, would have been missed if ERCP had not been performed in the setting of a negative MRCP. Aydelotte et al.[10] found similar results evaluating the use of MRCP for discovery of choledocholithiasis, duct strictures, and duct injuries. The group advocated for the removal of MRCP as a diagnostic tool for the work up of biliary duct pathology.[10] It is also important to note that two patients had Roux-en-Y hepaticojejunostomy biliary reconstructions. Both of these patients had anastomotic strictures on ERCP. The remaining patients included in this study all had duct-to-duct biliary reconstructions.

Several studies claim that MRCP is equally as accurate as ERCP; however, these studies focus on the evaluation of biliary duct pathology in the general population.[5,11,12] Katz et al.[13] studied 27 post liver transplant patients and found a much better sensitivity and specificity between MRCP and ERCP (93% and 97.6%, respectively). However, only 18 of these patients underwent both procedures. They found one false negative and one false positive in the 18 selected patients.[13] Aufort et al.[14] studied 27 patients who underwent MRCP and ERCP after liver transplantation and found a sensitivity and specificity of 85% and 81%, respectively. However, their study included a much more detailed review of MRCP images at a later date by dividing the biliary tree into seven segments, and the study also had two blinded independent reviewers. Radiologists do not have the ability to perform such a detailed reading of MRCP images in clinical practice.[14]

Missing a stone or a delay in diagnosing a malignancy will typically result in continued pain and worsening cholestatic laboratory findings, eventually leading to diagnosis via ERCP at a later time in the general population. In the liver transplant population, delay in discovering biliary pathology, especially anastomotic strictures, can lead to severe infections, organ failure, and potentially death.

In this study population, MRCP was found to be not as useful as anastomotic evaluation, and the procedure is difficult in most patients due to duct mismatch post-transplant, which makes it difficult to determine whether anastomotic narrowing is significant. During ERCP, the endoscopist has the option of timing drainage or sizing the anastomosis with occlusion balloons to more accurately determine presence and severity of stricture and, ultimately, determine need for intervention. Additionally, the subgroup analysis carried out here was intended to determine if the severity of certain laboratory abnormalities would help clinicians predict the possibility of anastomotic stricture; however, none proved to be a reliable enough predictor.

Limitations

A limitation of this study is that it was performed as an observational retrospective study. The goal of this study was to determine diagnostic accuracy, but despite the 273 OLT patients evaluated at our centre, 83 of which had an MRCP, only 52 fit the study criteria of undergoing MRCP followed by ERCP and so were selected for in-depth study.

Table 4: Average values of laboratory findings in patients with normal and abnormal endoscopic retrograde cholangiopancreatography.

	Mean for anastomotic stricture, N=37	Mean for normal, N=19	p value	Mean difference	Confidence interval
Temperature (°F)	98.17	98.14	0.894	0.034	-0.47–0.54
AST (U/L)	140.89	137.79	0.935	3.100	-72.72–78.92
ALT (U/L)	237.61	192.21	0.468	45.580	-79.54–170.91
Total bilirubin (mg/dL)	5.39	5.56	0.886	-0.240	-3.58–3.10
Alkaline phosphatase (U/L)	498.84	245.21	0.048	253.630	2.45–504.81
GGT (U/L)	648.17	646.01	0.990	2.120	-329.75–333.98

ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyltransferase.

This generated a selection bias, as a small number of patients with normal MRCP scans (n=6) did undergo a subsequent ERCP due to significant clinical suspicion for biliary abnormality. While a prospective study would reduce the level of bias, it was not feasible, and to subject all patients with any suspicion of biliary disease to both procedures would be unnecessary, costly, and potentially dangerous. Additionally, a single clinician performed the chart review and outcomes were measured based on procedure notes, which can result in reader error.

CONCLUSION

In conclusion, MRCP does not appear to be either sensitive or specific in discovering biliary tract pathology in the post liver transplant population. ERCP should be used to confirm all positive MRCP findings, and even in normal MRCP cases if there are other clinical signs to suggest biliary abnormalities.

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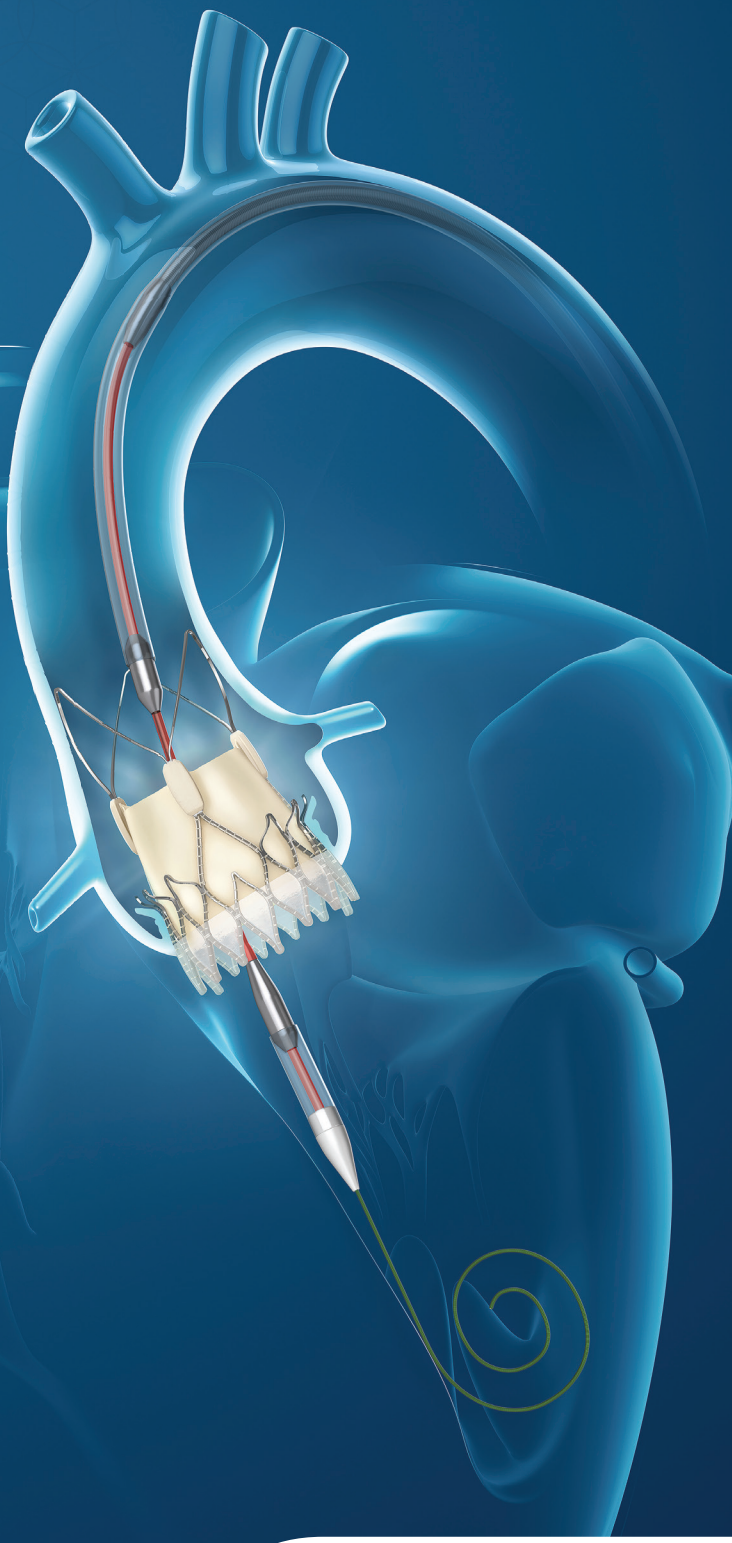

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Systemic Expression of Oxidative DNA Damage and Apoptosis Markers in Acute Renal Graft Dysfunction

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Disclosure: The authors have declared no conflicts of interest.

Acknowledgements: The authors would like to thank colleagues at the Specialties Hospital of the National Occidental Medical Centre at the Mexican Social Security Institute (Hospital de Especialidades del Centro Médico Nacional de Occidente, Instituto Mexicano del Seguro Social), Guadalajara, Mexico, for their support.

Received: 13.12.17

Accepted: 06.06.18

Keywords: Acute renal graft dysfunction (AGD), apoptosis, oxidative DNA damage, renal transplant (RT).

Citation: EMJ. 2018;3[3]:66-73.

Abstract

Background: Acute renal graft dysfunction (AGD) is one of the primary complications after kidney transplantation. The aim of this study was to identify the systemic oxidative DNA damage and apoptosis markers in patients with AGD, which will aid the understanding of the underlying processes of the complication.

Methods: A cross-sectional analytical study was conducted in renal transplant (RT) recipients with and without AGD. The follow-up time of patients was <1 year. Using the ELISA technique, the markers of oxidative DNA damage (8-hydroxy-2-deoxyguanosine and 8-oxoguanine-DNA-N-glycosylase-1) and apoptosis (caspase-3, caspase-8, soluble TNF receptor 1, and cytochrome C) were determined.

Results: Donor age was significantly higher in patients with AGD versus those without AGD (43±11 years versus 34.1±10.6 years, respectively; p<0.001). Levels of 8-hydroxy-2-deoxyguanosine were also significantly higher in AGD patients than those without AGD (624.1±15.3 ng/mL and 563.02± 17.4 ng/mL, respectively; p=0.039) and the DNA repair enzyme 8-oxoguanine-DNA-N-glycosylase-1 was significantly diminished in AGD patients versus non-AGD patients (7.60±1.8 ng/mL versus 8.13±1.70 ng/mL, respectively; p=0.031). A significant elevation of soluble TNF receptor levels in AGD patients was also found versus those without AGD (1178.6±25.2 ng/mL versus 142.6±39 ng/mL, respectively; p=0.03). Caspase-3 levels were higher in patients with AGD (1.19±0.21 ng/mL) versus

those without AGD (0.79±0.11 ng/mL; p=0.121) and was also significantly augmented in AGD versus healthy control subjects (0.24±0.1 ng/mL; p=0.036). Cytochrome c in AGD patients was 0.32±0.09 ng/mL and 0.16±0.03 ng/mL in those without AGD versus 0.08±0.01 ng/mL in healthy controls (p=0.130 and p=0.184, respectively).

Conclusion: These findings suggest that oxidative DNA damage with insufficient DNA repair and higher levels of caspase-3 compared to controls are markers of apoptosis protein dysregulation in AGD patients.

INTRODUCTION

Renal transplantation (RT) is the best management strategy for end-stage renal disease.[1] Although adequate function of the kidney is the aim of RT, diverse factors can trigger acute renal graft dysfunction (AGD): acute rejection, toxicity by calcineurin inhibitors, infections, and recurrence of the original disease.[2] Graft transplantation involves an ischaemia/reperfusion process during the surgical procedure, which is continuous with pathophysiological mechanisms, such as immunosuppressive infections, and is associated with the production of free radicals with the capacity to oxidise carbohydrates, lipids, proteins, and nucleic acids.[3]

8-hydroxy-2-deoxyguanosine (8-OHdG) is a marker of oxidative DNA damage that increases in the presence of oxidative stress[4] and reflects the magnitude of oxidative DNA damage.[5,6] 8-OHdG is a potentially mutagenic compound that participates as an inducer of apoptosis through activation of initiator and executor caspases.[7-9] 8-OHdG is therefore considered a marker of DNA damage severity.[10,11] The response to DNA damage involves repair, regulation of the cell cycle, and activation of apoptosis.[12] Oxidative DNA damage can be repaired primarily by the endonuclease enzyme 8-oxoguanine-DNA-N-glycosylase-1 (hOGG1).[13,14]

The stimulation of apoptosis can be produced in response to severe DNA damage or by severe shortening of the telomeres.[15] Apoptosis involves condensation of chromatin, fragmentation of the nucleus, vesiculation of the plasma membrane, and creation of apoptotic bodies through activation of the intrinsic or extrinsic pathways.[16] The extrinsic pathway is activated by death receptors belonging to the super family of TNF receptors (TNFR), which contain the death domain. The TNFR1/TNF-α or Fas/CD95 receptors are activated when they bind with ligands specific for trimerisation and transduce signals through the cytoplasmic death receptors. Interaction with TNFR1 activates the extrinsic pathway of apoptosis by activating caspase-3.[17] The intrinsic mitochondrial pathway participates in the liberation of pro-apoptotic proteins from the mitochondrial intermembrane space to the cytosol (permeabilisation of the outer mitochondrial membrane).[18] When severe DNA damage occurs it induces the activation of both pathways of apoptosis. In response to DNA damage, signals of phosphorylation of other proteins are activated and transduced.[19] Caspase-3 is activated in the presence of oxidative DNA damage and its activation increases during dysfunction of the graft.[20-22] The objective of this study was to identify systemic oxidative DNA damage and apoptosis markers in patients with AGD.

METHODS

A cross-sectional analytical study was performed in 90 patients >18 years of age who underwent RT at the Specialties Hospital of the National Occidental Medical Centre of the Mexican Social Security Institute (Hospital de Especialidades del Centro Médico Nacional de Occidente del Instituto Mexicano del Seguro Social [IMSS]), Guadalajara, Mexico; follow-up was <1 year and patients were managed with a triple immunosuppressive regimen (tacrolimus, mycophenolate mofetil, and prednisone). Two study groups were formed: 45 patients with AGD characterised by elevation of creatinine ≥30% from normal levels (average creatinine level per patient during the first post-transplant months) and 45 patients without AGD with serum creatinine <30% who attended the hospital for routine follow-up. Recipients of grafts from perished donors >55 years of age,

recipients with renal comorbidities at the time of the study (blood dyscrasias or infections), patients who were undergoing a second or third RT, and patients who had ingested nonsteroidal anti-inflammatory drugs, antineoplastic drugs, or angiotensin-converting enzyme inhibitors were excluded for the study. The blood samples of 10 clinically healthy subjects (volunteer blood donors) as a healthy control (HC) group were included to establish the normal values of the reagents.

Blood Samples

A total of 5 mL of venous blood was obtained immediately prior to RT in a tube containing ethylenediaminetetraacetic acid (EDTA) and another 5 mL in a dry tube. The serum and plasma were separated at 1,800 rpm for 10 minutes and the samples were stored at -80°C until final processing.

8-hydroxy-2-deoxyguanosine

Levels of the 8-OHdG marker were determined using ELISA (ELISA kit; Abcam®, Cambridge, UK), following the manufacturer's instructions. The samples, buffer, and standards were added to all wells except the blank. The 8-OHdG monoclonal antibody was added and the plate was incubated for 18 hours at 4°C. The plate was then washed with buffer for the recommended time and 200 µL of Ellman's reagent was added to each well. The absorbance was read at 405 nm with the automated reader (BioTek Instruments Inc., Winooski, Vermont, USA).

Human 8-oxoguanine-glycosylase-1

Levels of the hOGG1 enzyme were determined by an ELISA Kit (Elabscience®, Wuhan, China), following the manufacturer's instructions. The samples and standards were added to each well and the plate was incubated for 90 minutes at 37°C. The corresponding washes were performed and 100 µL of the biotinylated antibody was immediately added. The plate was incubated for 1 hour and later the enzyme conjugate and substrate were added. The optical density was read at 450 nm.

Caspase-3 and Caspase-8

Quantification of caspase-3 and caspase-8 in the serum was performed using the commercial ELISA kit (Caspase-h3 ELISA Kit and Caspase-h8 ELISA kit [MyBioSource®, San Diego, California, USA], respectively). Before performing the assay, the samples and reagents were kept at room temperature for 30 minutes. The standards and serum were added to the wells with 50 µL of the conjugate reagent and the plate was incubated at 37°C for 1 hour. The wells were washed five times and 50 µL of substrate solution A and 50 µL of substrate solution B were added, and then incubated at 37°C for 15 minutes. Then, 50 µL of stop solution was added and the absorbance was read at 450 nm.

TNF Receptor 1 Levels and Cytochrome c

The concentration of soluble TNFR1 (sTNFR1) was measured using the commercial ELISA kit (MyBioSource). Fifty microlitres of the standards and serum were pipetted into the 96-well plate and covered with the antibody, together with 100 µL of the horseradish peroxidase conjugate reagent and the resulting solution was incubated at 37°C for 1 hour. The wells were washed four times with buffer wash and 50 µL of the chromogen solution A and 50 µL of the chromogen B solution were added, and then incubated for 15 minutes at 37°C; 50 µL of the stop solution was added and the absorbance was read at 450 nm. The serum levels of cytochrome c were quantified using the commercial ELISA kit (Abcam). One hundred microlitres of serum and standards were added to the corresponding wells on the plate. Then, 50 µL of the biotinylated antibody was added and the plate was incubated for 2 hours at room temperature. The plate was washed and 100 µL of the streptavidin-horseradish peroxidase enzyme was added. The plate was incubated for 1 hour and the TMB substrate and the stop solution were added at the corresponding times. The absorbance was read at 450 nm.

Statistical Analysis

The Kolmogorov–Smirnov test was used to determine the distribution of the study variables. Variables are expressed as mean±standard deviation and in frequencies (%) according to their type. To compare biomarkers among AGD patients versus patients without AGD, and HC versus patients with AGD and without AGD,

a Mann–Whitney U test was used. Chi-squared was used to compare qualitative variables. A p≤0.05 value was considered statistically significant. The data were analysed with SPSS software (v20, SPSS Inc., Chicago, Illinois, USA).

Ethical Considerations

This study was performed in agreement with the norms established by the Declaration of Helsinki (64th General Assembly, Fortaleza, Brazil in October 2013) and the Best Clinical Practices Guide (International Conference on Harmonization for Research in Human Beings). Signatures were obtained for informed consent and the confidentiality of the patients was maintained. The study was approved by the Research and Ethics Committee of the Mexican Social Security Institute (R-2015-1301-83) and by the Research Registry in the State of Jalisco (59/E-JAL/2015).

RESULTS

Clinical and Demographic Data

There were no differences in the demographic data between the study groups except for sex frequency: more RT (84%) were performed in males, and females presented with significantly less AGD (p=0.007). The average age of patients with AGD was 25.7±6 years (range: 17–52 years) and 28.0±9 years (range: 18–58 years) without AGD. As expected, the levels of creatinine were significantly higher in AGD patients (p<0.001) versus those without AGD since it was the variable used in making the groups. The levels of baseline creatinine were significantly increased in AGD patients (p=0.007) versus those without AGD. The donor age was significantly higher in AGD patients versus those without AGD (43.0±11.0 years versus 34.1±10.6 years; p<0.001) (Table 1).

Oxidative DNA Damage and DNA Repair Enzyme

The level of the 8-OHdG marker of oxidative DNA damage in the HC was 427.03±32.2 ng/mL, while the average was found to be significantly elevated in AGD patients (624.1±15.3 ng/mL; p=0.005). 8-OHdG levels were also increased in patients without AGD (563.02±17.4 ng/mL; p=0.063) versus HC and were significantly increased (p=0.039) in AGD patients compared to non-AGD patients. Levels of the hOGG1 enzyme in HC were 12.23±0.30 ng/mL, while the enzyme levels in AGD and non AGD patients were significantly diminished (7.60±1.8 ng/mL and 8.13±1.70 ng/mL, respectively; p<0.001) compared to HC and between AGD patients and those without AGD (p=0.031) (Table 2 and Figure 1).

Table 1: Demographic and clinical features of the study population.

	AGD (n=45)	Without AGD (n=45)	p value
Male, n (%) Female, n (%)	38 (84.4) 7 (15.6)	26 (57.7) 19 (42.3)	0.007*
Smokers, n (%)	11 (22.0)	10 (19.6)	0.131
Age (years)	25.7±6.1	28.06±9.1	0.321
Weight (kg)	70.4±19.0	62.2±13.3	0.186
Age of donor (years)	43±11	34.1±10.6	<0.001†
Time post-transplant (months)	4.6±3.4	8.3±3.8	<0.001†
Creatinine (mg/dL)	1.9±0.65	1.1±1.0	<0.001†
Baseline creatinine (mg/dL)	1.18±0.30	1.01±0.39	0.003†
Habitual creatinine (mg/dL)	1.19±0.21	1.06±0.27	0.007†

*p≤0.05, chi-squared test was used to compare frequencies between groups; †p≤0.05, for comparison of quantitative variables using a Mann–Whitney U test.

AGD: acute renal graft dysfunction.

Table 2: Oxidative DNA damage and apoptosis markers.

