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GcMAF: a polemic or a highly promising molecule?

Eric Matamoros Morales

INS Ramon Berenguer IV, S/Mestre Sunyer 1-37- Amposta, Spain

E-mail address: ericmatamoros1999@gmail.com

ABSTRACT

Vitamin D Binding Protein (DBP) is a multifunctional protein which main role is to carry vitamin D and its metabolites, but it also acts as an actin scavenger and is the precursor of the macrophage activating factor molecule (GcMAF), which has reported highly promising results against cancer, HIV, and neurological disorders including autism, Alzheimer disease, Chronic Fatigue Syndrome (CFS), among others. DBP leads to the formation of GcMAF due to the loss of the O-glycosylated oligosaccharide moiety of the peptide by glycohydrolysis mediated by T and B cells. Some of the current noticed diseases have got increased levels of α-*N*-acetylgalactosaminidase (Nagalase), a molecule that deglycosylates DBP so it cannot drive to GcMAF, leading to immunosuppression. In this review we take a close look at the state of art strategies and trials using GcMAF as well as the controversies that have emerged during the last decade with this 'polemic' molecule.

***Keywords*:** vitamin D binding protein; Vitamin D binding protein-macrophage activating factor; α-*N*-acetylgalactosaminidase; HIV; cancer; neurological disorders; controversies

1. INTRODUCTION

VITAMIN D BINDING PROTEIN: THE 'CONTROVERSIAL' MOLECULE

The vitamin D binding protein (DPB), also known as group-specific protein, is a 51-58 kDa multifunctional serum glycoprotein which is synthesized by a single copy of the GC gene and it's widely secreted by hepatic parenchymal cells to the blood as a final 458 residues

peptide with three well differentiated domains as revealed by the crystal. [1,2,3] It completely differs from its homologues Human Serum Albumin (HSA) or α-fetoprotein (AFP) by its domain organization due to different connections with two α-helices.[2,4] DPB has several unrelated functions which include vitamin D transport, a mediator-associated for the complement activation peptide C5a caused by its extracellular binding with G-actin from necrotic cells, and one of the most controversial and polemic roles within the last decade, the macrophage and osteoclast activating factor, which will be lately discussed about the state-of-art benefits of this promising molecule as an anti-tumoral, HIV, and neurological disorders such as autism, among others. [1,2,5]

2. GC GENE ENCODES FOR PDB: RELATION BETWEEN COMMON ISOFORMS AND FUNCTION

The Gc gene (NCBI ID: 2638) encodes for PDB and belongs to the albumin gene family that also includes albumin (ALB), fetoprotein (*AFP*), and α-albumin/afamin (*AFM*). [6] It has got a sequence of 35 kb length and it's located in the Chromosome 4 (4q13.3) with a total of 15 exons and 12 introns, displaying a wide range of isoforms due to its mRNA alternative splicing and single-nucleotide polymorphisms (SNP's). [6,7]

SNP's and PDB isoforms have also been described with a total of 120 considerable alleles.[8,9] Among these, there are three common variants: Gc 1F (pI 4.94-4.84), Gc 1S (pI 4.95-4.85) and Gc 2 (pI 5.1), which are determined by two SNPs in *Gc*; rs7041(Asp432Glu) and rs4588 (Thr436Lys). They can be distinguished by electrophoresis gels.[8-13] The main difference between those isoforms is their binding affinity to serum and 25(OH)D metabolite (a type of vitamin D). [9,12,13] Serum levels did not differ between Gc1F – Gc1S alleles, instead, Gc2 showed significant lower values when compared with both Gc1F and Gc1S combined ($p = 0.001$). Higher values were found in Gc1-1 isoforms (72 ± 2 mg/L) and the lowest in Gc2-2 (226 ± 2 mg/L). [2,14]

Studies also suggest DBP phenotype as an independent predictor of 25(OH)D concentration, as differences of 25(OH)D metabolite concentrations in plasma have been reported within all three isoforms, being Gc1F homozygotes the isoform with the highest affinity (60.7 nmol/L) when compared to the other two isoforms combined (56.6 nmol/L). [11,12,15] Another cross-sectional study with 741 premenopausal French-Canadian women proved that additional copies of 432D or 436 K alleles reduced 25(OH)D concentrations. [15]
By knowing this correlations, large-scale studies have been performed to determine that population with 436 K allele genotype is less prevalent in Africans (15%), Hispanics (20%), Asians (26%) or Caucasians (23%) due to different metabolic processing of vitamin D as a result of their geographic location and the adaptive process.[16]