	Healthy controls	AGD (n=45)	Without AGD (n=45)	p value	p value	p value
Oxidative DNA damage						
8-OHdG (ng/mL)	427.03±32.20	624.10±15.30	563.02±17.40	0.005*	0.063	0.039**
hOGG1 (ng/mL)	12.23±0.30	7.60±1.80	8.13±1.70	<0.001*	<0.001†	0.031**
Apoptosis						
Caspase-3 (ng/mL)	0.24±0.10	1.19±0.21	0.79±0.11	0.036*	0.070	0.121
Cytochrome c (ng/mL)	0.088±0.010	0.32±0.09	0.16±0.03	0.130	0.184	0.430
Caspase-8 (ng/mL)	0.79±0.23	1.25±1.20	0.90±0.75	0.983	0.538	0.166
sTNRF1 (ng/mL)	150.81±62.60	178.60±25.20	142.60±39.00	0.944	0.588	0.03**

*AGD versus healthy controls; †without AGD versus healthy controls; **AGD versus without AGD.

8-OHdG: 8-hydroxy-2-deoxyguanosine; AGD: acute renal graft dysfunction; hOGG1: 8-oxoguanine-DNA-N-glycosylase-1; sTNFR1: soluble TNF receptor 1.

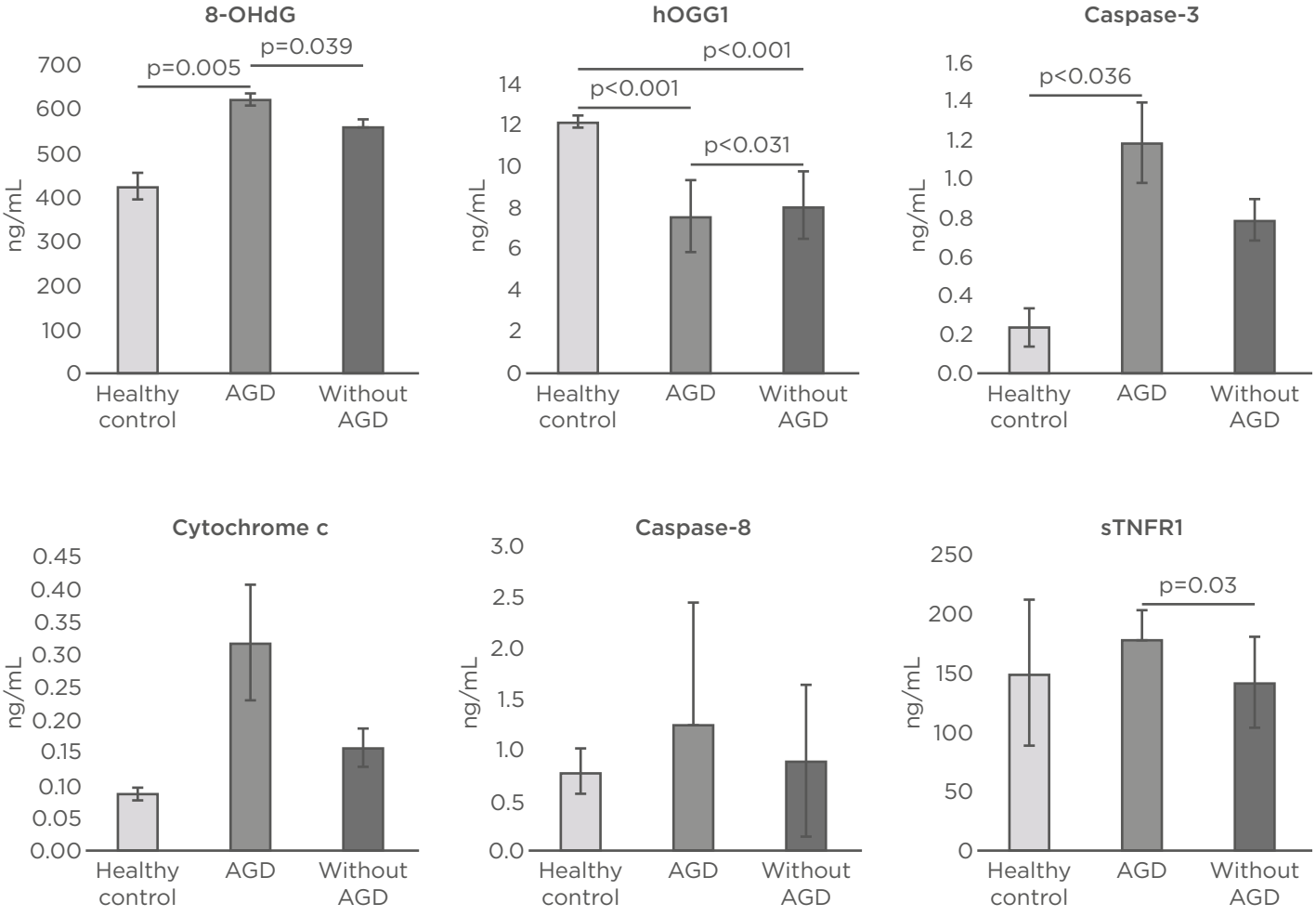

Figure 1: Oxidative DNA damage and apoptosis markers in healthy controls and patients with and without acute renal graft dysfunction.

8-OHdG: 8-hydroxy-2-deoxyguanosine; AGD: acute renal graft dysfunction; hOGG1: 8-oxoguanine-DNA-N-glycosylase-1; sTNFR1: soluble TNF receptor 1.

Caspase-3, Caspase-8, Cytochrome c, and Soluble TNF Receptor 1

The levels of caspase-3 were increased in AGD patients (1.19±0.21 ng/mL) versus those without AGD (0.79±0.11 ng/mL; p=0.121). Levels of caspase-3 were also significantly augmented in AGD patients versus HC (0.24±0.1 ng/mL; p=0.036). Levels of caspase-8 in HC were 0.79±0.23 ng/mL, 1.25±1.2 ng/mL in AGD patients, and 0.90±0.75 ng/mL in those without AGD (p=0.166). The levels of cytochrome c in HC were 0.08±0.01 ng/mL, 0.32±0.09 ng/mL in AGD patients, and 0.16±0.03 ng/mL without AGD (p=0.130 and p=0.184, respectively). The sTNFR1 levels were 150.81±62.6 ng/mL in HC, similar to patients with AGD (178.6±25.2 ng/mL) and patients without AGD (142.6±39 ng/mL) (p=0.944 and p=0.588, respectively). Nevertheless, when comparing the sTNFR1 levels between the AGD patients and without AGD, the plasma levels were significantly greater in AGD patients (p=0.03) (Table 2 and Figure 1).

Correlation Between Clinical Parameters and Apoptotic Markers

The association between the study variables and clinical parameters was evaluated. Donor age was an important factor in AGD patients, with a positive correlation to serum creatinine levels (Rho=0.36; p<0.001) and caspase-3 levels (Rho=0.29; p=0.01). There was a significant correlation between serum creatinine levels and sTNFR1 levels (Rho=0.25; p=0.017), and a positive association between the levels of serum creatinine and baseline creatinine levels (Rho=0.27; p=0.01).

DISCUSSION

The demographic characteristics of the patients included in this study demonstrate the important relationship that exists between donor age and AGD, in agreement with a previously published paper that indicates that kidneys from donors >55 years of age have a higher risk for AGD.[23] In the present study, donors >55 years of age were not included but it is noteworthy that as donor age increased it was related to an increase in serum creatinine levels in recipients; this result is consistent with other published reports.[24] Despite the fact that the effect of donor age on survival of the renal graft is well known, older donors are still used due to the scarcity of organs.[25] The levels of caspase-3 positively correlated with donor age; however, previous reports have demonstrated that caspase-3 levels increase in the process of damage and acute kidney graft rejection, but in our results patients were acute rejection-free and higher levels of caspase-3 were found in AGD subjects compared to HC, as well as a trend to those without AGD. In the case of caspase-8, no differences were found, which suggests higher protein apoptosis in AGD.[26] In 2015, Lorente et al.[27] reported the novel finding of an association between serum caspase-3 levels and mortality in patients with cranium trauma. Another study has suggested a novel mechanism of caspase-8 involvement in promoting inflammation via the release of exosomes by cancer cells that contain lysyl-tRNA synthetase to induce the migration of macrophages and secretion of TNF-α.[28]

The results of this study revealed a significant increase in the levels of the 8-OHdG marker in patients with AGD and 8-OHdG was found to be higher in acute graft rejection patients. Matsumoto et al.[29] concluded that 8-OHdG can be considered a prognostic biomarker of the graft,[29] and the increase in 8-OHdG in end-stage renal disease patients subjected to haemodialysis, peritoneal dialysis, or acute renal graft rejection has been reported.[30]

The results of this study are also consistent with those that have been previously described since the study found that patients with AGD present more oxidative DNA damage, possibly mediated by a significant decrease in the levels of the repair enzyme hOGG1. This finding relates to a report by Xu et al.,[31] who described an increase in oxidative DNA damage mediated by a decrease in its repair.

Other markers of apoptosis, such as cytochrome c liberated by the cellular mitochondria in apoptosis, are considered a key factor in the activation of the intrinsic pathway of apoptosis. This study discovered increased levels of cytochrome c in AGD patients, relating to reports from the experimental study by Tanaka et al.,[32] which demonstrated that the regulation of the anti-apoptotic BCL-2 proteins diminish the liberation of cytochrome c and that this

decrease favours graft maintenance. Other studies have highlighted the important role that TNF-α plays in graft rejection, and the TNF-α receptors are widely implicated in the function of this cytokine. Previous reports have suggested the relevant role of the TNFR in the development of acute rejection in RT.[33,34] In this paper, the serum levels of sTNFR1 were elevated in AGD patients and the increase found correlated positively with the clinical data of renal graft prognosis. The systemic expression of oxidative DNA damage and repair and some apoptotic markers could be potential biomarkers for the follow-up of patients with AGD.

CONCLUSION

This study provides one of the first sets of evidence showing a strong association between oxidative stress, apoptosis, and acute graft dysfunction in a RT setting. Despite this, the cross-sectional nature of the study is its major limitation because causality cannot be determined; however, this study paves the way for other researchers to explore this topic. In conclusion, these findings suggest that AGD patients experience oxidative DNA damage with insufficient DNA repair, possibly mediated by apoptosis dysregulation.

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Switching from Reference to Biosimilar Products: An Overview of the European Approach and Real-World Experience So Far

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Disclosure: The authors have declared no conflicts of interest.

Received: 27.03.18

Accepted: 31.05.18

Keywords: Biosimilar, interchangeability, post-marketing studies, real-world evidence.

Citation: EMJ. 2018;3[3]:74-81.

Abstract

Switching patients from a reference to a biosimilar product has become a primary topic of interest, with different approaches being undertaken by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA). In European countries, substitution of a reference medicine with a biosimilar product is encouraged for treatment-naïve patients. However, a more cautious approach has been taken with regard to switching patients on the reference product to a biosimilar product, with differences across countries. In general, there is a tendency to encourage the switch to biosimilars if conducted under the supervision of a clinician, with a few exceptions for substitution at the pharmacy level being permitted. There is also a general agreement that no further clinical trials are needed to allow any kind of switching, including automatic substitution, which differs from what is required by the FDA. With massive numbers of non-medical switches taking place in some European countries, as well as an increasing number of post-marketing studies being conducted, a growing amount of data on switching from originator to biosimilar products are becoming available. The data recorded so far suggest that switching is not detrimental for patients both in terms of safety and efficacy, although there have been some reports of increased treatment discontinuation rates after switching. Therefore, large-scale and long-term data are warranted to provide a more robust assessment of the effects of single or multiple switching. In addition, in Europe, the use of biologics has increased since their emergence, in particular in countries with historically poor access to biological medicines, and the tendency to promote the use of cheaper biological drugs is expected to increase further in the future. A communication strategy involving the patient and all other stakeholders that focusses on the patient's specific circumstances and information needs will play a crucial role in the conduction of a successful switch. An overview of switching policies across Europe together with outcomes from clinical trials and real-world evidence data is presented in this review.

INTRODUCTION

The number of biosimilar medicines approved in Europe, USA, and other regions has been steadily increasing over the past years, in parallel with the expiration of marketing exclusivity rights of originator molecules. More recently, this phenomenon has led to major concerns about the similarity of monoclonal antibody-based biosimilar products to the originator and related potential safety and immunogenicity risks. As of June 2018, the European Medicines Agency (EMA) has granted marketing authorisation to 43 biosimilar products, including some molecules with different brand names, with nearly half of those being monoclonal antibodies mainly indicated for the treatment of inflammatory diseases.[1] The USA trails behind Europe in this regard due to delays in implementing a clear regulatory pathway. However, as of June 2018, 11 biosimilar products have been authorised by the U.S. Food and Drug Administration (FDA), with 8 of them monoclonal antibodies, suggesting that the USA is rapidly progressing.[2] In addition, the FDA has approved two insulin treatments as follow-on products (approved as biosimilars in Europe) via the abbreviated 505(b)(2) pathway, due to differences in the FDA pathway regulating authorisation of biological medicines.

With an increasing number of biosimilars reaching the market, hence allowing wider access to biological medicines, switching patients from treatment with an originator molecule to its biosimilar is becoming a hot topic in the clinic. Appropriate strategies to achieve the right balance between the need for patients to be optimally treated without additional risks and the control of public expenditure are warranted.

DIFFERENCES BETWEEN THE EUROPEAN UNION AND THE USA

Differences in Terminology

Differences between the FDA and EMA terminology for switching have contributed to some confusion on this topic. The EMA has recently released an information guide for healthcare professionals on biosimilars[3] that includes definitions of terms related to replacement of medicines. According to the guide, interchangeability is a comprehensive medical and scientific term referring to the medical practice of changing a medicine for another one expected to have the same clinical effect, including replacement of an originator with its biosimilar or vice versa, as well as of a biosimilar with another biosimilar. Such a replacement can be carried out by switching or by automatic substitution; switching is an exchange performed by the prescriber, while automatic substitution is the replacement of a medicine with another equivalent drug performed by the pharmacist, as done for generic drugs, without intervention of the prescriber.

The confusion arises from the FDA's definition of interchangeability. According to Section 351(i) of the Public Health Service Act and the FDA draft interchangeability guidance,[4] the term interchangeable means that "the biological product may be substituted for the reference product without the intervention of the healthcare provider who prescribed the reference product", thus corresponding to the EMA's definition of automatic substitution.

Differences in Regulations

Interchangeability or substitution is regulated differently in the USA and in Europe. In 2017, the FDA released draft guidance on biosimilar interchangeability entitled 'Considerations in Demonstrating Interchangeability with a Reference Product'.[4] According to the guidance, sufficient information showing that a biosimilar product "can be expected to produce the same clinical result as the reference product in any given patient" has to be provided to get approval for interchangeability. To that end, an adequately powered switching study should be performed in a sensitive patient population. The design of such a study should include a parallel arm comparison, with one treatment arm alternating at least three times between the reference and the biosimilar product (multiple switching) and the other arm continuing with the reference product. Intensive pharmacokinetics sampling is to be conducted with clinical pharmacokinetics and pharmacodynamics, when applicable, representing the primary study endpoints, and immunogenicity and safety being key secondary endpoints. Once a product is FDA-approved as interchangeable, the pharmacist receiving the

prescription may substitute the interchangeable product for the reference product without consulting the prescriber, depending on the individual state regulation. To date, no biosimilar has been granted interchangeability (in addition to biosimilarity) by the FDA. To the authors' knowledge, only one study complying with FDA interchangeability guidance has been initiated, and this involved psoriasis patients with the recently approved adalimumab biosimilar Cyltezo® (Boehringer Ingelheim, Bracknell, UK).[5] A multiple switch design has been incorporated into adalimumab[6] and etanercept[7] biosimilar efficacy and safety studies in psoriasis, as well as in the filgrastim[8] biosimilar study, but without performing intensive pharmacokinetic sampling as recommended by the FDA.

Since the release of the guidance, a number of comments from different organisations have been submitted to the FDA. The Biosimilar Medicines Group, a sector group of Medicines for Europe, noted that the draft guideline did not take into account the wealth of evidence gathered in Europe where biosimilar medicines have been used in clinical practice.[9] Based on current knowledge of switching, derived from post-marketing surveillance and clinical studies, the group recommended developing a more pragmatic guidance document balancing theory with learnings from clinical practice to date.

In the European Union (EU), the EMA gave decision-making authority regarding interchangeability or substitution to each national agency of its member states. To date, not all member states have released guidance or implemented a law regulating substitution; however, it appears that all states share the opinion that further clinical studies dedicated to assessment of switching are not required, differing from the opinion of the FDA. The thorough comparability study conducted in the development of a biosimilar, combined with the growing amount of data about switching from reference products to biosimilars in clinical practice and clinical trials, is considered sufficient. However, there also tends to be general agreement that automatic substitution, with the exception of a few selected cases, is not possible for biological medicines and should be considered with caution and carried out under medical supervision.

For example, the Italian Drug Agency (AIFA) has recently released a second position paper[10] encouraging switching based on clinical judgment in patients being treated with the reference product, while excluding automatic substitution altogether. In the Netherlands, the Dutch Medicines Evaluation Board (MEB) released a revised opinion on biosimilar use,[11] concluding that there was enough evidence to support the use of biosimilars in clinical practice, provided this occurs with caution and under certain conditions. Substitution of one biological medicine for the other, both for originator biologicals and biosimilars, is possible according to the MEB, as long as it is adequately monitored clinically and the patient is well informed. In the UK, NHS England released a document in September 2017[12] providing guidance on how to achieve the objective of treating 80% of existing patients with the best-value biological medicine within 12 months of the launch of a biosimilar medicine. A switch programme involving all stakeholders has been outlined, although it was stated that any decision to conduct such a switch should be done with the approval of a physician and in consultation with the patient, since automatic substitution for biologics is not permitted in the UK. In addition, France took one of the most favourable positions with regard to the uptake of biosimilars and substitution.[13] Based on the 2017 French Social Security Finance Act (PLFSS), automatic substitution from a reference product to its biosimilar is allowed in the course of treatment provided the physician has not marked the prescription as 'non-substitutable' (similarly to generics). In practice, however, automatic substitution can only occur after an implementing decree has been adopted defining the precise conditions for biosimilar substitution by a pharmacist. In some countries, such as Germany and Sweden, automatic substitution is possible only for specific groups of biosimilars (e.g., those with the same manufacturer as the reference product).[14]

Availability of biosimilars is of particular interest for countries with limited access to expensive biological medicines due to financial constraints. Within Europe, this primarily concerns countries from Central and Eastern Europe. The highest increase in the use of different classes of biologics after the enrolment of biosimilars

has been observed in Central and Eastern European countries, including Romania, Bulgaria, Slovakia, Slovenia, and Poland.[15] Pharmacy-level substitution for both biologic-naïve patients and patients on treatment is possible in the Czech Republic, Estonia, Latvia, Poland, and Serbia with different modalities.[16] In some cases, the physician can opt out, but this may require a justification. In Poland there is no distinction between generics and biosimilars, making automatic substitution possible in the absence of any specific law.[14] In Bulgaria, Poland, and Serbia, tendering procedures are applied for purchasing biologics and the physician cannot opt out, thus forcing the patient to switch.[16] If tenders are conducted frequently, as in Bulgaria, patients might be forced to undergo multiple switches. Further to this, the requirement to prescribe drugs by their international non-proprietary name in Estonia and Latvia favours prescription of biosimilars even if physicians can opt out.

Therefore, it appears that the current situation in Europe is highly dynamic; however, in general, prescription and use of biosimilars is encouraged to control public expenditure, and substitution under the supervision of a healthcare professional in patients on treatment with the reference product tends to be allowed or even forced in some countries. As previously described, in certain countries automatic substitution may occur in the absence of or against specific regulations or recommendations if the biological product is prescribed by its international non-proprietary name or if products are purchased by a bulk tender. For this reason, several non-medical switches have taken place in Europe. A non-medical switch is a switch conducted for reasons other than the patient's health or safety, including economical or supply availability reasons, even if conducted with the agreement of the treating physician. For example, this has been the case in Denmark, where in 2015 a national guideline was implemented mandating non-medical switching of all patients treated with the infliximab reference product Remicade® (Janssen Ltd., High Wycombe, UK) to its biosimilar product Remsima® (Celltrion Healthcare Co. Ltd., Incheon, South Korea).

WHAT REAL-WORLD EVIDENCE IS SHOWING

As mentioned previously, a number of non-medical switches to biosimilar products have taken place in Europe within recent years. The majority of these have been single switches, i.e., a transition from the reference product to its biosimilar once the biosimilar was launched in the market. This generated a considerable number of reports of real-world experience, registry, and post-marketing data that were published or presented at international conferences, confirming that biosimilar switching continues to be a topic of great interest.

A recent literature review of switching studies with any biosimilar product found a total of 57 studies that included at least 20 switched patients, from inception to June 2017.[17] Thirty-four of these studies were observational; 50 studies in total (27 observational) reported a non-medical reason for switching. Overall, it was observed that the number of large studies with adequate statistical power evaluating switching was very low and that long-term post-switch data were missing. In addition, information on immunogenicity was missing from most of the observational studies. Nonetheless, the overall conclusion was that switching to the biosimilar product did not raise special concerns in terms of either efficacy or safety. In a more recent review[18] including all types of biosimilars, attention was also paid to multiple switching, which will certainly become more frequent in the future, including switches among different biosimilars. It was noted that data on multiple switching are still quite limited; only the three multiple switch studies of adalimumab,[6] etanercept,[7] and filgrastim[8] previously mentioned were found, but it is expected that more data will be generated within the coming years.

One of the largest post-marketing studies that explored the effects of switching is the NOR-SWITCH study,[19] sponsored by the Norwegian government, which randomised nearly 500 patients with various inflammatory diseases to either continue on the originator infliximab Remicade or to switch to its biosimilar product CT-P13 Remsima for 52 weeks. The study was double-blinded, with neither the study staff

nor patients knowing the assigned treatment. Switching was demonstrated to be non-inferior to remaining on the originator treatment in terms of disease worsening after 1 year and did not present any notable safety issues. A 4% drug discontinuation rate was reported in the two groups, primarily due to adverse events. Changes in patient-reported outcome measures were also comparable between the two groups. It should be noted that switched patients experienced various inflammatory diseases, with numbers ranging from 16 patients with psoriatic arthritis to 77 patients with Crohn's disease. Disease worsening was assessed differently for each disease, but the study was statistically powered to demonstrate non-inferiority only for the pooled groups of patients; therefore, no conclusion could be drawn on individual diseases.

A massive switch from reference to biosimilar products occurred in European Nordic countries, providing some robust long-term follow-up data. In Denmark, >800 patients with inflammatory arthritis and receiving stable therapy with infliximab were switched from the originator product (Remicade) to infliximab biosimilar CTP-13 (Remsima) and followed for >1 year in an observational study.[20] No impact on disease activity was found, while retention rates after 1 year were slightly lower than those of an historical Remicade cohort, totalling 83.4% and 86.8%, respectively (p=0.03), with about half of withdrawals due to lack of effect. Patients who had received Remicade for >5 years exhibited longer retention with Remsima.

In a prospective observational study, 260 patients receiving maintenance therapy with infliximab (Remicade) at a single French hospital were switched to Remsima.[21] Approximately half of the patients had axial spondyloarthritis. At the time of the third infusion, the retention rate was 85%. During a follow-up period (an average of 34 weeks), 18% of patients discontinued Remsima due to lack of efficacy; however, no changes in objective measures of disease condition or in other biological or safety parameters were observed. In subjects with axial spondyloarthritis, a significant worsening of Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score was found. Since BASDAI is a patient-reported outcome, it is believed that this discontinuation rate was related to wrong causal attribution effects rather than pharmacological differences. Similar results were reported in an observational study conducted in the Netherlands, where 192 patients with inflammatory arthritis were mandatorily transitioned from Remicade to Remsima.[22] Although no changes in efficacy, safety, and immunogenicity during 6 months follow-up were found, 24% of patients discontinued treatment, mainly due to an increase in subjective features such as tender joint count, or patient's global assessment of disease activity, and/or subjective adverse events such as arthralgia, fatigue, pruritus, or myalgia. Several small single-centre observational studies have consistently reported that switching from Remicade to Remsima/Inflectra® (HOSPIRA Enterprises B.V., Almere, Netherlands) does not lead to loss of efficacy or safety concerns.[18]

Following approval and launch in Europe during 2016 of the first etanercept biosimilar SB4 (Benepali® [Biogen, Cambridge, Massachusetts, USA]), a rapid and significant uptake took place. Prescription data from biologic registries showed that massive non-medical switches occurred in a number of European countries. In Denmark, >1,500 patients with rheumatic diseases were switched from Enbrel® (Amgen, Cambridge, UK) to Benepali in 2016. A 3-month follow-up after the switch showed that disease activity was largely unaffected,[23] although 9% of patients stopped treatment during a 5-month follow-up period. After 1 year, the withdrawal rate was roughly 18%, with about half of these withdrawals due to lack of efficacy.[24]

Data from Sweden showed that 55% of >5,000 rheumatology patients who initiated treatment with Benepali had undergone a non-medical switch from Enbrel; 11% of these switched back to Enbrel after a median time of 55 days. Follow-up data showed no overall worsening in the condition of patients, although a proportion of patients interrupted treatment and, in some instances, returned to the originator drug.[25]

Furthermore, a recent analysis of discontinuation rates among rheumatoid arthritis patients recorded in the Swedish Biological Registry who received TNF-inhibitors during 2003–2011 found that 25% of patients on Enbrel discontinued the drug after 1.3 years. However, it was highlighted that overall biologic discontinuation

rates tended to increase in the more recent years; therefore, comparison with historical rates from the past may no longer be valid.[26] This is consistent with the outcome of the recent Dutch BIO-SPAN study.[27] In the study, >600 patients with inflammatory arthritis were requested to transition from originator Enbrel to Benepali following a structured strategy with an opt-out option. Patients on treatment with Enbrel in 2014 were recruited as a historical cohort. The 6-month crude retention rate in the transitioned cohort was 90% versus 92% in the historical cohort, with an increased risk of drug discontinuation in the transition cohort (adjusted hazard ratio: 1.57) that was considered not clinically significant. The main reasons for discontinuation were lack of effect (more frequent in the historical cohort) and adverse events (more frequent in the transition cohort).

The Nocebo Effect

The above findings that suggest a tendency toward higher discontinuation rates following non-medical switch to a biosimilar product need further confirmation and investigation about possible causes. A nocebo effect has been frequently stated as a potential reason for this phenomenon, based on the observation that most of the patients who discontinued biosimilar treatment due to a perceived lack of efficacy or subjective complaints did not show objective signs of disease worsening or specific safety issues.

A nocebo effect occurs when a patient's negative perception of a therapy causes an unexpected and unexplained worsening in treatment outcome. Regarding the use of biosimilars, a nocebo effect could be ascribed to a negative perception associated with transitioning from high-cost biologics to new lower-cost products without a medical reason. Consequently, the importance of a proper communication strategy to explain to patients the reasons for and benefits of the switch as well as the importance of adherence to therapy has been given increasing attention, particularly when planning large switch programmes. The crucial role of a proper and correct communication strategy involving healthcare professionals, patients, and payers was emphasised at the third multi-stakeholder workshop on biosimilar medicinal products organised by the European Commission,[28] where the adoption of a collaborative approach to successfully switching to biosimilars represented a primary topic for discussion, with positive examples provided by Sweden, Denmark, and the Netherlands.

A collaborative approach was also fundamental to the switch programmes implemented at Southampton University Hospital, Southampton, UK.[29] A total of 143 patients with inflammatory bowel disease were switched to Remsima through a managed switching programme that actively involved all stakeholders.[29] No changes in the incidence of side effects, objective disease parameters, immunogenicity, or drug persistence were observed. Following the success of the programme, a similar study was implemented to transition patients with rheumatological diseases from Enbrel to Benepali. The strategy included a comprehensive education and support programme. As a result, 92 patients (99% of those requested) accepted switching and within the following 6 months only 8 discontinued treatment; 7 of these cases were due to reported lack of efficacy and 1 for an adverse event. The rate of discontinuation was low compared with the discontinuation rate in the 6 months preceding the switch.[30]

Studies primarily aimed at investigating patients' and clinicians' characteristics and beliefs in relation to drug persistence and treatment outcome following biosimilar switch, like the BIO-SPAN study,[27] are being initiated and it is hoped that they will provide a better insight into the multiple aspects of a successful (or unsuccessful) switch to biosimilars.

CONCLUSION

The need for controlling public expenditure while making biological medicines accessible to a wider patient population means that the adoption of biosimilars is currently encouraged in Europe, even if individual countries take different approaches. To facilitate this process while also reinforcing patient confidence in switching and guaranteeing that any potential safety or efficacy signal is promptly recognised, it is of fundamental importance to collect real-world data derived from large-scale observational or registry studies. At the

same time, specific collaborative switching programmes should be implemented that involve all stakeholders, with physicians maintaining a leading role. A properly managed switching strategy, combined with long-term follow-up of the patient's condition and immunogenicity, will help to clarify all aspects of switching to biosimilars, including the concerns about reported higher rates of drug discontinuation.

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Knee Infection After Anterior Cruciate Ligament Reconstruction

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Disclosure: The authors have declared no conflicts of interest.

Acknowledgements: The first author acknowledges Leonel Barahona for his support in writing this article.

Received: 15.01.18

Accepted: 12.06.18

Keywords: Anterior cruciate ligament (ACL) reconstruction, antibiotics, arthroscopy, infection, knee.

Citation: EMJ. 2018;3[3]:82-89.

Abstract

Knee infection is a challenging scenario. One way to classify the infection would be as spontaneous or post-surgery, the latter having a particular relevance given the presence of synthetic materials such as screws or prostheses surrounding the joint. Open surgery has a higher rate of infection than arthroscopic procedures. Periprosthetic infection is a complication that follows arthroplasty, with an incidence that varies between 0.4% and 2.0%, while arthroscopic procedures have an incidence varying between 0.001% and 1.100%. Anterior cruciate ligament (ACL) reconstruction complication rate is low, with septic arthritis one of the most frequently seen. Early diagnosis of complications is vital to improve functional outcome. In these cases, knee pain, decreased range of motion, fever, and high C-reactive protein levels should alert any physician, and infection must not be ruled out. This article presents a case of infection after ACL reconstruction and discusses risk factors, treatment choice, antibiotic treatment length, and functional outcomes, proposing a guide for the treatment. The clinical case presented is a chronic infection due to *Staphylococcus aureus* that resulted in extensive cartilage damage and graft loosening; delayed diagnosis was an essential modifiable risk factor in this case. Treatment success is defined as the eradication of the infection without the need to remove the ACL graft. Risk factors for a worse outcome after ACL reconstruction infection are allograft compared to autograft and *S. aureus* or polymicrobial infection compared to coagulase-negative staphylococcus infection. Functional outcome is compromised by infection; however, if early diagnosis and treatment are performed, good functional results and a return to sports activities can be expected.

INTRODUCTION

Acute septic arthritis is a common clinical problem seen in emergency departments, and the knee is the most frequently affected joint. Despite advances in medicine, septic arthritis is still a significant cause of morbidity and sequelae.[1] Knee infection can be classified as spontaneous or post-surgery, with the latter commonly due to the presence of osteosynthesis or prosthesis material. Open surgery has a higher rate of infection than arthroscopic procedures. Periprosthetic infection is a complication that follows arthroplasty and the incidence varies

between 0.4% and 2.0%, while arthroscopic procedures have an incidence that varies between 0.001% and 1.100%.[2,3]

The anterior cruciate ligament (ACL) is an intra-articular and intrasynovial structure of the knee, which plays a crucial role in joint stability.[4] ACL tear incidence is increasing; the most frequent mechanism of ACL tear is an indirect, involuntary torsion known as pivot shift, which consists of a valgus and internal force applied to a knee at a flexion degree of 10–20°.[5] General consensus is that anatomical ACL reconstruction can restore anteroposterior and rotatory stability,[6] but graft choice depends on different factors, with the most important being the surgeon's preference and patient's preference, patient activity, and history of prior surgery.[7] The most commonly used grafts are bone-patellar tendon, hamstrings (HT), bone-quadricipital tendon, and allograft.[8] Although rare, ACL reconstruction has complications. Cvetanovich et al.[9] reviewed the complications of ACL reconstruction in the first 30 days and found the major complication rate to be 0.55%, with deep vein thrombosis the most frequent; pulmonary embolism and infection were also observed.[9] Other studies report similar complication rates and show that infection is one of the most frequent complications after ACL reconstruction, with an incidence between 0.14% and 1.70%.[10-13]

This article presents a case of infection after ACL reconstruction and discusses the risk factors for infection, treatment choice, antibiotic treatment length, risk factors for treatment success, and functional outcomes, concluding with a guide for treatment.

CASE REPORT

A 40-year-old male was evaluated in the emergency department complaining of 2 weeks of intermittent fever, quantified up to 38.5°C, and left knee pain associated with articular effusion and a loss of range of motion. His clinical record showed a left knee ACL reconstruction with bone-patellar tendon-bone (BTB) graft 4 months prior to emergency department admission. After surgery, he did not recover normal articular range and had intermittent severe pain. Two months after surgery, he was diagnosed with articular stiffness and an arthroscopic fibrous tissue debridement, and mobilisation under anaesthesia were performed. No cultures were taken on this occasion. Both procedures were performed in other institutions.

RISK FACTORS

Many variables have been studied to determine if they are a risk factor for infection after ACL reconstruction. In a recent meta-analysis performed by Murphy et al.,[14] a multivariate logistic regression was estimated in a cohort of 1,397 ACL reconstruction patients for risk factors for infection. Significant factors noted were age >20 years, male sex, connective tissue disease, consumption of immune suppressive medications, and HT graft. Diabetes and previous ipsilateral knee surgery were not statistically significant variables.[14] Despite this finding, a study by the MOON Knee Group showed a higher incidence of infection in diabetic patients following ACL reconstruction.[15] Krutsch et al.[16] compared infection rate by sport and found that football had a higher rate of infection than skiing, suggesting that ambient temperature must affect infection rates.[16] In addition, Westermann et al.[17] performed a multivariate analysis in a cohort of 6,389 ACL reconstruction patients comparing infection rates between outpatients and hospital-admitted patients. The authors found a significant increase in the rate of infection in admitted patients and concluded that efforts must be made to improve outpatient care.[17]

A recent meta-analysis performed by Bansal et al.[18] showed a lower infection rate when a BTB graft was used compared to HT autograft, reaching a relative risk of 0.230 (95% confidence interval: 0.097–0.540) and a heterogeneity of 0%. This meta-analysis did not find a significant difference in infection rates for autograft versus allograft, achieving a relative risk of 1.035 (95% confidence interval: 0.589–1.819) and a heterogeneity of 0%.[18] Likewise, another recent cohort study of 10,190 ACL reconstructions performed with allograft showed an infection rate of 0.15%, highlighting that the newest evidence shows no increased risk of infection if an allograft is used.[19]

Graft contamination is a risk for the development of septic arthritis.[20,21] Accidental contamination of the graft is a modifiable risk factor and is important to consider in graft contamination prevention. If this happens, there are protocols for treating the contaminated graft, which include the use of gentamicin and chlorhexidine, obtaining negative cultures of >90%.[22] The current consensus in cases of contamination, regardless of the type of autograft (BTB or HT), is to perform a washing procedure with saline solution and an antibiotic or disinfectant for 8 minutes. The recommended method is to use gentamicin or 2% chlorhexidine, achieving negative cultures of approximately 100%. There are no established guidelines for allografts.[12,23,24]

In addition to accidental contamination, such as incidence of the graft falling to the ground, there is concern about the contamination of the graft as it was placed on the surgical table. Alomar et al.[25] performed a case-control study in which they took cultures of a dropped graft (case) and a graft that was always kept on the surgical table (control). The dropped graft showed a trend towards more positive cultures; nevertheless, no significant difference was achieved. In both groups, *Staphylococcus epidermidis* was the most commonly found bacteria, followed by *S. aureus* in the control group and by *Bacillus* species in the case group. Notably, the control group showed epidemiology more likely to be an ACL reconstruction infection than the control group.[25]

In the authors' own experiences, after preparation, the graft is kept in a glass container, submerged in a saline solution with 80 mg of gentamicin; this is maintained until it is time to start the fixation, and the process does not alter the biomechanics of the graft. Also, all patients receive 2 g of cefazolin before surgery and two doses of 2 g cephazolin after the surgery. Referring to the aforementioned case study, except for age (40 years), the patient did not present other risk factors for infection. It is noteworthy that there was no history of what happened to the patient between the two previous surgeries, such as accidental contamination of the graft, for example.

DIAGNOSIS

The most common symptoms of infection following ACL reconstruction are fever, knee effusion, loss of knee flexion, and pain.[26] According to Wang et al.,[27] the median number of days after surgery for symptom presentation is 13. It is important to emphasise the transcendental nature of early diagnosis since it increases the possibility of retaining the graft and avoids severe cartilage damage and ankylosis.[28,29]

Laboratory tests are essential to confirm the diagnosis. In a study by Wang et al.,[30] comparisons between C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) in noninfected and infected ACL reconstruction patients at 5 days post-surgery demonstrated that a CRP >41 mg/dL had a sensitivity of 94.1% and a specificity of 97.6%, while ESR >32 mm/hour had a sensitivity of 91.2% and a specificity of 80.5%.[30]

Infections are classified by time after surgery as acute (<3 weeks after surgery), subacute (3 weeks to 3 months after surgery), and chronic (>3 months after surgery), with acute and subacute the most common. The most frequent infection-causing agents were the *Staphylococci*, with the most common, *S. epidermis*, responsible for 50% of incidences (coagulase-negative staphylococcus [CNS]), followed by *S. aureus*. Fungus infections by *Candida* and *Aspergillus* have also been reported and are associated with high rates of graft removal and worse functional outcomes compared to *Staphylococcus* infection.[31] Also, tubercular infection cases have been reported, so endemic zones must include this infection in diagnosis algorithms.[32]

In the reported case, physical examination showed an axillary temperature of 37.8°C, a significant increase in local temperature, 10° of passive articular range of motion (-20° extension and 30° flexion), and a moderate amount of effusion. This was recognised as a classic presentation of joint infection and it was probably a chronic presentation due to the time elapsed since surgery and beginning of the symptoms. Knee X-rays and laboratory tests were performed; the X-rays showed osteolysis around the femoral metallic screw and loss of

the definition of the distal edge of femur, especially in the lateral condyle (Figure 1), confirming that the infection was not acute. The CRP and ESR values were 175 mg/L and 50 mm/hour, respectively, values that are above the cut-off values described by Wang et al.[30]

TREATMENT

Arthroscopic irrigation and debridement, graft retain, and antibiotic therapy are the treatments of choice, reaching a success rate of 85%.[33] Graft debridement is only encouraged if a loss of tension is found during arthroscopy procedure, if purulent exudation has adhered to the graft, or in cases of persistent infection after 2–3 arthroscopic irrigation and debridement.[34] Treatment must be monitored clinically and using laboratory examinations. A 50% decrease in CRP value must be expected after 48 hours of treatment and normal values must be reached between 10 and 14 days.[30]

Some authors suggest no surgical treatment. Viola et al.[35] proposed only antibiotics; meanwhile, Monaco et al.[36] proposed antibiotics and joint irrigation. These authors report a rate of change to the surgical treatment of 42.9% and 30.0%, respectively.[35-36] A literature review by Wang et al.[37] showed that 153 out of 176 (86.9%) patients with infection underwent surgery, and 39.8% of them required more than one surgical procedure. The need for a second surgical procedure depends on the evolution of symptoms; if fever, knee effusion, or decreased range of motion persist or laboratory exam results do not improve within 48–72 hours of treatment, the patient must undergo another surgical procedure.[38,39] When the decision to perform graft debridement is made, all screws or other devices must be removed because keeping sutures or such devices can maintain symptoms even after graft removal.[39]

The duration of intravenous (IV) antibiotic administration and the total length of antibiotic treatment are topics of debate. Some authors recommend IV antibiotics for 14–21 days; another recommendation is to use antibiotics until the CRP value reaches ≤50 mg/dL and no fever is present, which normally occurs after 5–7 days. On the other hand, Pérez-Prieto et al.[40] proposed an oral treatment with levofloxacin and rifampicin as soon as cultures are available, a treatment which should be continued for 6–8 weeks, with no more than 3–4 days of IV treatment. In their report, Pérez-Prieto et al. only included acute cases, and reported a normalised ESR and CRP after 3 weeks of oral treatment plus surgery. Their graft removal rate was 1 of 15 patients (0.6%).[40]

Since the evidence available is only from consensus and case series, we should be very critical of the available information in the literature. The data show a trend that patients who are treated with longer periods of IV antibiotics are associated with surgical irrigation and debridement, have a shorter duration of symptoms, and experience an early decrease in CRP and ESR.[13,17,34,37]

The authors of this review recommend surgery as soon as possible after diagnosis is made, starting empirical treatment once the cultures are taken. The authors use cephalosporin as empirical treatment and adjust by culture results and antibiogram. If the symptoms persist or the inflammatory parameters remain stagnant using the correct antibiotic according to antibiogram, a second surgical procedure is performed as soon as possible. In the case presented, the authors were even more prompt with the indication of the second procedure, given that it was a chronic presentation that caused much joint damage and had extensive fibrotic tissue.