3. COMMON DBP VARIANTS: A ROLE AS ACTIVE BIOMARKERS

Some studies have been performed in order to associate the most common variants of DBP with diseases. Those studies suggest that people with DBP phenotype 1f/1f have got a 23-36% reduced risk to suffer from cancer when compared to normal phenotypes. [17,18] There is no biological explanation but it could be used as a genetic marker of cancer. Other studies

suggest that different polymorphism and the different levels of vitamin D have got a direct implication in the progression of Multiple Sclerosis (MS) due to increasing serum levels of vitamin D which might have an opposite-effect of MS. There is no current clinical relevance on which phenotypes are more present in the disease. [2,19,20]

Amyotrophic lateral sclerosis (ALS) is a neuropathy characterized by the loss axons and neurons in the central nervous system (CNS) and the dorsal cord, leading to muscle atrophy, debilitating and paralysis. [21] Gc-2 polymorphisms are described to play an increased role in actin-scavenging when compared to other variants like Gc1, that might increase the likelihood to progress with ALS. [21,22,25] Several studies also suggest that excess of Gc2 and low levels of Gc1 (increased levels of vitamin D) are directly associated with schizophrenia (neurological disorder) due to high levels of vitamin D being a risk factor, but there is a lack of association. [23,24] Liver disease clinical outcome also come worse with Gc-1 carriers due to inability to sequester cellular actin released, which increases risk of intravascular coagulation (Figure 1). [21,25]

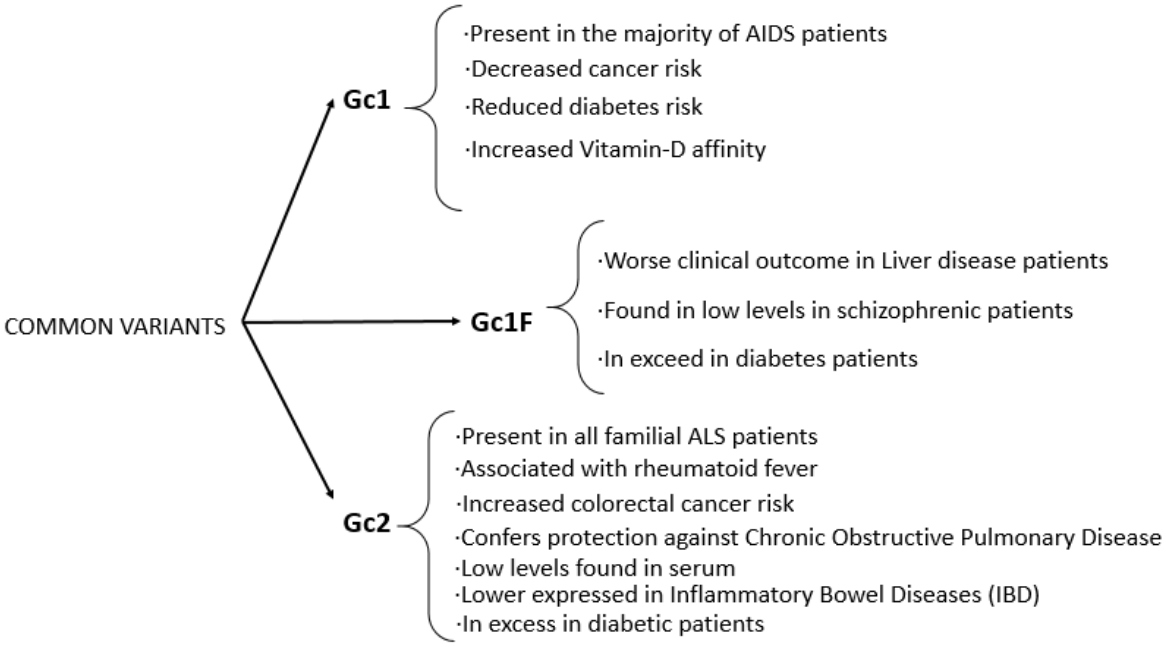

Figure 1. Current known associations between Gc most common variants and related diseases. Notably Gc2 variant is the one that is associated with a major range of diseases or properties, probably because of some atypical isoform conformational changes in structure or the lack of the site for O-linked trisaccharide glycosylation as reported in following points (See *DBP GLYCOHYDROLISIS BY LYMPHOCYTES T AND B…)*

4. TRIDIMENSIONAL STRUCTURE OF DPB: INSIGHT INTO BINDING DOMAINS

Taking an insight into DPB domains revealed by crystallography (PDB ID: 1J78), domain I ranges between 1-191 amino acid and it's stabilized by 5 disulfide bonds whereas Domain II is slightly smaller with a total of 186 amino acids (192-378) and 6 disulfide bonds.