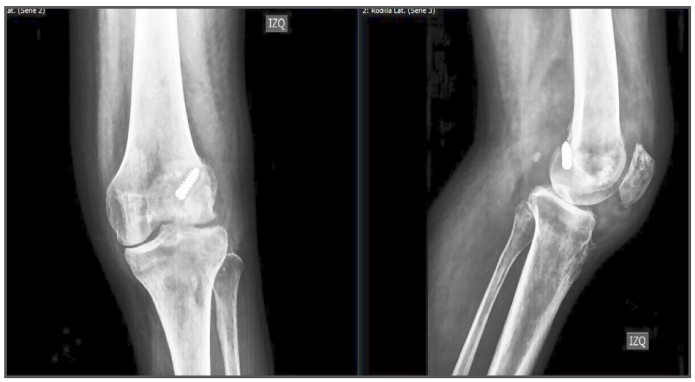

Figure 1: X-rays of the damaged knee joint taken at the emergency department.

Osteolysis around the femoral screw and loss of the definition of the articular edge of the femur, more significant in the lateral condyle, were the most notable findings.

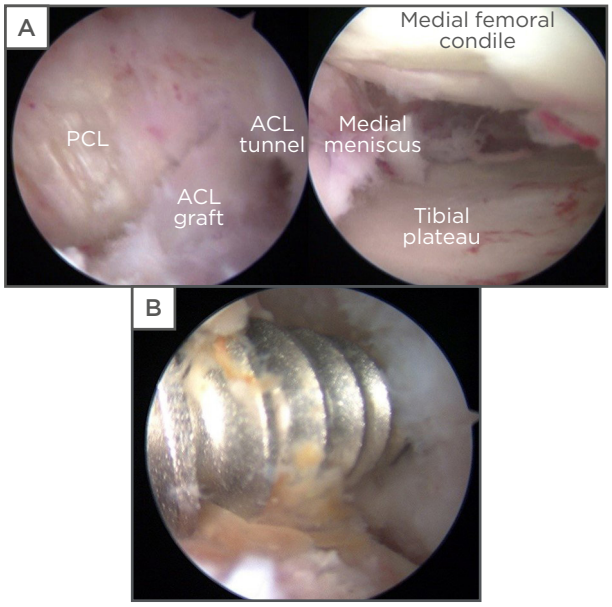

Figure 2: Arthroscopic images obtained during irrigation and debridement.

A) After debridement of an extensive anterior fibrous tissue, the ACL graft was identified. The graft was completely loose, the femoral tunnel was widened, and the screw was removed with a grasper clamp, without the help of a screwdriver, and purulent exudate surrounded it. B) The medial compartment. Extensive cartilage and meniscus damage was observed in both the lateral and medial compartments.

ACL: anterior cruciate ligament; PCL: posterior cruciate ligament.

Regarding antibiotic treatment, the authors' policy is to maintain IV antibiotic treatment until CRP reach normal values (<20 mg/dL), which normally occurs between Day 10 and 14; following this, oral antibiotics are administered until 6 weeks of treatment have been completed.

In the case presented, arthroscopic irrigation and debridement was performed at 3 hours after admission. Arthroscopy showed extensive compartmentalisation of the knee due to fibrous tissue and a rotten and loose graft. Also, the femoral screw was completely loose and was easily removed with a grasper without any help of a screwdriver. In the medial and lateral compartment, an important loss of meniscal tissue and extensive and diffuse Grade 3 International Cartilage Research Society (ICRS) classification chondral damage in both condyles and tibial plateaus was observed (Figure 2). Irrigation was completed with 24 L of saline solution and debridement of fibrous tissue with a shaver was performed. Four tissue samples were cultured, as well as a sample of articular liquid and the metal screw of the femur. After taking cultures, 2 g of cefazolin every 8 hours was started.

Treatment success is defined as the eradication of the infection without the need to remove the ACL graft. Various factors have been studied to predict the success of treatment. When arthroscopic irrigation and debridement and antibiotics are used for treatment, no difference in treatment success exists between autografts (bone-patellar tendon, CT, or HT). Meanwhile, allograft has a higher failure rate compared to autograft. Another risk factor for treatment failure is *S. aureus* or polymicrobial infection compared to CNS infection. As noted, fungal infections have a high risk of sequelae;[31] on the other hand, negative cultures are a protective factor.[33]

The patient reported in this case had many factors of a bad prognosis: infection with *S. aureus*, chronic presentation, and substantial joint damage, so immediately the graft and screws were removed. After 2 days of treatment, the patient recovered 50° of knee range of motion (-20° extension and 70° flexion), and his pain decreased considerably. However, the local temperature still increased, and inflammatory parameters remained unchanged (Figure 3). As a consequence, a second arthroscopy irrigation and debridement was performed. Irrigation with 18 L of saline solution was conducted, and two perforations with a 4.5 mm drill were performed in the tibia, guided with a tibial compass in 60° to decompress the tibial tunnel.

FUNCTIONAL OUTCOMES

Return to any sporting activities after ACL reconstruction occurs in 80% of cases and return to the same level of activity is experienced by 65% of patients.[41] Makhni et al.,[22] in a recent meta-analysis, reported a 67% return to sports, 13% knee instability, and a median score of 82.1 in the Lysholm Knee Scoring System after infection following ACL reconstruction.[22] Windhamre et al.,[38] in a recent study, demonstrated that all patients achieved

an improvement over their functional score before reconstruction; however, they tended to have lower functional outcomes to similar groups that were not infected.[38]

In cases of graft removal, ACL revision, in which a new reconstruction of the ACL occurs, is a treatment option. Most authors recommend revision after 6–12 months.[13,37] Hantes et al.[34] published a case series of ACL revision after infection with excellent functional outcomes with a follow-up of 6.3 years (4–9 years). They performed revision with an ipsilateral BTB autograft after 5–9 months of treatment in 4 of 6 patients. They achieved a Lysholm Knee Scoring System score of 92 (87–95) and a difference of anterior translation of the tibia between the two knees of the patient of 1.4 mm (measured by the KT-1000 test).[34]

The medical literature regarding post-infection ACL revision is limited to case reports. The authors of this report follow an algorithm similar to prosthetic infection scenario, in which it is requisite that the infection is controlled for the following surgical procedure, in this case, ACL revision. The minimum required to establish control of the infection is to maintain CRP and ESR at normal values after 1 month of having completed the antibiotic treatment, and, at any minimum suspicion of infection, an arthrocentesis for synovial fluid cultures must be performed.[42] The procedure itself must be addressed and planned like any ACL revision. The decision to perform revision as either a one or two-step procedure time depends on the size of the tunnels (femoral and tibial). Two-step revision firstly requires the filling of the tunnels with a bone graft, then waiting 4–6 months for graft incorporation, and in the second procedure, ACL reconstruction is carried out.[43]

After the second arthroscopy irrigation and debridement, the patient's condition improved, achieving a joint range of -10° extension and 80° flexion, and considerably less pain. After 10 days of IV treatment, the patient achieved a normal CRP of 18 mg/L and was discharged with cefadroxil 1 g every 12 hours until 6 weeks of treatment were complete.

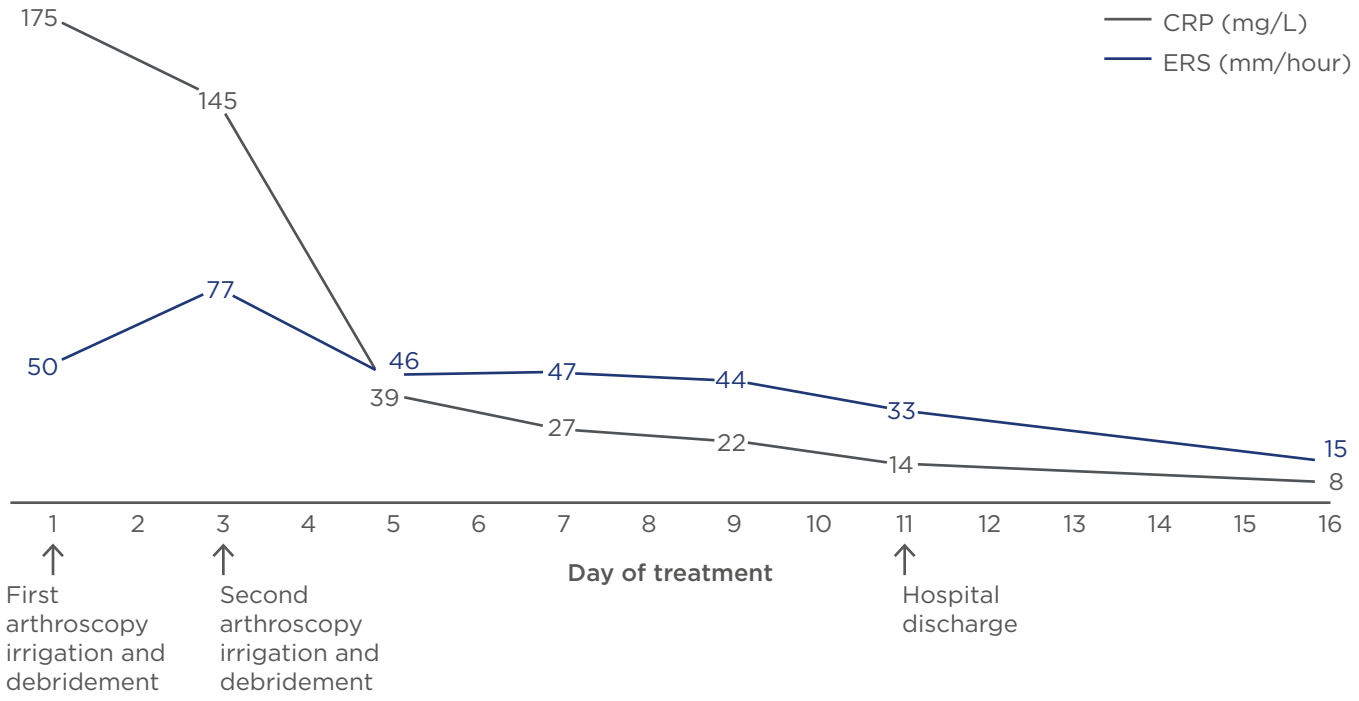

Figure 3: C-reactive protein (mg/L) and erythrocyte sedimentation rate (mm/hour) behaviour from Day 1–16 of treatment.

On Day 1, the first arthroscopy irrigation and debridement was performed. On Day 3, after CRP and ERS results, the second arthroscopy irrigation and debridement was performed. Hospital discharge was on Day 11.

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.

One week after discharge, the patient had significantly less articular effusion, a functional knee range of motion (-5°/90°), and was walking with partial weight bear and two canes. His inflammatory parameters continued to decrease (CPR 8 mg/L and ESR 17 mm/hour) (Figure 3). Six months after surgery, the patient resumed his work activities (desk work), had no complaints of instability in daily life activities, had normal CPR and ESR levels, and had a full range of motion. For now, there are no plans to carry out an ACL revision on this patient.

CONCLUSION AND RECOMMENDATIONS

Delayed diagnosis of infection after ACL reconstruction is catastrophic, compromising functional outcome and cartilage survival, as documented in the case reported in this article. Therefore, the authors recommend a prompt and comprehensive diagnosis before any suspicion of infection. Early treatment improves the functional result and is very important to preserve the graft. The gold standard treatment is arthroscopic irrigation and debridement with graft retaining and antibiotics, achieving 85% success. On the other hand, in cases like the one reported in this review, in which factors of bad prognosis are present, the authors recommend removal of synthetic material, such as a screw, and graft removal. Among the risk factors for infection reported in the literature, the most consistent is the use of HT graft. Other reported risk factors are age >20 years, diabetes, and the use of immunosuppressive drugs.

The most common agents of infection are *Staphylococci*; as a result, cephalosporin or vancomycin should be used as initial IV treatment until cultures are available. The duration of IV antibiotic administration and total length of antibiotic treatment are topics of debate, and the authors strongly recommend that antibiotics are used until the CRP is <20 mg/dL. Infection compromises functional outcome; however, if treatment is started early, good functional results and a return to sports activities can be achieved. If the graft must be removed, ACL revision requires no signs of infection for at least 1 month after having completed antibiotic treatment.

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The Role of Nicotinamide Adenine Dinucleotide in the Pathogenesis of Rheumatoid Arthritis: Potential Implications for Treatment

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Disclosure: The authors have declared no conflicts of interest.

Acknowledgements: This study was supported in part by the grants from National Natural Science Foundation of China (81701600), and the Natural Science Foundation of Zhejiang Province (LQ17H100001, LGF18H100001).

Received: 02.03.18

Accepted: 07.06.18

Keywords: ADP-ribosyltransferases (ART), CD38, nicotinamide adenine dinucleotide (NAD^+), poly-ADP-ribose polymerases (PARP), rheumatoid arthritis (RA), sirtuins.

Citation: EMJ. 2018;3[3]:90-97.

Abstract

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory, autoimmune disease characterised by small joint swelling, deformity, and dysfunction. Its exact aetiology is unclear. Current treatment approaches do not control harmful autoimmune attacks or prevent irreversible damage without considerable side effects. Nicotinamide adenine dinucleotide (NAD^+), an important hydrogen carrier in mitochondrial respiration and oxidative phosphorylation, is the major determinant of redox state in the cell. NAD^+ metabolites act as degradation substrates for a wide range of enzymes, such as sirtuins, poly-ADP-ribose polymerases, ADP-ribosyltransferases, and CD38. The roles of NAD^+ have expanded beyond its role as a coenzyme, linking cellular metabolism to inflammation signalling and immune response. The aim of this review is to illustrate the role of NAD^+-related enzymes in the pathogenesis of RA and highlight the potential therapeutic role of NAD^+ in RA.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterised by synovial inflammation, synovial hyperplasia, pannus formation with subsequent joint swelling, space narrowing, and destruction of articular cartilage and bone. The exact causes of RA are still unclear. However, it is well recognised that a combination of factors, including abnormal autoimmune response, genetic susceptibility, and some environmental or biologic triggers, such as viral infection or hormonal changes, are involved in the development of RA.[1] Despite the use of biological disease-modifying antirheumatic drugs, such as anti-TNF-α inhibitors, and targeted synthetic disease-modifying antirheumatic drugs, such as JAK inhibitors,[2] there are still a significant number of RA patients who have poorly controlled disease. Therefore, the development of new therapies is urgently needed.

T cell immune responses to self-antigens are known to play an important role in the development and progression of RA. T cells

can differentiate toward the T helper (Th) 1 or Th17 lineages, imposing a hyper-inflammatory phenotype.[3-5] The activation of T cells, which produce proinflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-8, and IL-17, is involved in the pathogenesis of RA.[6,7] NFκB, which regulates the activity of many genes that code for cytokines, contributes to rheumatoid synovial inflammation. Therefore, the NFκB pathway is one of the most important signalling pathways involved in the development of synovitis.[8]

Recently, scientists found that abnormal energy metabolism is associated with the development of RA.[9] During preclinical RA, when autoreactive T cells expand and immunological tolerance is broken, the main sites of disease are the secondary lymphoid tissues. Naïve CD4+ T cells from patients with RA have a defect in glycolytic flux due to the upregulation of glucose-6-phosphate dehydrogenase.[3] This therefore leads to high levels of NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate) and thus depleted levels of intracellular reactive oxygen species, which facilitates T cell hyperproliferation and development of proinflammatory effector functions. In clinical RA, immune cells coexist with stromal cells in the acidic milieu of the inflamed joint. This microenvironment is rich in metabolic intermediates that are released into the extracellular space to shape cell–cell communication and the functional activity of tissue-resident cells.[10] However, it is still unclear how energy metabolites influence the pathogenic behaviour of T cells and regulate signalling pathways in RA.

BIOLOGICAL EFFECT OF NICOTINAMIDE ADENINE DINUCLEOTIDE IN ENERGY METABOLISM AND IMMUNE RESPONSE

NAD^+, an important hydrogen carrier in mitochondrial respiration and oxidative phosphorylation, is the major determinant of redox state within the cell.[11] NADPH is the phosphorylated form of NAD^+. Broadly speaking, proton carriers are required for energy metabolism. NAD^+ can be synthesised from five major precursors and intermediates: nicotinamide (Nam), nicotinamide mononucleotide (NMN), nicotinamide riboside (NR), nicotinic acid (NA), and tryptophan.[12] NAD^+ is mainly synthesised by two pathways (the *de novo* synthesis and salvage synthesis pathways). Nicotinamide phosphoribosyltransferase (Nampt) is a rate-limiting enzyme that plays an important role in the synthesis of NAD^+ via the salvage pathway.[13]

However, the roles of NAD^+ have been discovered to extend beyond its role as a coenzyme. NAD^+ and its metabolites also act as degradation substrates for a wide range of enzymes, such as the Class III NAD^+-dependent deacetylases (sirtuins), poly-ADP-ribose polymerases (PARP), ADP-ribosyltransferases (ART), and the cyclic ADP-ribose synthases (CD38 ectoenzymes).[13,14] Through its activities, NAD^+ links cellular metabolism to changes in inflammation signalling and immune response. It has been reported that NAD^+ is able to promote an impressive allograft survival through a robust systemic IL-10 production, suggesting IL-10 may be a key molecule involved in NAD^+-mediated immune regulation.[15] Administration of NAD^+ protects against experimental autoimmune encephalomyelitis (EAE) and reverses disease progression by regulating CD4+ T cell differentiation and apoptosis;[16] this suggests a potential role in the pathogenesis of NAD^+ and indicates potential therapeutic effects in RA by regulating the immune response. Here, the authors explore how NAD^+-related substrates contribute to the progress of RA and summarise the biological effects of NAD^+ in the treatment of RA.

Levels of Nam and tryptohan, the precursors of NAD^+, are decreased in patients with RA.[17-19] Nam has been shown to be a potent inhibitor of glucose-6-phosphate dehydrogenase, which may have benefits for conditions like RA.[20] However, tryptophan has been shown to be a poor NAD^+ precursor *in vivo*.[21] NR has been found to reduce obesity-related inflammation, which may apply to other inflammatory diseases, such as RA.[22] Nampt has been shown to play a major role in inflammatory arthritis because expression of Nampt is increased in both the sera and in the arthritic paw in a collagen-induced arthritis (CIA) mouse model. Furthermore, a specific competitive inhibitor of Nampt was shown to effectively reduce arthritis severity and progression of arthritis with comparable

activity to the TNF inhibitor etanercept. Moreover, Nampt inhibition has been shown to reduce intracellular NAD^+ concentration in inflammatory cells and circulating TNF-α level during endotoxaemia in mice.[23,24] However, no papers have been published on the targeting of Nampt in human patients with RA.

SIRTUINS INVOLVED IN SYNOVITIS AND T CELL DIFFERENTIATION IN RHEUMATOID ARTHRITIS

The mammalian sirtuins family of proteins has seven members, named Sirt1–7. Sirtuins have NAD^+-dependent deacetylase activity and belong to the Type III histone deacetylase. They are involved in the regulation of various biological processes, such as cell survival, apoptosis, proliferation, lipid metabolism, senescence, and systemic inflammation, as well as in bone and cartilage remodelling.[25,26] Three sirtuins are located in the mitochondria (Sirt3, 4, and 5), while Sirt1, 6, and 7 are predominantly located in the nucleus and Sirt2 is found in the cytoplasm.[27] Sirt1 is the most important member of the sirtuins family. Sirt1 is activated during times of energy deficit and reduced carbohydrate energy sources, such as during exercise or when hungry. Herranz et al.[28] showed that Sirt1 overexpression helps to reduce metabolic and age-related complications in mice, promoting healthy ageing.[29]

Besides exerting an anti-ageing effect, Sirt1 is also involved in the development of synovitis. Sirt1 is upregulated in synovial tissues, human synovial fibroblasts, and chondrocytes in patients with RA.[30] Sirt1 has been shown to decrease apoptosis in synovial cells and to promote the production of proinflammatory cytokines. TNF-α-induced Sirt1 overexpression contributes to chronic inflammation by promoting inflammatory cytokine production and inhibiting apoptosis in RA synovial cells. Knockdown of Sirt1 results in a reduction in proinflammatory IL-6 and IL-8 and proliferation of RA fibroblast-like synoviocytes (FLS).[30,31] However, Sirt1 exhibits anti-inflammatory properties in RA by enhancing macrophage polarisation to an anti-inflammatory phenotype or inhibiting NFκB signalling.[32,33]

Sirt1 can mediate the differentiation of inflammatory T cell subsets in an NAD^+-dependent manner.[34] Sirt1 is highly expressed in the thymus, suggesting the involvement of Sirt1 in T cell development. Furthermore, T cell-specific Sirt1 deletion and treatment with pharmacological Sirt1 inhibitors has been shown to suppress Th17 differentiation and exert a protective effect in a mouse model of multiple sclerosis.[35] The loss of Sirt1 has been shown to compromise the survival of regulatory T (Treg) cells, resulting in antigen-induced T cell proliferation and inflammation in two mouse models.[36] A deficiency of Sirt1 in mouse or human T cells has been shown to enhance IL-9 production, suggesting that Sirt1 negatively regulates Th9 cell differentiation.[37] Myeloid deletion of Sirt1 impairs Th1 and Th17 cell differentiation and dendritic cell maturation in CIA.[38] In contrast, Gardner et al.[39] found that Sirt1 activators contribute to the suppression of T cell proliferation. Oral Sirt1 activator treatment has been shown to suppress antigen-specific T cell responses and the production of proinflammatory cytokines, including IL-6, IL-17A, and IFN-γ, in experimental autoimmune uveoretinitis mice.[39] Overall, the role of Sirt1 in controlling synovitis and the differentiation of effector T cells in RA is still controversial.

Sirt6, another NAD^+-dependent protein lysine deacetylase, is known to interfere with the NFκB signalling pathway and thereby has an anti-inflammatory function.[40] An adenovirus containing Sirt6 complementary DNA delivering Sirt6 to human RA FLS *in vitro* was shown to suppress TNF-α-induced NFκB target gene expression; additionally, this adenovirus containing Sirt6 complementary DNA was also used to deliver Sirt6 to mice with collagen-induced arthritis, which resulted in reduced arthritis severity.[40] In contrast, Sirt6 has been shown to enhance the proinflammatory and matrix-destructive potential of RA-FLS through TNF-α.[41] Sirt6 has also been shown to increase the intracellular levels of ADP-ribose, an activator of the Ca^{2+} channel. Sirt6 can also enhance the production of IL-8 and TNF-α via a Ca^{2+}-dependent mechanism, showing that cell metabolism can connect with inflammatory responses through a Sirt6-mediated pathway.[42]

POLY-ADP-RIBOSE POLYMERASE-1 IS INVOLVED IN INFLAMMATION AND T CELL DIFFERENTIATION IN RHEUMATOID ARTHRITIS

PARP-1 catalyses 80% of cellular poly(ADP-ribosyl) action, while the other PARP family members, PARP-2, PARP-3, PARP-4, and tankyrases (PARP-5 a and b) account for the remaining 20%. PARP-1 has been shown to increase the risk of RA and promote the development of arthritis inflammation.[1,43] PARP-1 deficiency or inhibition suppresses the activation of JAK, activator protein-1, and NFκB signalling.[1,44,45] PARP-1 inhibition has been shown to decrease TNF-α-induced RA-FLS proliferation and significantly reduces expression of cytokines and chemokines in FLS from patients with RA.[44] Furthermore, PARP-1 inhibitor treatment has been found to significantly attenuate the severity of experimental arthritis by downregulating inflammation, Th1, and Th17 cells, and upregulating Treg cells.[45,46] The deletion of PARP-1 in mice (*PARP-1−/−*) has been shown to lead to T cells generating more thymic Treg cells and converting more naïve T cells into induced Treg cells both *in vitro* and *in vivo*.[47] Additionally, the inhibition of PARP-1 enzyme activity was found to result in an increased expression of *FOXP3* and *TGF-β* receptor I genes in human CD4+ T cells.[47]

ADP-RIBOSYLTRANSFERASES ARE A CRUCIAL REGULATOR OF T CELL FUNCTION

ADP-ribosylation is a post-translational modification regulating protein function in which amino acid-specific ART transfer ADP-ribose from NAD^+ to specific target proteins. Five paralogs (ART1–5) have been cloned, but only four of them are expressed in humans due to a defective *ART2* gene, and six in mice as the result of *ART2* gene duplication.[48] NAD can activate the purinergic receptor P2X7 via ART1 and cause cell depletion in murine models; however, NAD^+ does not induce a cell death in human CD4+CD39+ Treg cells. Further experimentation showed that the expression of P2X7 is lower in human CD4+ CD39+ Treg cells than in CD4+CD39-Treg cells, while the expression of ART1 is relatively higher.[49] This implies that ART1–P2X7 signalling participates in the resistance against cell death of Treg cells induced by NAD^+.[49]

In patients with RA, Treg cells are functionally defective or unstable and are converted to Th17 cells in the presence of inflammatory cytokines, such as IL-6.[50] ART1 may be related to the stability of Treg cells in patients with RA.[49,50]

ART2 could activate the cytolytic purinergic receptor in turn to affect T cell differentiation in mice. For instance, the conversion of Treg cells into Th17 cells is promoted in the presence of IL-6, primarily through the NAD^+-ART2.2-P2X7 pathway. Activation of P2X7 in T cells by ATP or by NAD^+-dependent ADP-ribosylation initiates a cascade of events, including the influx of calcium, the shedding of the L-selectin homing receptor, the externalisation of phosphatidylserine on the outer leaflet of the cell membrane, DNA fragmentation, and, ultimately, cell death.[51,52] This mechanism is called NAD^+-induced cell death.[53] It has been shown that ART2.2-overexpressing mice with normal T lymphocytes are sensitive to NAD^+ and prone to death. It has also been shown that the fewer B cells that express ART2.2, the lower the amount of cell death. Bannas et al.[54] showed that ART2.2 transgenic T cells, but not B cells, are sensitive to NAD^+-induced cell death. NAD^+ can also induce apoptosis of naïve CD4+ $CD62L^{high}$ T cells. Conversely, activated $CD44^{high}$ $CD69^{high}$ T cells are resistant to NAD^+-induced cell death.[55] A study has shown that CD4+ CD25+ FoxP3+ Treg cells express the ART2.2 enzyme and high levels of P2X7, and that these Treg cells can be depleted by intravenous injection of NAD^+.[54] This can be used to promote an antitumour immune response.[54] This mechanism may provide a means by which NAD^+ released during immune diseases controls T cell functions. This suggests that the NAD^+-ART2.2-P2X7 signalling pathway is an important part of T cell death in mice. However, ART2 is not expressed in human T cells.

CD38 IS INVOLVED IN T REGULATORY CELL HOMEOSTASIS AND THE PRODUCTION OF THE PROINFLAMMATORY CYTOKINES IN RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLAST CELLS

CD38 and CD157 are two prominent enzymes that catalyse the synthesis and hydrolysis of cyclic ADP-ribose, a Ca^{2+} messenger molecule responsible for regulating a wide range of cellular functions.[56] The catalytic efficiency of CD38 is significantly higher when compared with CD157. CD38 is an important NAD^+ consumer; loss of CD38 function in mice led to a 30-fold increase in NAD^+ levels in different tissues.[56] CD38 is expressed by lymphocytes, endothelial cells, and several other cells.[57] CD38 is the main enzyme involved in the degradation of the NAD precursor NMN *in vivo*, indicating that CD38 has a key role in the modulation of NAD-replacement therapy for aging and metabolic diseases. Cells from CD38-deficient mice do not metabolise NAD^+ efficiently.[58]

Most importantly, CD38 can be used as a marker for mice Treg cells with a high suppressive activity. CD38 is expressed mainly in a subset of Foxp3+ CD25+ CD4+ T cells. $CD38^{high}$ Treg cells have a superior suppressive activity compared to $CD38^{low}$ Treg cells.[59] Lower Treg cell numbers are found in CD38-deficient mice, indicating the role of CD38 in Treg cells homeostasis. Chang et al.[60] found that CD38 expression is increased in the synovial membranes of patients with RA. IL-1α and IL-β levels are significantly decreased after treatment with siRNAs targeting the *CD38* or *E2F2* genes.[60] Mice deficient in CD38 develop attenuated collagen-induced arthritis.[61] In addition, CD38 is a surface marker for regulatory B cells in human disease. However, the number and the frequency of regulatory B cells do not change in patients with RA compared to healthy controls.[62]

POTENTIAL VALUE OF NICOTINAMIDE ADENINE DINUCLEOTIDE AND RELEVANT CONSUMING ENZYMES IN THE TREATMENT OF RHEUMATOID ARTHRITIS

One study showed that NAD^+ promotes the conversion of effector Th1 cells (CD4+ IFNγ+) into Type 1 regulatory T cells (CD4+ IL-10+ IFNγ+, Tr1) and blocks chronic inflammation independently of the cytokine milieu.[16] Furthermore, after NAD^+ administration, mast cells exclusively promote CD4+ T cell differentiation *in vivo* and *in vitro*, both in the absence of antigen and independently of major antigen-presenting cells. Moreover, mast cell-mediated CD4+ T cell differentiation is independent of major histocompatibility complex II and T cell receptor signalling.[63] It has been reported that NAD^+ promotes Treg cell conversion into Th17 cells *in vitro* and *in vivo*.[15] NAD^+ has been shown to promote allograft survival by promoting a robust systemic IL-10 production, which suggested that IL-10 is a key molecule involved in immune tolerance and immune regulation.[15] However, Elkhal et al.[15] did not consider that NAD^+ is associated with apoptosis of Treg cells, which may affect the conversion of Treg into Th17. The other factor is that Treg cells are unstable in the anti-graft-dependent inflammatory state and are easily converted into Th17.[17] Therefore, whether NAD^+ promotes the differentiation of Treg cells towards Th17 cells and whether they directly affect Th17 cells and facilitate their differentiation remains to be further studied.

Several drugs are in development or are already available in clinics that could be useful to suppress inflammation in autoimmune diseases. Nam, a precursor of NAD^+, is able to inhibit activation and modulate the activity of B lymphocytes, suggesting a potential role of this agent in regulating antibody-mediated autoimmune disorders like RA.[64] There are abundant sources of Nam, NR, and NA in natural food and milk, suggesting they are generally safe.[65,66] Indeed, new studies have demonstrated the therapeutic potential of supplementing NAD^+ intermediates, such as NR and NMN, providing a proof of concept for the development of an effective intervention.[12] NR is widely used as an NAD^+ precursor vitamin.

Single doses of 100, 300, and 1,000 mg of NR produced dose-dependent, safe increases in the blood NAD^+ metabolome in the first clinical trial of NR pharmacokinetics in humans.[67] Also, re-establishing cellular NAD^+ levels with NAD^+ or Nam has been shown to exert a protective effect against axonal degeneration in EAE.[16,68] Restoring NAD^+ levels with NR or PARP inhibitors has been shown to have a therapeutic effect on nonalcoholic steatohepatitis.[69,70] Jonas et al.[71] found that Nam treatment improved the global symptoms of patients with osteoarthritis, joint flexibility, and reduced inflammation when compared to placebo in patients with osteoarthritis.

NAD^+ plays a crucial role in inflammatory response and autoimmune diseases through Sirts, PARP, ART, or CD38. Although most studies suggest that Sirt1 plays a proinflammatory role in the development of RA, the role of Sirt1 in synovitis and T cell differentiation remains unclear and is controversial.[30,32,33,35-39] Intracellular NAD^+ levels regulate tumour necrosis factor protein synthesis in a Sirt6-dependent manner.[72] Sirt6 overexpression suppresses the expression of NFκB target gene in RA FLS and significantly decreases arthritis severity. Intra-articular injections of an adenovirus containing Sirt6 complementary DNA was shown to decrease arthritis severity in mice.[40] This demonstrates that the NAD^+-Sirt6-NFκB pathway may be an important target for the treatment of RA.

Additionally, PARP-1 inhibiton can reduce the production of proinflammatory cytokines through physically interacting with NFκB. A study showed that PARP-1 inhibition either with specific inhibitors or by siRNA transfection significantly reduced TNF-α-induced proliferation, cytokine, and chemokine expression in RA-FLS via suppressing NFκB signalling. This suggests that PARP-1 inhibitors could have therapeutic benefits in RA.[44,45,47] In fact, many clinical trials using PARP-1 inhibiton have been carried out for the treatment of different tumours. For instance, PARP-1 inhibition has been shown to suppress tumour progression in breast cancer patients by limiting the rate of cell proliferation and activation of NFκB, which results in the suppression of inflammation and the expression of genes related to tumour progression.[73] Common side effects, such as nausea and vomiting, should be monitored.[74]

Studies have shown that ART2 is specifically expressed on T cells in mice. NAD^+ can regulate murine CD4+ T cell differentiation through the NAD^+-ART2.2-P2X7 signalling pathway.[51-53] Due to inactivated ART2 pseudogenes in the human genome, ART2 is deficient in humans. Recently, scientists reported a higher expression level of ART1 in human CD4+ CD39+ Treg cells. ART1 participates in the resistance against cell death of Treg cells induced by NAD^+.[49] It is known that Treg cells are functionally defective or unstable in patients with RA and are converted to Th17 cells in the presence of proinflammatory cytokines, such as IL-6.[50] ART1 may be related to the stability of Treg cells in patients with RA.

CD38 is highly expressed in synovial membranes and plasma cells from RA patients.[75] The IL-1α and IL-β levels are significantly decreased after treatment with siRNA targeting CD38.[59] Mice deficient in CD38 develop an attenuated collagen-induced arthritis.[59,61] Inhibitors or therapeutic antibodies targeting CD38 should be tested for their ability to raise the concentration of NAD^+.[76,77] It is not easy to benefit from therapy through simply targeting CD38 from bench to bedside because targeting CD38 may impair the function of Treg cells and regulatory B cells.

CONCLUSION

Many studies have revealed the importance of NAD^+ biosynthesis in energy metabolism and in the immune response process. It is clear that levels of NAD^+ precursors, Nam and tryptophan, are decreased in patients with RA. Nam, NR, NMN, and NA are promising candidates to replenish NAD^+ and reduce inflammation in patients with RA and experimental arthritis model. IL-10 may be a key molecule involved in immune tolerance and immune regulation after treatment with NAD^+. Administration of NAD^+ protects against autoimmune reaction and reverses disease progression by regulating CD4+ T cell differentiation and apoptosis. The authors argue that the focus of study should be moved from other diseases, like EAE, nonalcoholic steatohepatitis, or OA, to RA and the therapeutic effect of NAD^+ and its precursors should be explored.

Different NAD+-consuming enzymes, such as Sirt1, Sirt6, PARP-1, ART-1, and CD38, are involved in T cell differentiation and homeostasis and synovial inflammation in RA pathogenesis. NAD+ has diverse biological functions through these consuming enzymes. Therefore, NAD+ and Sirt1/Sirt6, NAD+ and PARP-1, NAD+ and ART-1, and NAD+ and CD38 signalling pathways are more affected after NAD+ supplementation. These enzymes can be targeted to efficiently improve arthritis through inhibition of synovitis or regulation of T effector cells differentiation. Published data show that Sirt6 overexpression and PARP-1 inhibitor both have therapeutic benefits in RA animal models. In the near future, human clinical studies are needed to further confirm the therapeutic effect of NAD+ biosynthesis, especially regarding NAD+ precursors and their related consuming coenzymes in RA.

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Epstein–Barr Virus: A Biological Overview and Clinicopathological Changes of Two Epstein–Barr Virus-Related Lymphoproliferative Disorders in a World Health Organization (WHO) 2017 Report

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Disclosure: The author has declared no conflicts of interest.

Received: 19.03.18

Accepted: 15.06.18

Keywords: Diffuse large B cell lymphoma (DLBCL), Epstein–Barr virus (EBV), Epstein–Barr virus mucocutaneous ulcer (EBVMCU), non-Hodgkin's lymphoma.

Citation: EMJ. 2018;3[3]:99-107.

Abstract

Epstein–Barr virus (EBV) is one of the most important viral causes for the development of tumours. The global geographical epidemiology of EBV shows prevalence differences between rich and poor countries across the world, and the impact on health suggests EBV should be an important target of research worldwide. This article will discuss the biology of the virus with an emphasis on its latency types, vital to understanding the possibilities of viral detection. The main objective is to discuss two lymphoproliferative diseases that are associated with EBV and appear in the World Health Organization (WHO) 2017 Classification of Tumours of Haematopoietic and Lymphoid Tissues: EBV-positive diffuse large B cell lymphoma and EBV mucocutaneous ulcer. The name of the former was changed to support the better understanding of infection pathology, while the second was recently described and made its debut in the WHO classification. Pathologists must have knowledge on these diseases and how to investigate them, and oncologists and clinical doctors must be informed on the guidelines.

INTRODUCTION

Viral aetiology for tumours was described for the first time in 1911.[1] Almost 56 years later, a relationship between Epstein–Barr virus (EBV) and lymphoid neoplasms was reported by studies involving Burkitt's lymphoma (BL).[1] Nowadays, other viruses and their influences on the development of tumours are the focus of research; some examples of such viruses include hepatitis B virus, hepatitis C virus, herpes virus 8, human papilloma virus, and human T cell lymphotropic virus Type 1. In the context of lymphoproliferative disorders, EBV is a major contributor.[1]

In humans, EBV is the most common persistent virus infection, with approximately 95% of the world's population presenting with an asymptomatic life-long carrier status.[2] EBV has a geographical epidemiology of around 10–15% prevalence among individuals from Asia and South America, and a lower prevalence of 5% in

the USA and Europe.[3] Geographical differences are interesting in the study of EBV infection because there is an association with social and economic aspects. Countries from North Africa and China have higher endemic rates than countries from Northern Europe, such as Denmark and the Netherlands.[4] One study of Hodgkin's lymphoma (HL) reported EBV in 100% and 88% of mixed cellularity subtype patients from northeast Brazil and southeast Brazil, respectively.[4] Brazil has a continental size and the regions are very different in relation to culture, social aspects, and economic levels. Despite recent advances, the inequality seen in Brazil is recognised as a global microcosm of the EBV infection status worldwide.