Finally, Domain III starts from the 379 amino acid residue until the end, which is the amino acid 458. It's quite smaller when compared to the other domains and it's only stabilized by 2 disulfide bonds.[26-28] Two binding regions are described, a vitamin D/fatty acid binding domain located between residues 35-49 (Domain I), where Trp 145 and His (out of 6) play a key role as responsible amino acids for the proper binding of vitamin D sterol.[27,28,30] The other binding domain is localized between residues 350-403 (Domain II-Domain III) and acts as a scavenger site for G-actin binding site to be removed from damaged cells.[26,29] Recently, another binding domain in the N-terminal between amino acids 130-149 has been identified as a key pocket for the protein to act as a chemotactic cofactor for C5a. [31,32] Further studies also notice that there are two amino acid sequences located in both N-terminal (amino acids 150-172 of Domain I) and C-terminal (amino acids 379-458 of Domain III) which lets DBP to bind with surface molecules of neutrophils (white blood cells) (Figure 2). [32]

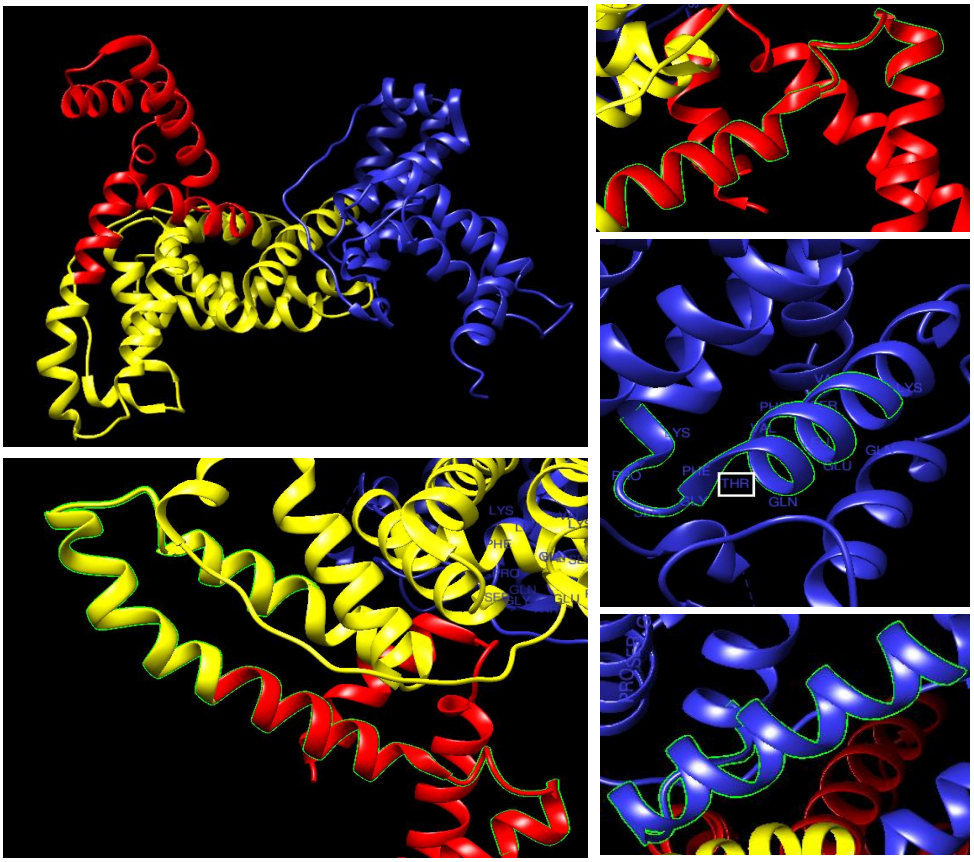

Figure 2. Representation of a monomer of DBP. A) Tridimensional structure of the 458 amino acids length DBP. Domain I (1-191 amino acid) is highlighted in blue, Domain II (192-378) is coloured in yellow and Domain III (379-458) is represented in red. **B)** Cell binding region sequence located between 379-402 amino acids. **C)** Vitamin-D binding site in residues 35-49. There's pointed out the Thr residue, which plays a key role for its proper binding with DBP. **D)** Another described cell binding site, mediated by residues 150-172. **E)** G-Actin binding site in DBP (residues 350-403), which form a scavenging system to remove acting from the damaged cells.