Malignancies associated with EBV represent approximately 200,000 new cases and 143,000 deaths from cancer worldwide every year.[5] With regard to the high public health and economic impact, the National Institutes of Health (NIH) indicates EBV as an important target for cancer prevention, stimulating studies and research on the virus, predictive markers, a therapeutic vaccine, and immune correlations in EBV-related malignancies.[5]

Generally, the first contact with EBV occurs at a young age. The virus starts its replication cycle in the oropharynx, affecting epithelial cells and B cells of lymphoid tissues associated with the mucosa. EBV may produce three reactions: replication in permissible B cells or epithelial cells, latent infection in B cells, and stimulation or immortalisation of B cells.[6,7]

The scientific history of EBV began when virions were detected in B cells of BL biopsies. After many years, it was discovered that EBV is the main parasite of B lymphocytes and, in tissue culture models, the virus stimulates B cell growth and immortalisation.[6,7] The definition of B lymphocyte tropism is supported by CD21, an EBV-specific receptor that also binds C3d. These receptors are found on epithelial cells from the oropharynx and nasopharynx of human and monkey B cells. The viral structure includes EBV nuclear antigens (EBNA) 1, 2, 3A, 3B, and 3C; latent membrane protein (LMP)1 and LMP2; and RNA molecules produced by EBV (EBER1 and EBER2). However, the capacity of protein synthesis is higher and there is variation in the infection phases or types (such as permissible or non-permissible).[6,7]

Among these proteins, LMP1 and EBNA1 have a potential role in tumour pathogenesis. While EBNA1 expression is related to replication and maintenance of the viral genome during the dividing process, LMP1 mainly enables neoplastic cells to avoid apoptotic mechanisms;[6] however, the complete sequence from EBV infection to EBV-related malignancy is still being studied. The number of B cells infected in the germinal centre from the lymph node may also be important. For example, comparisons between tonsils from Brazilian and German patients showed that the infection rate was similar but the number of infected cells was different, with higher numbers in the Brazilian samples ($p<0.0007$). Thus, there are other pathways to explain the EBV–neoplastic interaction.[8]

Tsai et al.[9] investigated other factors that may explain the diversity of tumours related to EBV. Focussing on the differences between EBV strains, the authors showed that the strains B95-8, Akata, and GP202 induce more cellular growth than YCCEL1, SNU719, and M81. Akata and B95-8 had a significant tropism for B cells, which was not observed for YCCEL1 and M81; therefore, different EBV strains induce lymphoid tumours with distinct pathogenic aspects and there is a relationship between viral strain and tumour type and progression.[9]

In some individuals, the first contact with EBV produces a feverish state called infectious mononucleosis (IM). During this phase, a lytic and latent response involving T cells characterises the interaction between the patient and the virus.[6] First described in the 19th century, IM is characterised by polyclonal expansion of infected B cells, which are triggers for a cytotoxic T cell response. The intensity of the CD8+ T lymphocyte response to EBV is key to controlling the infection.[2] Patients usually have a fever with enlarged tonsils, cervical lymphadenopathy, reactive lymphocytosis, and variable splenomegaly. Lymphoid tissues show a mixed follicular, parafollicular, and sinusoidal monocytoid proliferation. In general, the paracortex has lymphocytes, plasma cells, and immunoblasts, sometimes in aggregates; it is also possible to find Hodgkin and Reed–Sternberg-like cells. Immunohistochemistry (IHC) is helpful because it demonstrates a reactive pattern with CD30+/CD15- cells in the middle of a polymorphous infiltrate.[2]

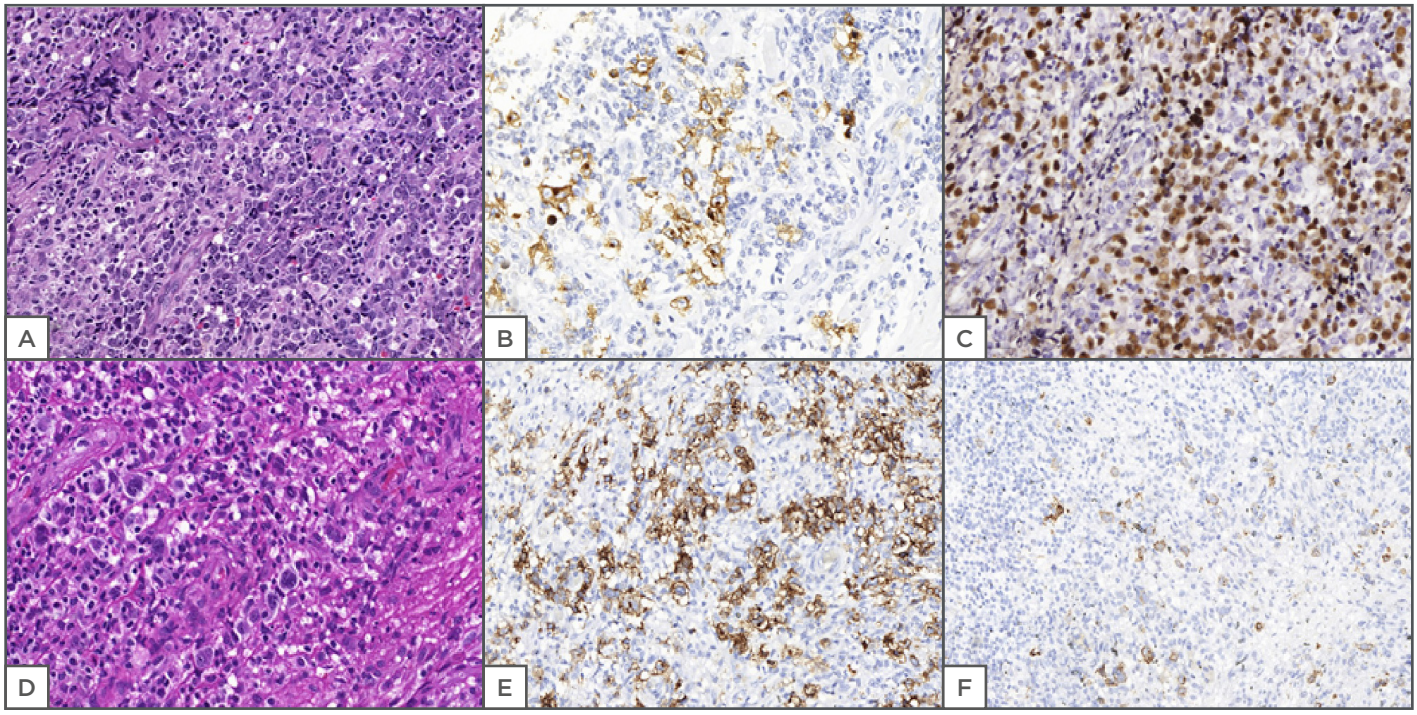

Figure 1: Examples of Epstein–Barr virus-positive diffuse large B cell lymphoma (A, B, C) and Epstein–Barr virus mucocutaneous ulcer (D, E, F).

A) (400x, HE stain) diffuse pattern with pleomorphic cells; B) (400x, CD30 antibody staining) neoplastic cells positive for CD30; C) (400x, CISH for EBV) nuclear staining using CISH to determine EBV infection; D) (400x, HE stain) EBVMCU is quite similar to HL: inflammatory background with atypical cells and sometimes Reed–Sternberg-like cells; E) (400x, CD30 antibody staining) other disease with atypical cells positive for CD30; F) (400x, LMP1 stain) EBV status is positive by immunohistochemical evaluations with LMP1.

CISH: chromogenic *in situ* hybridisation; EBV: Epstein–Barr virus; EBVDLBCL: EBV-positive diffuse large B cell lymphoma; EBVMCU: EBV mucocutaneous ulcer; HE: haematoxylin and eosin; HL: Hodgkin's lymphoma; LMP1: latent membrane protein 1.

Following IM, lower viral levels persist in lymphoid tissues, especially in the oropharynx. This viral memory represents a risk of reactivation to the organism, which depends on specific factors.[6] In this context, immunosuppression and chronic antigenic activation are possible triggers for the beginning of a new viral cycle and a neoplastic process.[3] EBV may interact with key molecular pathways controlling the cell cycle, such as the NFκB pathway. The interaction between the virus and these pathways induces cytokine release with proliferative effects, as well as inhibition of apoptosis.[2] Other findings are activation of MYC, BCL2, and NOTCH1, and induction of genomic instability.[9]

Viral latency is a strategy to avoid T cell immunosurveillance. EBV downregulates antigen expression, allowing a viral reservoir in memory B cells. In this context, there are four latency types:

1. Latency Zero: Viral antigen is almost totally suppressed, despite viral genome being carried by B cells.
2. Latency Type 1: There is an expression of EBNA1 in all virus-infected cells, which maintains replication of the episomal EBV genome.
3. Latency Type 2: With the exception of EBNA2, many viral proteins are produced; LMP1 expression in the absence of EBNA2 confirms Type 2 latency.
4. Latency Type 3: Unrestricted expression of nine latent genes, including *EBNA1*, *EBNA2*, *EBNA3A*-C, *LP*, *LMP1*, *LMP2A*, and *LMP2B*.

The importance of these latency types is that they only occur during an acute EBV infection or in the context of immunosuppression, situations in which there are strong cytotoxic T cell responses due to these viral proteins being highly immunogenic. Regardless of the latency type, levels of EBER1 and EBER2 RNA are constant, which indicate that they are a gold standard for the identification of EBV in tissue sections.[2]

EBV detection during routine pathology examinations is of increasing importance because the impact of the aetiology for diagnosis and prognosis has changed in recent years. Thus, the accuracy of detection is also important and the DNA and RNA integrity are valuable. For high-quality (mainly molecular) examination, fixation, post-fixation processing, and preparation of sections must be the priority of pre-analysis team members.[10]

Molecular tests are used for latent EBV infection diagnosis and include *in situ* hybridisation (ISH) and PCR. The membrane latency proteins can also be detected by IHC (Figure 1). In general, PCR sensitivity is higher than both ISH and IHC; comparisons of PCR and ISH analysis have indicated that both methods can produce similar results, depending on the probe mark and quality. Considering the similar results obtained, the cost of the techniques is often the determining factor and ISH has the best cost–benefit ratio so is recommended where possible. The use of IHC is also possible, but negative results must be interpreted with caution because the latency protein is not expressed in these cases. Thus, as discussed above, other proteins, including nuclear proteins, may be expressed.[10]

Another method for EBV detection is through plasma and peripheral blood mononuclear cell (PBMC) analysis. Kanakry et al.[11] studied the clinical significance of PBMC detection, using viral quantitative real-time PCR of plasma and PBMC. Among patients with EBV-positive disease, the viral detection and quantification had greater specificity and sensitivity for diagnosis in the plasma than in PBMC. In the plasma, EBV-positive results were observed in 99% of patients.[11] In addition, the authors demonstrated that the number of EBV copies was different in untreated EBV-positive lymphomas, EBV-positive lymphomas in remission, and EBV-negative lymphomas.[11] Therefore, according to this group, the analysis of plasma for EBV detection represents a useful resource. In cases of EBV detection in histological samples, the correlation with plasma value is also important because the disease can have an atypical presentation and laboratory confirmation by different methods is a differential for clinicians.[11]

The objective of this article is to discuss EBV and lymphoid diseases, mainly the clinicopathological aspects. The main examples of lymphoproliferative disorders associated with EBV are classical HL, BL, diffuse large B cell lymphoma (DLBCL), plasmablastic lymphoma, primary effusion lymphoma, extranodal T and natural killer cell lymphomas, post-transplant B cell lymphoproliferative disease, mucocutaneous ulcers, and HIV-related B cell lymphoproliferative disease.[2,5] In the new edition of the WHO classification of haematological and lymphoid tumours,[12,13] two EBV-related diseases were highlighted due to changes in nomenclature after clinical observation and the description of a new entity: EBV-positive DLBCL and EBV-related mucocutaneous ulcer, respectively.[2,5,12,13]

EPSTEIN–BARR VIRUS-POSITIVE DIFFUSE LARGE B CELL LYMPHOMA

The WHO definition of DLBCL is a lymphoid neoplasm with diffuse pattern and neoplastic B cells characterised by nuclei larger than histiocyte nuclei. The classification evolution began with a morphological description and is now a molecular approach with important impact on prognosis of the most common non-HL worldwide. According to this evolution, subtypes of DLBCL were described. In the WHO 2017 classification,[12,13] there are many specific subtypes, such as not otherwise specified (NOS); T cell or histiocyte-rich large B cell lymphoma; primary DLBCL of the central nervous system; primary cutaneous DLBCL, leg type; and EBV-positive DLBCL.[12,14-16]

Until 2008, EBV-positive DLBCL was called EBV-positive DLBCL of the elderly. The initial reports included patients with morphology of DLBCL and expression of EBER in malignant

cell nuclei. The patients were elderly, had a poor response during therapy, and had a short survival time with standard combination chemotherapy.[3] This subtype was known as a neoplasm that occurs in people >50 years old without any identifiable immunodeficiency or prior lymphoma. The tumour was understood in the context of immunologic deterioration related to the ageing process. Frequency of this subtype is estimated at 3–4% in the USA and Western Europe, and 8–10% in Asian countries.[17]

EBV infection is associated with immunosuppression and chronic antigenic activation, both of which are important components of the neoplastic process. In general, EBV-positive DLBCL patients have EBV latency Type 3 with expression of latent membrane proteins and nuclear antigens. Immunosenescence is characterised by thymic atrophy, T cell response dysregulation, anergic memory cells, and deficiencies in cytokine production. EBV may accelerate this process and provides the genesis of this subtype of aggressive lymphoma.[3]

Patients with this EBV-positive DLBCL have a worse survival rate than patients with EBV-negative DLBCL, with high International Prognostic Index scores, extranodal involvement, bone marrow infiltration, and an advanced clinical stage. From 2008–2016, studies demonstrated that EBV-positive DLBCL is not only found in individuals >50 years old;[1,3,12] younger patients without immunodeficiency were also diagnosed with this pathology.[12] In fact, older people have poor survival rates and therefore it may be the virus and other diseases that affect the elderly and immunosenescence.[1,3]

EBV-positive DLBCL is on the spectrum of activated B cell (ABC) diseases, which is a necessary classification after the WHO 2017 report.[12] Using IHC markers, the lymphoma is classed as B germinal centre or ABC disorders. Initially, this process was a molecular classification supported by gene expression, but now it is an IHC classification based on algorithms by, for example, Hans et al.[18] Detection by IHC results in a similar prognosis to gene expression and, in general, ABC patients have a poor survival rate. Recent articles and the WHO 2017 classification[12] indicate that pathologists must perform an IHC test for calculation of Hans' algorithms because the results are similar to the molecular findings; this methodology is controversial when using other similar algorithms and other markers.[18-20]

The monoclonality of Ig rearrangements is documented by analysis of the EBV terminal repeat copy number. In general, translocations involving *MYC*, *BCL2*, and/or *BCL6* have not been found.[19] On the other hand, copy number gains of these genes have been reported. EBV-positive DLBCL presents constitutive NFκB pathway activation and chronic active BCR signalling in a more pronounced way than EBV-negative patients with lymphoma.[19] Other connections reported include high levels of expression of immune and inflammatory gene pathways in addition to NFκB, such as *JAK/STAT, NOD* receptor, and toll-like receptor signalling.[19-21] The morphological presentation is an important disease indicator but does not influence the prognosis. The polymorphous subtype is more associated to EBV-positive DLBCL and has centroblasts, immunoblasts, and plasmablasts mixed with reactive cells such as lymphocytes, plasma cells, and histiocytes. Neoplastic cells are Hodgkin and Reed–Sternberg-like, often with geographic necrosis.[3]

Regarding IHC, CD30 expression is more common in EBV-positive DLBCL than in the other types of DLBCL;[22,23] almost 40% of EBV-positive DLBCL patients are positive for CD30, but there is no cut-off for a positive definition and the intensity and extension staining are variable among patients.[3,11] EBV-positive and CD30-positive patients have poor survival rates compared to EBV-positive and CD30-negative or EBV-negative and CD30-positive individuals.[22,23] Considering viral proteins, 94% of cases have expression of LMP1, 10% have expression of EBNA1, and 28% have expression of EBNA2. Other B cell positive markers are CD20, CD19, CD79a, and PAX5. As mentioned, this subtype has an ABC phenotype with positive cells for IRF4/MUM1 and negative cells for CD10 and BCL6. Generally, CD15 is negative, but the differentiation with HL is derived from morphological aspects;[12] in approximately 68% of cases, CD30 and CD15 are positive, making it difficult to differentiate from HL.[20] However, a very important aspect for the differentiation is that extranodal HL

are rare, representing an exclusion form for diagnosis.[3] The WHO classification recommends ISH for EBER as a mandatory exam for an EBV-positive DLBCL diagnosis. In these cases, the positivity rates are 80% of the atypical cells.[12] Expression of NFκB and pSTAT3 are more common in EBV-positive DLBCL than in EBV-negative lymphomas, which is evidence of the distinct gene expression profile of EBV-positive DLBCL.[3] These lymphomas have an immune response in the context of a virus-induced inflammatory microenvironment with Ig heavy domain rearrangements and a B cell-activated phenotype. It is possible that the pathogenetic mechanism of this lymphoma in young people potentially has other aspects; for example, the EBV latency is more closely associated with immune evasion than a decrease in host immune competence. In this context, it is possible to compare EBV-positive DLBCL in young HL patients and EBV-positive patients. In both pathologies, genes related to the B cell receptor signalling pathway are supplemented by EBV-mediated activation of NFκB and upregulation of PDL2, for example, with immune evasion and poor prognosis.[3,20]

Treatment does not differ between DLBCL and the NOS subtype. Both subtypes are treated with the rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) regimen.[11,13] New therapeutic strategies include treatments targeting EBV and drugs against other pathways, with the objective of improving and/or modulating the immune response against EBV. One possibility found in the literature is the use of antiviral therapy combined with EBV lytic phase induction.[11-13] This consists of the use of inducers of the lytic phase, such as methylase transferase inhibitors, histone deacetylase inhibitors, and proteasome inhibitors. Instead of typical antiviral therapies, such as those used for herpes virus, EBV requires a treatment that targets the latent phase, stimulating a lytic process.

Studies regarding conjugate antibodies, similar to rituximab, suggest brentuximab vedotin as an example of an anti-CD30 drug.[21,22] Therefore, diagnosis using immunostaining is very important for CD30 in DLBCL, both for future treatment and for the correct diagnosis of EBV infection. Other options being studied are tyrosine kinase inhibitors and T cell therapy, stimulating cytotoxic T cells.[3]

EPSTEIN–BARR VIRUS-POSITIVE MUCOCUTANEOUS ULCER

In 2010, Dojcinov et al.[24] described a series of 26 patients with immunosuppression due to medication, autoimmune disease, or immunosenescence, who had self-limited ulcers. All cases had similar pathological patterns characterised by Hodgkin-like features. The lesions were indolent, with good responses to conservative treatment. EBV status was examined through IHC using LMP1 and ISH using EBER. All individuals were positive for EBER, with good correlations with LMP1.[24]

The authors named the disease EBV-positive mucocutaneous ulcer (EBVMCU) and, in the 2017 WHO report, it was included as a category.[13] Since the first series,[24] 22 new papers about this ulcer have appeared in the literature.[25] The ulcer is described as occurring in patients with age-related or iatrogenic immunosuppression, often with a Hodgkin-like pattern and an indolent course, including spontaneous regression in some situations. There is currently no specific disease frequency recorded because this category is new to the scientific community. In general, it is classed as a lesion present in elderly patients at an estimated age >70 years; however, a history of immunological defects or changes has resulted in the diagnosis of patients at younger ages.[13]

More causes of EBV-lymphoproliferative disorders have been revealed in recent years and immunosenescence, primary immune deficiency, HIV infection, post-transplant setting, and the use of methotrexate and TNF antagonists have been suggested as possible aetiologies.[24] In the context of HIV infection, oral ulceration is more common in the end stage of disease during AIDS and results in a decreased number of CD4+ T cells. Regardless of the similarities, in general, these patients have poor survival rates and EBV is not detected.[26] In the cases of EBVMCU, for example, in elderly patients, the pathogenic point mutation induced the change in T cell response causing an accumulation of clonal or oligoclonal restricted CD8+ T cells with a functionality defect.[12] Malignancies, haematological or otherwise, can also represent a setting for EBVMCU, independent of treatment status. The lesion

may appear concomitant to a neoplasm, for example, an acute lymphoblastic leukaemia, reported by Vatsayan et al.[27] as an indication to oncologists.[28]

In general, in EBV-lymphoproliferative disorders, the pathogenic focus is infection of the B cell. Approximately 39% of patients present findings of B cell rearrangements; however, a study by Dojcinov et al.[24] showed 38% of EBVMCU patients had monoclonal *TCR* gene rearrangements, 31% had evidence of decreased T cell activity, and monoclonal Ig rearrangement was detected in 3 of the 26 cases. In addition to a B cell effect, there is also a deficiency in T cell control in EBVMCU patients.[24]

EBVMCU presents as ulcerated lesions, generally solitary and well demarcated in the oral mucosa, such as the tonsils, tongue, and buccal mucosa.[5] Cases in the large intestine and rectum have also been reported[27] and skin lesions are common, especially at sites such as the lips, arms, and torso. Lymphadenopathies appear near the ulcer infrequently, but systemic findings have not been observed.[5]

Histologically, ulcers have adjacent epithelial borders with pseudoepitheliomatous changes and acanthosis/epithelial hyperplasia. The inflammatory infiltrate presents a mixture of lymphocytes, plasma cells, eosinophils, and histiocytes. Large cells, such as atypical immunoblasts, and cells with two nuclei or irregular nuclei, similar to Hodgkin cells and Reed–Sternberg cells, have been detected.[5] Apoptosis, angioinvasion, and necrosis may be present in the background.[12] IHC examination shows the large, Hodgkin, and Reed–Sternberg-like cells are positive for CD20, ranging from weak to strong; the cells are generally heterogeneous. These cells are also positive for PAX5, IRF4/MUM1, and OCT2, and negative for BOB1, CD10, and BCL6. Those negative for BCL6 and CD10 and positive for IRF/MUM1 show the ABC phenotype. Generally, in these patients there is strong staining for CD30; CD15 may be expressed, but the subset of cells with this stain is not significant. Reactive T cells are presented in the background, with predominance of CD8+ T lymphocytes. Regarding EBV status, LMP1 is consistently positive, mainly in the large cells of immunoblastic type, and EBER is also positive in all types of cells, from lymphocytes to bigger cells, including the Hodgkin and Reed–Sternberg cells.[2,5,12]

In cases where HL is a consistent possibility, knowing about differential diagnosis is fundamental. Cutaneous or mucosal involvement by classical HL is the most probable possibility for these patients. However, careful consideration should be taken before diagnosis because HL manifestations outside the lymph nodes, with main involvement of skin and oral or gastrointestinal mucosae, are very rare. EBV-positive DLBCL is also a differential diagnosis. This neoplasm normally has CD15-negative neoplastic cells and a prominent diffuse pattern.[5] In spite of the limited number of cases and series, different authors have affirmed the benign course of the lesions, with reports of spontaneous disappearance or good responses with reduction of immunosuppression therapy.[12,23]

In general, patients have a good prognosis with a self-limited disease. Other treatments include the use of rituximab in association with CHOP, local surgical excision, local radiation therapy, and, similar to EBV-positive DLBCL therapeutics, CD30-directed antibody therapy in the future.[28,29] The therapeutic approach in EBV lymphoproliferative diseases, in general, is influenced by a variety of aspects, including the presence and/or the type of immunosuppression. Patients with age-related EBVMCU may receive aggressive treatments, such as chemotherapy, mainly with rituximab. Nevertheless, the current understanding of EBVMCU is that the disease is a self-limited condition in which a conservative treatment approach is sufficient.[27-29] In fact, there is no guide for management of these cases because there are only a few cases published in the literature and opinions are based on self-experience only. Optimal clinical practice involves obtaining a detailed clinical history and detecting immunosuppressive conditions, associated to a biopsy analysed with the perspective of these differential diagnoses, in addition to a long follow-up with periodic evaluations of new lesions.

CONCLUSION

EBV and neoplastic disorders are key pathologies in the field of medicine, especially

the pathology and oncology aspects of EBV infection. Epidemiologic data indicate this high importance, especially in Latin American, Asian, and African countries, where access to molecular methods for EBV detection in the context of public health is often difficult. For pathologists, it is important to learn about these diseases to better identify and investigate them. This article highlights some specificities about EBV-related lymphoproliferative disorders.

Firstly, when DLBCL presents atypically in Hodgkin or Reed–Sternberg-like cells, it is important to investigate EBV-positive DLBCL. One possibility is the inclusion of CD30 in the first IHC panel. If the cells stain for CD30, CD15 should be performed and EBV investigated with LMP1 or EBER. Secondly, lesions in mucosae with a morphological Hodgkin-like appearance should concern a pathologist. HL in mucosae is rare and so the lesion could be an EBVMCU. For this diagnosis, it is fundamental to have a good clinical indication of a possible immunosuppression status. Again, CD30, CD15, and LMP1 identification by IHC is a useful technique. It is also important to know that EBER is the best marker because it is expressed in all EBV latency types.

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A Game of Clones: The Complex Interplay of Aplastic Anaemia, Myelodysplastic Syndrome, and Paroxysmal Nocturnal Haemoglobinuria

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Disclosure: Dr Wells has received honoraria and research support from Alexion, which markets a therapy for paroxysmal nocturnal haemoglobinuria. Ms Rho has declared no conflicts of interest.

Received: 21.03.18

Accepted: 05.06.18

Keywords: Aplastic anaemia (AA), bone marrow disorder, clonal haematopoeisis, immune escape hypothesis, myelodysplastic syndrome (MDS), paroxysmal nocturnal haemoglobinuria (PNH), relative growth advantage hypothesis.

Citation: EMJ. 2018;3[3]:108-115.

Abstract

Although idiopathic aplastic anaemia (AA), myelodysplastic syndrome (MDS), and paroxysmal nocturnal haemoglobinuria (PNH) are all associated with bone marrow failure, they have traditionally been understood as distinct diseases with sharply contrasting pathophysiologies. These three disorders show considerable overlap. In 10% of cases of MDS the bone marrow is hypocellular, resembling AA, while glycophosphatidylinositol-deficient PNH cells can be detected in up to 5% of MDS and in >50% of AA patients. Results of recent studies offer a resolution to this overlap: MDS pathogenesis commonly has an autoimmune component and clonal haematopoiesis can be demonstrated in most cases of AA. Two explanations have arisen to explain the association of PNH with these disorders. It is hypothesised that PNH haematopoietic stem cells are relatively resistant to T cell attack and therefore have a competitive advantage in this context. Alternatively, it has been demonstrated that mutations associated with MDS are commonly present in PNH stem cells; such mutations could provide the PNH clone with an autonomous growth advantage. The authors suggest that these mechanisms may be necessary for the development of PNH in all cases, even when PNH occurs in the absence of MDS or AA. Finally, identification of a PNH clone is a predictive and prognostic biomarker in AA and MDS, adding important information for treatment and follow-up.

INTRODUCTION

Bone marrow failure (BMF) occurs when the production of blood cells of one or more lineages by the bone marrow (BM) diminishes to such an extent that cytopenia ensues. Severe BMF is life-threatening, owing to the risks of infection and haemorrhage. Failure of haematopoiesis can result from a variety of causes. In the paediatric age group, 30% of cases of BMF are inherited syndromes (Shwachman–Diamond syndrome, Fanconi anaemia, the telomeropathies, and others). Exposure to ionising radiation, a wide variety of drugs, and certain toxins (e.g., benzene)

and systemic illnesses, such as infection and multisystem autoimmune disorders, can all result in transient or permanent BMF. In this review the authors consider three acquired BMF syndromes: aplastic anaemia, myelodysplastic syndrome, and paroxysmal nocturnal haemoglobinuria (PNH). These syndromes appear, on the surface, to be distinct disorders, but have been revealed to be closely interconnected.

PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA, APLASTIC ANAEMIA, AND MYELODYSPLASTIC SYNDROME: DISORDERS OF BONE MARROW FAILURE WITH DISTINCT PATHOBIOLOGY

Paroxysmal Nocturnal Haemoglobinuria

Approximately 1–2 people per million per year are diagnosed with PNH, a rare clonal disorder of haematopoietic stem cells (HSC). PNH is characterised by intravascular haemolysis, pancytopenia, and thrombosis, and, although it is considered a benign disease, can be life-threatening.[1] In PNH, somatic mutation of *PIGA* occurs in a single HSC, resulting in failure of biosynthesis of the glycophosphatidylinositol (GPI) anchor. This structure serves as the attachment point for diverse cell-surface proteins, including the complement defence proteins CD55 and CD59.

This detailed understanding of the biology of PNH has enabled the development of highly reliable flow cytometry assays for the detection of PNH cells in peripheral blood.[2] A simple two-colour assay using carefully selected monoclonal antibodies recognising CD235a and CD59 was developed to detect PNH red blood cells (RBC) at a sensitivity of 0.002%. PNH leukocytes are detected using specific monoclonal antibodies to gate monocytes or granulocytes combined with monoclonal antibodies that detect GPI-linked leukocyte antigens (e.g., CD157), along with FLAER, a reagent that binds directly to the GPI anchor. These assays have a lower limit of quantification of 0.02–0.05% for neutrophils, and a somewhat lower sensitivity for monocytes. The routine availability of these assays has revolutionised the diagnosis of PNH and has been crucial in uncovering the relationships of PNH with AA and MDS.

Expansion of the *PIGA*-mutant HSC clone leads to production of blood cells that lack CD55 and CD59 and are, therefore, very sensitive to lysis by complement proteins, resulting in intravascular destruction of RBC and depletion of nitric oxide, because this compound is scavenged by free haemoglobin. Reduction of the plasma nitric oxide concentration, combined with direct interactions between the complement and coagulation pathways, lead to the clinical complications of PNH: fatigue, dysphagia, abdominal pain, pulmonary hypertension, kidney injury, and a virulent hypercoagulable state. Although the clonal dynamics of PNH are not clearly understood, two hypotheses exist as to how PNH HSC clones survive and expand within the BM: relative growth advantage (intrinsic) and immune escape (extrinsic) hypotheses; these will be highlighted later.

Aplastic Anaemia

AA is a rare BM disorder (incidence: 2–3/1,000,000/year) characterised by destruction of haematopoietic stem and progenitor cells, leading to BMF.[3] Although some cases of AA are related to exposure to toxins (e.g., benzene) or to rare inherited mutations (e.g., dyskeratosis congenita), the majority of cases are the result of T cell-mediated autoimmune destruction of HSC, leading to uniformly reduced production of early progenitor cells and CD34+.[4] Thus, AA patients present with pancytopenia and histologically aplastic (empty) BM. The development of AA is promoted by augmented immune states, such as pregnancy, toxins, and viral infection.[5]

Myelodysplastic Syndrome

Myelodysplastic syndrome (MDS) is a heterogeneous group of malignant clonal BM disorders characterised by dysplasia of haematopoietic cells in the BM and peripheral blood, and ineffective haematopoiesis, leading to BMF and cytopenias, and progressing to acute myelogenous leukaemia (AML) in 25% of patients. MDS is more common than PNH and AA, with an incidence of 50 persons per million per year, but in individuals over the age of 70 years its incidence rises sharply to

200–400 per million per year.[6] The highly variable natural history of the disease presents challenges in diagnosis and effective and timely treatment.[6] Although MDS is typically associated with BM hypercellularity, in ~10% of cases the BM is hypocellular. This variant, known as hypocellular MDS (hypoMDS), is clinically and pathologically similar to AA (with the exception of the presence of dysplasia) and responds similarly to immunosuppressive treatment.

INTER-RELATIONSHIP BETWEEN PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA, APLASTIC ANAEMIA, AND MYELODYSPLASTIC SYNDROME: COMMON ASSOCIATIONS AMONG RARE DISEASES

With the development of reliable high-sensitivity flow cytometric assays, it is now clear that, despite the rarity of PNH, PNH clones can be detected in a surprisingly sizeable proportion of patients with AA and MDS.[2] The biology that underpins these associations has not been elucidated fully. However, observations on the clinical significance of the finding of PNH clones in AA and MDS, and emerging data obtained through the application of new genomics techniques, allow us to create a working model of the inter-relationship between these diseases.

Aplastic Anaemia and Paroxysmal Nocturnal Haemoglobinuria

AA is strongly associated with PNH. When highly sensitive flow cytometry protocols are used, PNH cells are detectable in 40–59% of AA patients.[7-9] The majority of the PNH clones are small, affecting <1% of granulocytes, and no correlation is seen between the presence or absence of PNH cells and the severity of AA. However, the presence of PNH cells is predictive of both response to immunosuppressive therapy and overall survival.

The behaviour of PNH clones in AA patients who undergo immunosuppressive therapy is worthy of note. In a series of 207 patients with severe AA who were treated with antithymocyte globulin and cyclosporine, 83 were found to have a detectable PNH clone.[8] In 30 of these patients, the PNH clone number increased after treatment, and treatment for PNH was required in 7 patients. Because of the risk of development of clinically important PNH, as well as the predictive and prognostic value of the detection of a PNH clone, flow cytometric analysis for the presence of PNH cells is recommended for all patients diagnosed with AA.[10]

Although AA is an autoimmune disease, it has long been understood that it possesses some clonal characteristics. Clonal cytogenetic abnormalities are observed in 10–15% of AA patients,[11] and in 15% of AA cases patients go on to develop the indisputably clonal diseases MDS or AML. Analysis by single nucleotide polymorphism (SNP) array shows loss of heterozygosity in 15% of AA cases, with many of these affecting the HLA locus, and X-chromosome inactivation studies show evidence of clonal haematopoiesis in 38% of female AA patients.[11]

Recent studies have used modern genomic techniques to detect somatic mutations in haematopoietic cells in AA. Targeted sequencing of genes, combined with SNP array karyotyping and PNH flow cytometry in 150 AA patients, revealed clonal haematopoiesis in 55% of patients,[12] while an approach combining whole-exome sequencing, targeted sequencing, and SNP array karyotyping in 439 patients showed clonal haematopoiesis in 47%.[4] In addition to mutations of *PIGA* and loss of heterozygosity in the vicinity of the HLA locus, somatic mutation was prevalent in genes known to be involved in myeloid malignancy: *ASXL1*, *DNMT3A*, *BCOR*, *TET2*, *JAKs*, *RUNX1*, *TP53*, and *MPL*. While *PIGA* and *BCOR* mutations, which tended to occur together, were associated with good response to immunosuppressive therapy (IST) and favourable prognosis, patients with myeloid mutations had poor response to IST, poor prognosis, and a higher rate of MDS and AML.[4,12]

Myelodysplastic Syndrome and Paroxysmal Nocturnal Haemoglobinuria

A strong association also exists between PNH and MDS, although the proportion of MDS cases in which a PNH clone is detectable is much smaller (at ~2-5%); as for AA, PNH clones found in association with MDS tend to be small (<1% in 45% of cases).[13] The strength of this association is stronger for the hypocellular

variant of MDS. While hypoMDS accounts for 10% of MDS, ~70% of PNH+ MDS is hypocellular,[14] and a PNH clone can be detected in 40% of cases of hypoMDS.[15] As for AA, the presence of a PNH clone in MDS has been reported to be predictive of response to IST,[16,17] although this association has not been evident in all studies.

DYNAMICS OF PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA IN BONE MARROW FAILURE: A GAME OF CLONES

In order for a PNH to arise, a *PIGA*-mutated haematopoietic clone must arise, persist, and expand. However, it appears that although *PIGA*-mutated clones commonly arise, most do not persist, but instead spontaneously extinguish over the following weeks.[18] So why do clones occasionally persist and expand to result in classical PNH?

To account for the persistence and expansion of PNH clones, two hypotheses have arisen: the 'intrinsic growth advantage' hypothesis and the 'immune escape' hypothesis. The plausibility of the intrinsic growth advantage hypothesis diminished significantly when experimental evidence showed that *PIGA*-mutant HSC did not possess an intrinsic growth advantage over wild-type stem cells *in vitro* or *in vivo*. Consequently, the immune escape hypothesis gained favour.[19] This hypothesis (Figure 1A) postulates that, in the context of T cell-mediated autoimmune attack on normal HSC, *PIGA*-mutant HSC gain a competitive advantage because they are less vulnerable to immune attack owing to loss of the GPI and its associated proteins. The precise mechanism of this reduced susceptibility has not been determined, but could be consistent with, for example, a scenario in which the autoantigen target of T cell attack or an important coregulatory molecule that modulates cell-mediated cytotoxicity is a GPI-linked protein. The immune escape hypothesis is exemplified by the highly prevalent emergence in AA of haematopoietic clones with loss of heterozygosity at the HLA locus.[20] This type of mechanism would fit well with the observed strong association between PNH and AA, and with hypoMDS.

Recent data have led to a resurgence of the intrinsic growth advantage hypothesis. Shen et al.[21] discovered unexpected complexity in the genomic landscape of classical PNH by executing whole-exome sequencing and targeted deep sequencing of 61 genes that are commonly mutated in myeloid malignancies.[21] Of 60 patients with a diagnosis of classical PNH, 24 were found to have somatic mutation of genes other than *PIGA*. These data supported an analysis of the clonal dynamics of PNH wherein additional mutations are present, of which three categories were defined. In the majority of cases, a *PIGA* mutation arose as a secondary event, creating a PNH subclone of an ancestral HSC clone. In other cases, the *PIGA* mutation was the founding event, and acquisition of myeloid mutations led to clonal evolution, or the *PIGA* and myeloid-mutant clones were independent of one another. These observations bolster the intrinsic growth advantage hypothesis by pointing to the possibility that PNH clones could gain a growth advantage by hitchhiking with certain myeloid mutations (Figure 1B).

The immune escape hypothesis is consistent with the coexistence of PNH clonal haematopoiesis in AA but is more challenging to reconcile with the expansion of PNH clone size that is frequently seen following treatment of AA with IST.[22] After IST, the BM is repopulated from a small number of HSC. In this situation, an HSC clone that possesses an intrinsic growth advantage or a contingent advantage related to immune escape will tend to expand. Immunosuppression would be expected to reverse the environment condition, such as an immune attack, that is responsible for the selective advantage enjoyed in this model by *PIGA*-mutant HSC.

New data may allow this apparent paradox to be explained without invoking a role for acquired mutations.[23] In this study, PNH clone behaviour was studied in 319 patients with AA who received IST. Clone expansion was seen only in patients who had detectable PNH cells prior to IST and was less frequent among patients treated with ATG than those treated with less intensive immune suppression. This supports a model in which successful and complete immunosuppression results in the disappearance of AA-associated PNH clones, but partially effective treatment supports their outgrowth. It should be noted that these data are consistent

with the expansion of a residual HSC possessing both a *PIGA* mutation and a myeloid mutation, such as the common *TET2* mutation, which confers a clonal advantage. The authors would expect this mode of clonal selection to be comparatively infrequent.

MDS is a disease characterised by clonal dominance but also, particularly in its hypocellular variant, is in part immune-mediated.[24] The persistence of small PNH clones in MDS may largely be sustained by immune escape, a notion supported by the observation that the presence of a PNH clone predicts response to IST. However, the authors conjecture that the intrinsic growth advantage model may also commonly apply: an MDS clone that already possesses an intrinsic growth advantage acquires a *PIGA* mutation, resulting in a persistent PNH subclone.

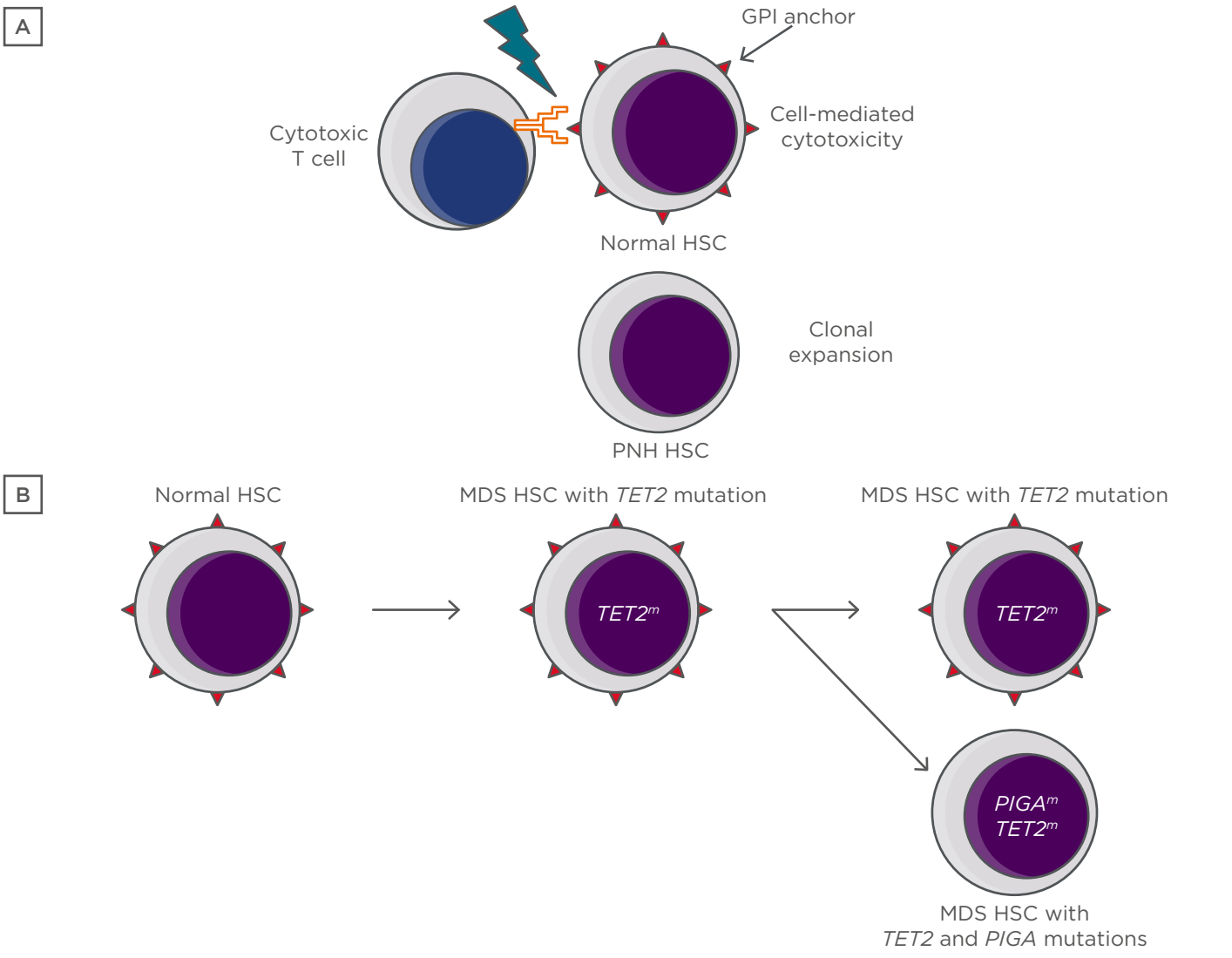

Figure 1: Depictions of the immune escape and intrinsic growth advantage hypotheses for the persistence and expansion of paroxysmal nocturnal haemoglobinuria clones.

A) The immune escape hypothesis. A GPI-dependent T cell-mediated autoimmune attack applies selective pressure on the HSC population. Lacking GPI and its associated proteins, rare *PIGA*-mutant HSC are relatively resistant to the attack and undergo clonal expansion. B) The intrinsic growth advantage hypothesis. An HSC that already carries a myeloid mutation that confers a growth advantage acquires a *PIGA* mutation, resulting in the creation of a PNH subclone.