5. DBP GLYCOHYDROLISIS BY LYMPHOCYTES T AND B PROMOTE FORMATION OF GC MACROPHAGE ACTIVATING FACTOR (GcMAF)

DBP can undergo posttranslational modifications which will convert the protein into a molecule that will activate the immune response of macrophages, mostly known as Gc macrophage activating factor (GcMAF). This process relies on the carboxy-terminal domain of DBP which contains an O-linked glycosylation site at the 420 threonine residue. This residue is attached to a mucin-type trisaccharide, which consists of an N-acetylgalactosamine with a dibranched galactose and a sialic acid. Further glycohydrolysis promoted by T and B cells results in the loss of the O-glycosylated oligosaccharide moiety in the DBP peptide, converting it into a Gc MAF (or DBP MAF) molecule and activating the immune system response. [34-36]

But not everyone is able to produce GcMAF. People with the T420K mutation which defines the Gc2 isoform lack of the site for O-linked trisaccharide glycosylation. [37,38] People having all the different 119 described alleles are able to produce GcMAF, for that reason studies suggest the low presence of Gc2 isoform caused by evolution. [36-38]

6. A NEW ERA FOR IMMUNOTHERAPY TREATMENTS: GcMAF AND CANCER: A 'NOVEL TREATMENT'?

Cancer is a disease which involves an uncontrolled cell growth with the potential to spread and invade other tissues and organs. Cancerous cells usually have mechanisms to avoid immune system response against them, and so many cancer cell types avoid the formation of GcMAF molecules by deglycosylating the DBP protein using serum α-*N*-acetylgalactosaminidase (Nagalase), leading to a dysfunctional protein that cannot be converted to MAF and achieving immunosuppression. [39,40] At that point, cancer has successfully evaded immune system, so it has no barriers impede it from proliferating. [42]

Treatment using GcMAF involves the administration of 100 ng of GcMAF to humans, resulting into maximal activation of macrophages 3.5 hours later. Macrophages activated by GcMAF develop a variation of cell membrane receptors that make them capable to recognize abnormalities in cancerous cell surface such as specific antigens, glycolipids or glycoproteins and kill them. [40-44] This clinical trials have been successful in many types of cancer: Prostate Cancer[40], Breast Cancer [42], Lung Cancer [45,46], etc. Recent trials also involve the combination of GcMAF with oral colostrum MAF and ozone therapy, which proved high effect on non-small cell lung cancer (NSLC). [46] Treatment with single macrophages does not provide a response, they need activation by GcMAF. Without presence of macrophage, GcMAF has provided an inhibitory effect of angiogenesis and so do proliferation and metastatic potential in breast cancer cells. Vimentin expression, which is overexpressed in breast cancer during the epithelial/mesenchymal transition decreased after GcMAF treatment, indicating a reverse cancerous-healthy cell process (Figure 3 & Figure 4).

7. HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Something similar to what happens with cancer is involved in the non- immune system response against certain viral infections, mainly Human Immunodeficiency Virus (HIV-1) and

influenza (also known as flu virus). A paper published in 2008 claimed that HIV virions released Nagalase due to the sum of some enzyme activities carried by their metabolism plus unassembled envelope proteins released from HIV-infected cells. This Nagalase activity inhibited the production of GcMAF like in cancer, so macrophages remained in a latent state. Inactivation of macrophages can be explained with the fact that even HIV virions infect more lymphocytes (T-cells mainly) than macrophages, they are not capable to recognize HIV virus as a pathogen and destroy them due to some possible evasion mechanism. The same study administrates doses of 100 ng to 15 non-anemic HIV-infected patients in order to stimulate their macrophage immune response. Results are very promising as macrophages immediately start synthesizing receptors including Fc-receptors and after the 4th week of therapy the viral load in plasma decreased by 5.1 x 10^{1}. After treatment viral loads were insignificant, proving the achievement of a functional cure against HIV. The same mechanism is described with influenza but there are no studies that are able to prove the effectiveness of the therapy. [47-49]