GPI: glycophosphatidylinositol; HSC: haematopoietic stem cells; MDS: myelodysplastic syndrome; PNH: paroxysmal nocturnal haemoglobinuria.

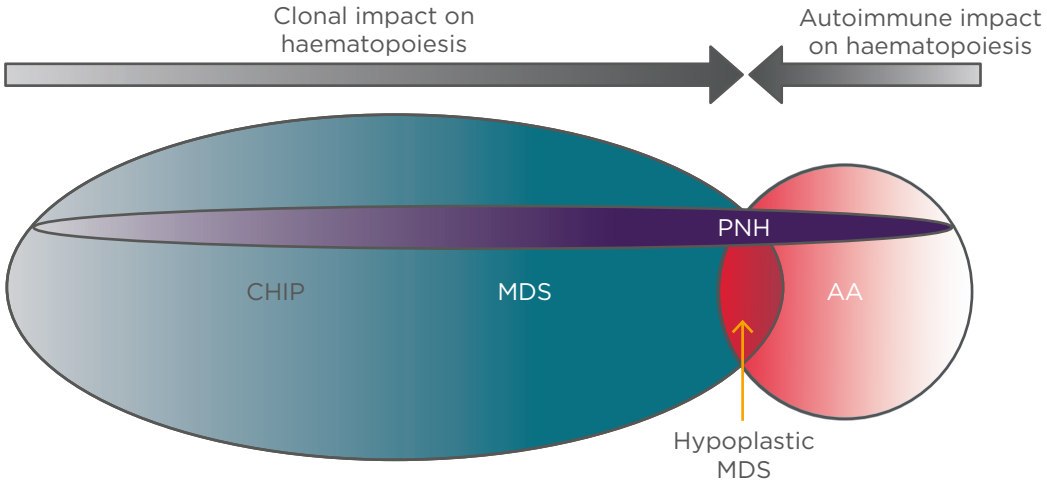

Figure 2: The interplay between the intrinsic and extrinsic influences on paroxysmal nocturnal haemoglobinuria clonal expansion.

In AA the dominant influence is T cell-mediated autoimmune attack, which permits the outgrowth of relatively resistant GPI-negative HSC. This mechanism may also apply in MDS, particularly in the hypocellular variant, which is clinically and pathologically very similar to AA. In MDS the presence of myeloid mutations that confer clonal advantage (e.g., by enhancing HSC self-renewal) also allow concurrent PNH mutations to be fixed and expand in the HSC population. Clonal haematopoiesis of indeterminate potential or subclinical autoimmune HSC attack may also provide support through the same mechanisms for expansion of PNH clones, leading to classical PNH, in which PNH clones expand in the absence of clinicopathological evidence of AA or MDS.

AA: Aplastic anaemia; CHIP: clonal haematopoiesis of indeterminate potential; GPI: glycophosphatidylinositol; HSC: haematopoietic stem cells; MDS: myelodysplastic syndrome; PNH: paroxysmal nocturnal haemoglobinuria.

These thoughts may also be applied to classical PNH that arises in the absence of any clinically apparent BMF (Figure 2). Here, the PNH clone must, in order to persist and expand, also gain a competitive advantage over normal HSC. The authors propose that in such cases, which account for most cases of PNH, there is always either a concurrent subclinical autoimmune suppression of normal haematopoiesis or clonal haematopoiesis of indeterminate potential/age-related clonal haematopoiesis. In the former scenario, the PNH clone expands via immune escape and in the latter by intrinsic growth advantage.

If it is the case, as the authors have suggested, that the presence of an intrinsic or extrinsic competitive advantage over normal HSC is a necessary condition for the persistence and expansion of PNH clones, then the application of advanced diagnostic techniques might support refined or novel management approaches in classical PNH. For example, patients who are shown by genomic analysis to have a PNH clone that also carry myeloid mutations would be followed more closely for the development of MDS or AML and may be advanced as candidates for curative treatment with allogeneic stem cell transplantation. Conversely, for patients with classical PNH in whom no myeloid mutations were found, it might be deduced that an underlying T cell-mediated autoimmune attack on HSC had supported the expansion of the PNH clone. In such patients, it may be predicted that aggressive IST could lead to the eradication of the condition supporting the persistence and expansion of the PNH clone by removing it, thus potentially curing the disease and removing the need for lifelong anticomplement therapy.

CLINICAL IMPLICATIONS OF PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA CLONES IN MYELODYSPLASTIC SYNDROME AND APLASTIC ANAEMIA

The detection of a PNH clone in AA or MDS patients is of established clinical value, since the presence of even minor populations of PNH

cells in patients with AA or MDS is an important predictor of a higher rate of response to IST and superior overall survival.[7,13,14,16,25] This is important in lower-risk MDS patients, in whom active therapeutic options are limited. In addition, PNH clones in AA may expand and cause life-threatening PNH. Therefore, identification and surveillance of PNH clones is recommended in all patients diagnosed with AA.[10,14] These principles are illustrated in the following brief clinical scenarios.

Scenario 1

A 33-year-old man, a recent immigrant with a history of severe aplastic anaemia diagnosed 8 years ago and successfully treated in his home country with IST, now presents with dark urine, dyspnoea, and severe anaemia. Initial investigations reveal pulmonary embolism and iliac vein thrombosis. His lactate dehydrogenase is elevated 9-times the upper limit of the normal range. Flow cytometry for PNH reveals GPI-deficient white blood cells (WBC) (FLAER-/CD157-): CD15+ neutrophils: 54.7%; CD64+ monocytes: 76.3%. Total GPI-deficient RBC (CD235a+/CD59-): 22.5% (Type III RBC: 14.4%; Type II RBC: 8.1%).

This case illustrates the importance of testing for the presence of PNH cells in all patients diagnosed with aplastic anaemia. In a significant proportion of patients who initially have a small PNH population, the size of the PNH clone expands after IST and may become clinically significant. In this patient, development of PNH led to a catastrophic clinical presentation that could easily have been fatal; this situation may have been avoided by surveillance, timely recognition of the expanding PNH clone, and implementation of anticomplement therapy.

Scenario 2

A 50-year-old man who is otherwise healthy and takes no medication presents with pancytopenia. Investigations, including a BM examination, lead to a diagnosis of severe aplastic anaemia. Flow cytometry for PNH reveals the presence of a small clone GPI-deficient WBC (FLAER-/CD157-): CD15+ neutrophils: 1.3%; CD64+ monocytes: 1.5%. Total GPI-deficient RBC (CD235a+/CD59-): 0.01% (Type III RBC: <0.01%; Type II RBC: <0.01%).

The best first-line therapy for this patient may be debated. Current recommendations are for allogeneic stem cell transplantation in patients <35 years old and IST for patients >50 years old, while either approach is acceptable for patients 35–50 years.[10] This patient has no comorbidities but is at the upper limit for his age range for stem cell transplantation and so is at higher risk for transplant-related morbidity and mortality. However, the detection of a PNH clone in this case indicates that this patient has a higher-than-average chance of responding to IST, and a better-than-average prognosis; these facts point toward choosing IST as first-line therapy. If he is treated with IST, this patient will require regular surveillance of the size of his PNH clone.

Scenario 3

A 57-year-old woman presents with pancytopenia (red cell transfusion dependence, platelets <20x10^9/L and neutrophils <0.5 x10^9/L). BM examination reveals multilineage dysplasia with markedly decreased BM cellularity (10%) and normal karyotype. The International Scoring System risk category is intermediate-1, and the Revised International Scoring System risk category is intermediate. Flow cytometry testing for PNH shows GPI-deficient WBC (FLAER-/CD157-): CD15+ neutrophils: 2.5%, CD64+ monocytes: 2.8%. Total GPI-deficient RBC (CD235a+/CD59-): 1.04% (Type III RBC: 1.03%; Type II RBC: 0.01%).

Patients with lower risk MDS who experience multiple cytopenias present a difficult therapeutic problem. Medical therapies for this condition are limited; some, like recombinant human erythropoietin and lenalidomide, are effective only in treating anaemia, while others, such as hypomethylating agents, are significantly inconvenient for patients who are expected to survive several years, since they require indefinite cycles of treatment. Allogeneic stem cell transplantation may be an option for some patients, although a decision analysis suggests that this modality of therapy results in a net loss of years of life.[26] The detection of a small PNH population in this patient suggests IST as an alternative approach. In this clinical situation, IST with ATG and cyclosporine can yield a durable trilineage response with acceptable toxicity.

CONCLUSION

Traditionally considered to be distinct BMF disorders with distinctive pathophysiology, current evidence suggests that PNH, AA, and MDS feature a complex interplay between clonal haematopoiesis and T cell-mediated autoimmune attack on normal HSC. The clonal development of PNH, both in the context of AA, MDS, or classical PNH, is currently explained by two hypotheses: the relative growth advantage (intrinsic) hypothesis and the immune escape (extrinsic) hypothesis. Evidence for each of these hypotheses comes from clinical observations and new data from next-generation sequencing analysis. These data have implications for the clinical management of AA and MDS, as well as for understanding the biology and potentially guiding the treatment of classical PNH. Future research will be required to test the contention that either co-operating mutations or autoimmune attack must always be present for PNH to develop and if in some cases PNH can be successfully eradicated by intensive IST.

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What's New

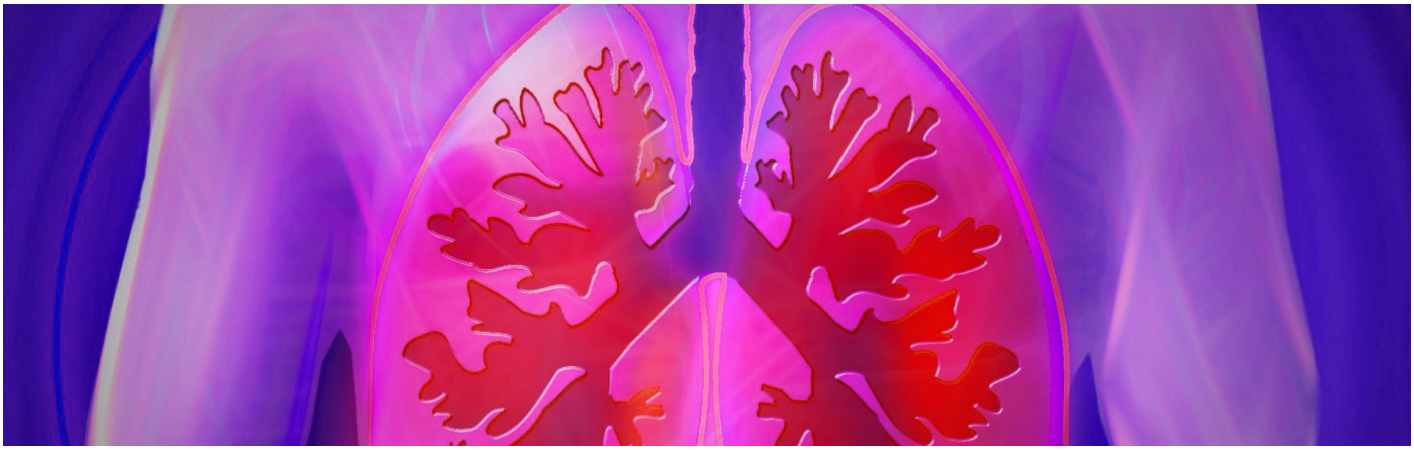
e-Cigarette Health Warnings

e-CIGARETTES have recently become the 'healthy' alternative to smoking conventional cigarettes, reported to give users the nicotine hit they crave without the harmful effects. However, according to new research from the University of Birmingham, Birmingham, UK, e-cigarettes do in fact cause negative health effects for those using them. With this knowledge, further investigation into the effect e-cigarette vapour can have on the immune cells of the lungs is warranted to ensure that e-cigarette users can make informed choices regarding their smoking habits.

"I do not believe that e-cigarettes are more dangerous than cigarettes, but I think we should have a cautious scepticism that they are as safe as we are being led to believe."

The negative effects of smoking e-cigarettes were discovered when immune cells were collected from lung samples from eight non-smokers who had not been diagnosed with chronic obstructive pulmonary disease (COPD) or asthma. Researchers at the University of Birmingham then exposed the extracted cells to different levels of e-cigarette fluid and condensed vapour. It was found that cell death rose 50-fold and there was an increase in inflammation-inducing chemical signals which disable the protective effect of the cells that usually clear the lungs of bacteria and other microbes. Over time, these changes can cause damage to lung tissue and eventually lead to the development of COPD. The researchers commented: "Importantly, exposure of macrophages to vaporised fluid induced many of the same cellular and functional changes in alveolar macrophage function seen in cigarette smokers and patients with COPD."

Prof David Thickett, Department of Respiratory Medicine, Birmingham's Institute of Inflammation and Ageing, University of Birmingham, stated: "I do not believe that e-cigarettes are more dangerous than cigarettes, but I think we should have a cautious scepticism that they are as safe as we are being led to believe." These results require more in-depth research to fully understand the effects of exposure to these vapours and to ensure that e-cigarette users can make more informed decisions about their habits, but there is no doubt that these results highlight the importance of continued investigation of already well-established products to ensure user and patient safety.



Mechanism of Fibrosis Discovered: A Potential Therapeutic Target

IDIOPATHIC pulmonary fibrosis (IPF) is an incurable lung disease of unknown origin. IPF is a particularly aggressive form of pulmonary fibrosis, a group of diseases associated with the excessive formation of connective tissue in the lungs that results in scarring of functional lung tissue, impeding gas exchange. Currently, there are drugs that slow the progression of IPF, yet there is still no permanent cure. However, according to researchers from Helmholtz Zentrum München, Munich, Germany, and the University of Denver, Denver, Colorado, USA, a recent discovery has uncovered the mechanism behind the development of fibrosis, which may lead to the development of a pharmacological biomarker and possible therapeutic target for IPF.

Researchers isolated extracellular vesicles (EV) from bronchoalveolar lavage fluid from patients with IPF, non-IPF interstitial lung disease, non-interstitial lung disease, and healthy volunteers. EV were characterised by transmission electronic microscopy, nanoparticle tracking analysis, and western blotting. Results showed that there was an increase in EV in bronchoalveolar lavage fluid from patients with IPF, particularly exosomes, which have been shown to act as carriers of the signalling molecule WNT5A. WNT5A is responsible for stimulating the proliferation of connective tissue cells in the lungs, causing a reduction in the surface area available for gas exchange.

"We were able to show in the study that increased levels of EV occur in IPF patients, which then act as carriers of WNT5A."

Before the release of these results, it was unclear how EV were involved in IPF development. "We were able to show in the study that increased levels of EV occur in IPF patients, which then act as carriers of WNT5A," explained lead author Aina Martin-Medina, Helmholtz Zentrum München. Researchers were also able to confirm the results in an experimental model in Petri dishes, where reducing the number of vesicles decreased the level of tissue scarring.

These results are incredibly interesting and are very much needed to help advance the treatment of IPF, an extremely debilitating disease. Further studies are still needed to assess the suitability of EV as a pharmacological biomarker as well as a potential therapeutic target.

What's New

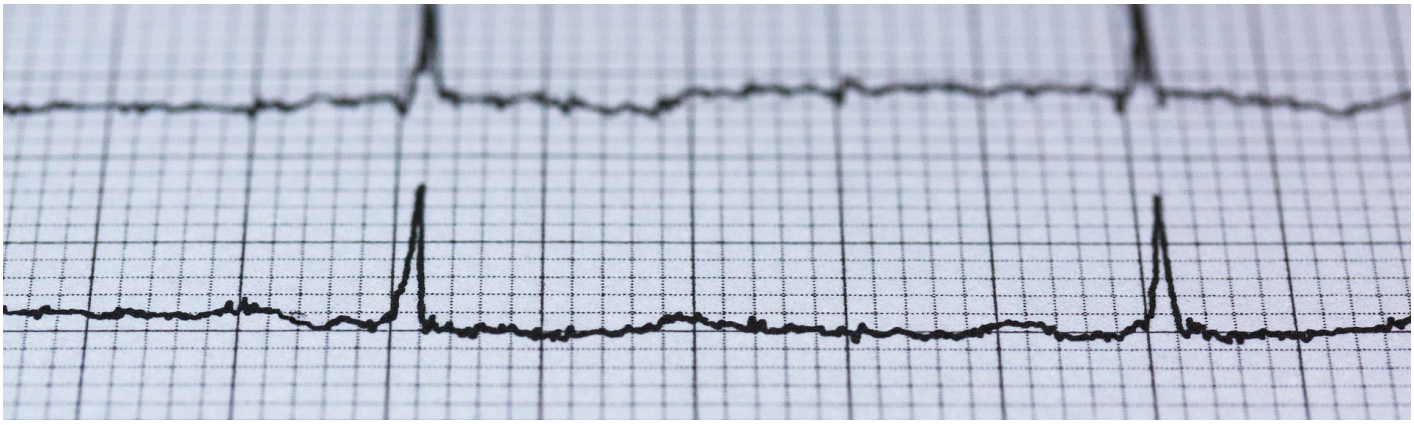
Epigenetic Reprogramming in Ischaemic Cardiomyopathy

REPROGRAMMING of the heart's metabolism and cellular remodelling have been shown to play an underlying role in ischaemic cardiomyopathy, the most common form of congestive heart failure. Believed to be a different causal mechanism to that of dilated cardiomyopathies, researchers at the University of Alabama at Birmingham, Birmingham, Alabama, USA are hopeful that this discovery will pave the way for personalised care of ischaemic cardiomyopathy patients.

Left ventricle heart tissue was collected from men with ischaemic cardiomyopathy (n=5) and non-ischaemic cardiomyopathy (n=6), all aged between 49 and 70 years. Using a wide range of bioinformatics tools, as well as cell culture experiments, the researchers found an epigenetic signature in the hearts of the ischaemic cardiomyopathy patients that represented a long-known metabolic change associated with the disease: the failing heart switches from aerobic to anaerobic metabolism.

Further investigation showed that a reduction in the expression of oxidative metabolism genes correlated with increased DNA methylation. The team noted that the KLF15 transcription factor, which regulates metabolic gene expression, was suppressed by epigenetic regulator EZH2, and inhibition of this regulator using small-molecular inhibitors may offer a therapeutic option for heart disease as well as various cancers. Commenting on the significance of these results, research team lead Dr Adam Wende, University of Alabama at Birmingham, summarised: "Altogether, we believe that epigenetic changes encode a so-called 'metabolic plasticity' in failing hearts, the reversal of which may repair the ischaemic and failing heart." Therefore, while the current treatments for congestive heart failure are symptomatic only, developments in the understanding of the causal mechanisms may lead to advances in life-saving treatments for a disease that affects nearly 6 million Americans.

"Altogether, we believe that epigenetic changes encode a so-called 'metabolic plasticity' in failing hearts, the reversal of which may repair the ischaemic and failing heart."



Touch-Free Monitoring of Heart Sounds

RADAR-BASED mobile devices could replace conventional stethoscopes for monitoring patient vital functions and the diagnosis of abnormal heart sounds, according to research by electronic engineers at Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany. This touch-free procedure could overcome the current issues associated with stethoscope use, specifically the subjectivity of the technique and its dependence on the examining doctor's experience.

Funded by the Federal Ministry of Education and Research, Bonn, Germany, the project involved a six-port continuous wave radar system to detect changes in movement of the skin due to the heartbeat, enabling researchers to calculate the strength and frequency of the heart sounds. By measuring the heartbeat of patients at rest and after sport, a direct comparison between the radar system and conventional stethoscopes and electrocardiograms (ECG) was made and showed a very high correlation. For example, the researchers noted that there was a 92% correlation between the radar system and ECG for measuring the first heart sound (S1), and an 83% correlation when signal shapes from a digital stethoscope and the radar system were compared. The slight variation was attributed to small deviations in the measurement location on the body, since the procedures could not be performed simultaneously, as well as the fact that the radar system measures surface area rather than focussing on a single location on the body.

"Touch-free and therefore stress-free measurement of vital parameters such as heart sounds has the potential to revolutionise clinical care and research… "

With the advantage that values are digitally recorded, hence minimising the risk of human error, the team are hopeful that the newly developed radar procedure could eventually replace conventional phonocardiology for investigating heart function. "Touch-free and therefore stress-free measurement of vital parameters such as heart sounds has the potential to revolutionise clinical care and research, for example, in palliative medicine," commented Prof Dr Christoph Ostgathe, Friedrich-Alexander-Universität Erlangen-Nürnberg. Other potential applications of the radar system include immediate recognition of changes in a patient's health and detection of painful symptoms in those who cannot communicate.