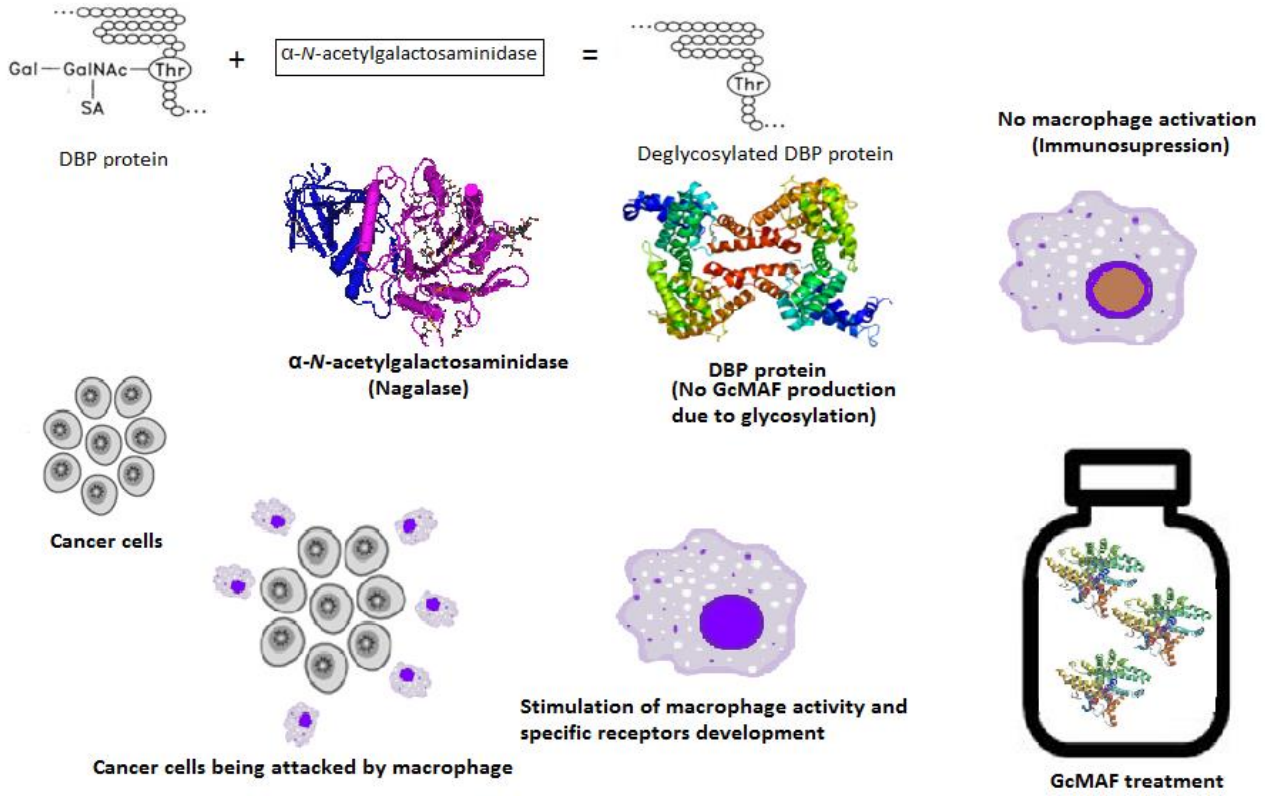

Figure 3. Schematic representation of cancer cells avoiding immune system method and GcMAF treatment against them. A) Cancer cells produce α-*N*-acetylgalactosaminidase (Nagalse) which deglycosylates DBP protein and the O-glycosylated oligosaccharide moiety of the peptide can't be lost, leading to no possible GcMAF production. **B)** Cancer cells production of Nagalase results in no macrophage activation and the capability of cancer cells to avoid immune system. Treatment with 100 ng of GcMAF stimulate and reactivate macrophage, which start collecting specific receptors (against certain antigens, glycoproteins and glycolipids) to detect and kill abnormal cells.

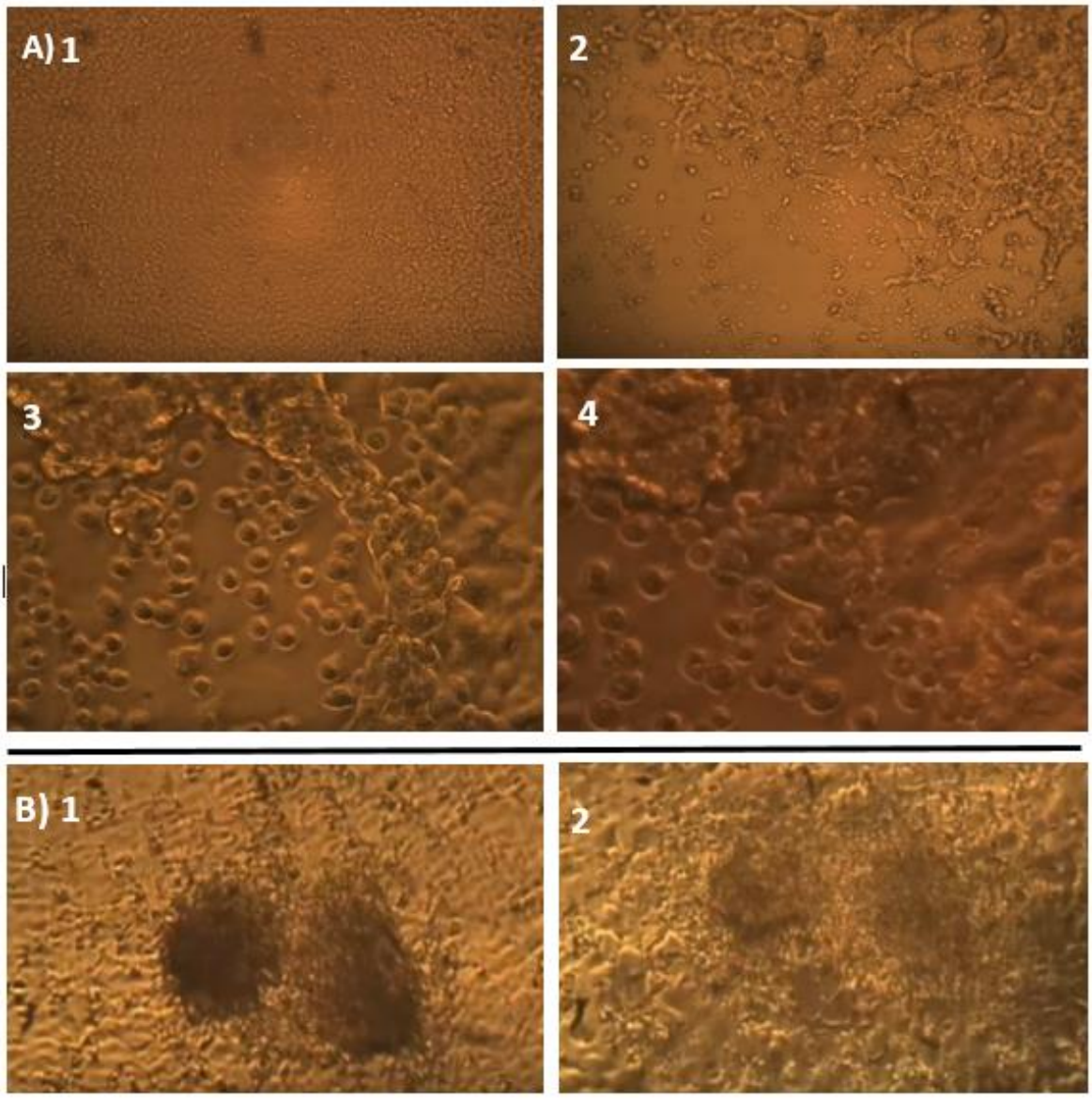

Figure 4. Effectiveness of macrophage activation by GcMAF against cancer cells.
A) 1. Monolayer culture of MCF7 breast cancer cells 48 hours post GcMAF seeding. **2.** Activated macrophages (small spheres) during the 72 hours phase. **3.** Zoom in image of macrophage surrounding cancer cells in the 72 hours phase as noticed with arrows. **4.** 60 hours post treatment, cancer cells are being killed and the monolayer culture is changing from corrugated to smooth due to cancer cells death**. B) 1.** MCF7 breast cancer culture. Brown holes are clumps of breast cancer cells. **2.** 90 hours post treatment, brown holes have nearly disappeared and the surface has been reverted to a healthy state with the cancer clumps gone.

8. HUMAN IMMUNODEFICIENCY VIRUS (HIV)

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Unfortunately, both papers published related with GCMAF have been recently retracted *due to irregularities in the documentation for institutional review board approval*, leading to an active controversy regarding the potential effect against GcMAF therapy, but there is no published proves that it doesn't work.

9. AUTISM

Autism is a neurological disease characterized by impaired social interaction, lack of verbal and non-verbal communication and repetitive behaviour. Immune system dysregulation is one of the main etiological causes of this disorder. Immune system is being alterated by a key immune system regulatory protein known as cannabinoid receptor type 2(CB2R). [50-52]

It's described that it plays an important role in activation of monocytes to trigger immune imbalances in normal-conditions. Otherwise, autistic people have got an overexpression of CB2R on macrophages and microglial cells, which results as an opposite-role effect by deactivating the response of macrophages and persist with the immune dysregulation. [50,51]

Vitamin D deficiency has been extensively investigated as one of the multifactorial causes of autism development in prenatal or early postnatal stages. [50,52] High levels of Nagalase activity has been observed in the serum of autistic group (1.93 nmol/min/mg of substrate). [49] Treatment with GcMAF has shown promising results by decreasing CB2R levels and posterior activation of blood monocyte-derived macrophages (BMDMs). Final post treatment results of the 22 autistic children sotmeted to study showed an 85% improvement and a 15% full recovery. [49,51,52]

In another pilot study, 40 autistic patients were taken and levels of Nagalase were measure before treatment and after GcMAF treatment (2/3 months aprox.). Results are very promising as Nagalase levels decreased in all patients, achieving a progressive recovery of the immune system that was disturbed. This reduction of Nagalase activity is also linked with some benefits and improvements in patients as they were less antisocial and more communicative. [51,52] Most individuals had higher Nagalase values than the expected healthy ones. [52] There's no current explanation on the higher decrease of Nagalase levels in some patients, probably it's due to different metabolisms (Table 1).

Table 1. GcMAF treatment decreases Nagalase levels in blood. Results have been taken from patients with higher and lower values pre-treatment. As seen, 2/3 months after treatment, Nagalase values decreased in all subjects, proving the effectiveness of GcMAF to restore immune system in autistic patients. It's still not known the conditions in which GcMAF is more effective in one patient rather than another.

Patients with higher-lower Nalgase levels	Nalgase Activity (nmol/min/mg of substrate)		
	Pre treatment	2/3 months post treatment	Pre/Post Nalgase diff.
m	7,80	4,40	**3,4**
m	4,00	1,40	**2,6**
f	3,90	1,60	**2,3**
m	3,00	1,00	**2**
m	2,90	1,20	**1,7**
m	1,00	0,44	**0,56**
m	1,00	0,90	**0,1**
m	1,00	0,90	**0,1**
f	0,92	0,62	**0,3**
m	0,90	0,47	**0,43**

9. NEURODEGENERATIVE DISORDERS: ALZHEIMER, PARKINSON, MULTIPLE SCLEROSIS (MS) AND CHRONIC FATIGUE SYNDROME (CFS). A CLAIM TO NOVEL NEURODEGENERATIVE TREATMENTS

Multiple Sclerosis (MS) is considered to be a disorder which involves an autoimmune, inflammatory and demyelinating degeneration of the central nervous system (CNS). The central role of the immune pathogenesis of MS is related with abnormal interactions between T cells and B cells. This miss-interactions lead to destruction of myelin by autoreactive T cells resulting in degeneration of axons and loss of neurons and autoantibodies production by B cells against myelin, causing a multiple attack against neurons. [53] Treatment with GcMAF has promoted rehabilitation of MS patients due to modulation of the immune system and the decrease of the immune system imbalance responses by T and B cells [54,55]. *Patient ID:004-C.Y* found a slightly recovery from her initial status and right now she recovered some leg mobility and is able to eat very well and perfectly talk and stand by herself for an hour holding on a chair.[54] Further mouse model experiments are required in order to understand the working mechanism of GcMAF in the immune modulation and determine if this is by GcMAF or any other resulting species. Overall, GcMAF treatment has proved beneficial results against autism

In other neurodegenerative diseases including Alzheimer's and Parkinson's disease Gc MAF therapy has also proved some benefits. [58] Most cases of Alzheimer and Parkinson involve the overproduction of Tumor necrosis factor alpha (TNF-α), a protein which promotes neuron death and interrupts synaptic communications, leading to the loss of memory or the uncontrolled spasms of the people that suffer this disorders. [55,56] GcMAF activate macrophages disable TNF- α interaction with neurons by occupying its receptors with stable-

bonds and avoiding neuronal death. Parkinson's symptoms dropped by 60% but continuous revisions of the patients revealed the needed of continue the treatment to conserve this results and avoid a rebound of the disease. [50]

Chronic Fatigue Syndrome (CFS) is also a neuronal condition characterized by long-term fatigue and other symptoms which relay on the decrease of the person's ability to carry out ordinary daily activities. [54,60] It's brought by different circumstances including weak immune system and stress conditions. People with CFS have elevate Nagalase levels up to 90%, meaning an immune system suppression. [55,59,60] Gc MAF treatment activated the macrophage response and reactivated again the whole immune system, leading to a partial recovery and improvement of near the 65% of the cases. [55,59,60]

10. OTHER IMMUNE RELATED DISEASES: TUBERCULOSIS, DENGUE FEVER, ENDOMETRIOSIS, CHRON'S DISEASE, ... AMONG OTHERS. TRUE FACT OR INVENTION?

Even the big potential of GcMAF, big companies claim the high effect of this molecule to threat other diseases which affect the immune system response. This makes sense as the possibility of reactivating immune system to attack *Mycobacterium tuberculosis,* the main tuberculosis agent, modulate it in proinflammatory and autoimmune diseases such as Chron's disease, or even attack viral infections including Dengue and Malaria. Unfortunately, there is no proven data and neither published reports in any known journal to contrast the effectiveness of GcMAF in those diseases, so further studies are required to prove it.

11. GCMAF AND CONTROVERSIES: LACK OF SIGNIFICANT RESULTS AND ADEQUATE METHODOLOGIES?

Since the last decade there has been a wide controversy involving GcMAF treatment and its claimed effectiveness. The main reasons against GcMAF is that clinical trials lack of significant data, because of the small-group of patients treated in the studies, involving fewer than twenty patients in each, rather than the hundreds or thousands that are required to obtain a reliable conclusion. Patients in many of those trials have previously received other treatments such as chemotherapy, surgery or derivate. [61,62]

Other controversies are related in the fact that during the studies only Nalgase levels are monitored during GcMAF treatment and change in tumoral markers are not taking into account. There is also no proven detail about the TNM (tumour, node, metastasis) status (measure to determine cancer spread in patients). [61]

The fact that GcMAF studies were published in a medium-journal instead of in a top-tier 'high impact' journal also disappoints scientists as it decreases credibility. Retraction also makes the paper dubious.[63] To sum up, a lot of web pages have claimed this molecule as the 'cure' that everyone expects to treat all type of diseases without providing journals to contrast their information. [62]

12. DISCUSSION AND CONCLUSIONS

In this review we describe the state-of-art strategies and biomedical applications of the novel molecule 'GcMAF' which is very promising to threat a wide range of diseases. The main focus of this paper is to provide a conclusion in two main topics: potential of GcMAF as a treatment and a view of the strongest scientific arguments against GcMAF therapies.

Let's first go insight the controversies relying GcMAF usage. One of the reasons proposed is that the first publications involving studies with this macrophage activator are published in medium-journals instead of 'high impact factor' journals despite of the importance of this discovery. It's a strange point for such a breakthrough but further publications regarding this topic have been published in higher order journals including *Anticancer research, International Journal of Cancer, J Med Virol. Journal of neuroinflammation or Journal of pathologies* since the first publications by Dr. N.Yamamoto. During this decade, policies in journals and restrictions have increased exponentially, so papers published within the last 8 years have to pass a lot of filters in other to achieve publication such as the possibility of the experiment to be replicated with similar results. All journals have got filters so there is no need to publish in a 'high impact' journal to prove reliability of the methods used.

Another proposed point is the small-group of treated patients in each study, and that's something which is true. None of the cited clinical trials with GcMAF have done large-scale trials, but proper similar results are obtained from each independent study that has been done around the world, so it might be a coincidence to obtain benefits using GcMAF therapy in all the trials with lack of adverse effects. Even that, bigger trials are proposed in this study in order to increase the reliability of this method and obtain significant results.

Monitoring of markers in cancer trials might also be recommended including TNF. Other trials involving HIV-1 or neurodegenerative disorders have quantified other markers a part of Nalgase activity such as p24 antigen ELISA assay (Enzyme-Linked ImmunoSorbent Assay) for HIV-1 trials and TNF-α levels in Alzheimer and Parkinson, but there are no evidences of other markers in CFS or the mentioned cancer trials.

In our opinion, information is wrong extended in websites because they propose a wide range of diseases that can be treated with GcMAF with no scientific approved content or references for it, so people shouldn't rely on those methods even they claim they are safe. Theoretically it's possible to treat a lot of neurological disorders with GcMAF due to the modulator effect of macrophage activation, but trials need to be done, published and approved in order to provide positive or negative results against a determined disease.

As a conclusion, GcMAF is highly promising molecule that provides benefits against a wide range of described diseases. However, further studies need to be done in order to determine its effectiveness against other pathological disorders and possible unnoticed long-term side effects. The study of GcMAF can potentially lead to 'novel' drugs with improved effects.

13. LIST OF ABBREVIATIONS

DBP: Vitamin D binding protein

GcMAF/DBP MAF: Gc Macrophage Activating Factor Molecule/ DBP Macrophage Activating Factor Molecule

HAS: Human Serum Albumin

AFP: α-fetoprotein (AFP)

SNP: Single Nucleotide Polymorphism

MS: Multiple Sclerosis

ALS: Amyotrophic Lateral Sclerosis

CNS: Central Nervous System

Nagalase: α-*N*-acetylgalactosaminidase

NSLC: Non-small cell lung cancer

HIV: Human Immunodeficiency Virus

CB2R: Cannabinoid receptor type 2

BMDM: Blood monocyte-derived macrophages

TNF-α: Tumor necrosis factor alpha

CFS: Chronic Fatigue Syndrome

TNM: Tumour, node, metastasis

ELISA: Enzyme-Linked ImmunoSorbent Assay

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