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(54) Title: USE OF ANTIBIOTICS FOR THE TREATMENT OF IMMUNOGLOBULIN A NEPHROPATHY

(57) Abstract: Immunoglobulin A nephropathy (IgAN) is the most common primary glomerulonephritis worldwide. IgA is mainly produced by the gut associated lymphoid tissue (GALT). Both experimental and clinical data suggest a role of the gut microbiota in this disease. The inventors aimed to determine if an intervention targeting the gut microbiota could impact disease development in a humanized mouse model of IgAN, the $\alpha 1^{KL}$ -CD89^{TG} mice. Four-week old mice were divided into two groups to receive either antibiotics or vehicle-control by oral gavage twice a week for eight weeks. Antibiotic treatment efficiently depleted the faecal microbiota, impaired GALT architecture and impacted mouse IgA production. However, while hlgA1 and mIgG serum levels were unchanged, the antibiotic treatment markedly prevented hlgA1 mesangial deposition, glomerular inflammation and the development of proteinuria. This was associated with a significant decrease in circulating hlgA1-mIgG complexes. These data support that use of antibiotics would be suitable for the treatment of immunoglobulin A nephropathy.



USE OF ANTIBIOTICS FOR THE TREATMENT OF IMMUNOGLOBULIN A NEPHROPATHY

5 FIELD OF THE INVENTION:

The present invention relates to use of antibiotics for the treatment of immunoglobulin A nephropathy.

BACKGROUND OF THE INVENTION:

10 Immunoglobulin A nephropathy (IgAN) is the most common primary glomerulonephritis worldwide (1). Most patients diagnosed with this condition are in their third or fourth decade of life (2). It is estimated that at least 10% of IgAN patients will require renal replacement therapy within 10 years of diagnosis (3). IgAN physiopathology mainly involves abnormally galactosylated IgA1 (galactose-deficient IgA1, Gd-IgA1) (4) which then becomes a target of anti-Tn antigen IgG (5). IgA1 complexes bind to an IgA Fc receptor (CD89)
15 expressed by monocytes and neutrophils, inducing shedding of this receptor (sCD89) and its integration into the complexes which are elevated in serum of IgAN patients (6). A decrease in serum levels of IgA-sCD89 as well as IgA1-IgG complexes have shown to be associated with disease progression or recurrence after transplantation (7-9). IgA1 complexes deposit into the mesangium, where they activate mesangial cells through IgA receptors. Three mesangial IgA
20 receptors have so far been described: the transferrin receptor, the integrin alpha1/beta1 and alpha2/beta1 and the 1,4-galactosyltransferase 1 (10-12). Among them, the mesangial transferrin receptor (CD71) is strongly upregulated in IgA nephropathy (13) being associated with transglutaminase 2 (TG2) (14). Activated by IgA1 complexes induces CD71 activation leading to kinase phosphorylation and cytokine secretion (15) which may serve as potential
25 factors for other lesions through cross-talk with podocytes (15, 16) and tubular cells (17). However, the origin of Gd-IgA1 and its mechanisms of production are still incompletely understood, leaving a lack of specific therapeutic targets for IgAN.

In humans, IgA is mainly produced by the mucosa associated lymphoid tissue (MALT) from where it is secreted into the lumen and participates in the barrier function of the mucosa.
30 Gut associated lymphoid tissue (GALT) is the most extensive MALT, and therefore the primary source of both subclasses of IgA. A relationship between the digestive tract and IgAN is strongly suggested by its association with inflammatory bowel diseases (IBD) (which share common genetic risk loci with IgAN) (18) and celiac disease (19). The latter shares common pathophysiological mechanisms with IgAN notably by overexpressing CD71 at the apical side

of enterocytes associated with TG2 (20, 21). On the other hand, depletion of gluten in the diet prevents IgAN development in mice humanized for IgA1 and CD89 (22). This link between the intestinal and IgAN has recently been consolidated by three different studies: 1) a GWAS has associated IgAN with polymorphisms of genes involved in gut mucosal immunity (23), 2) faecal microbiota dysbiosis has been reported in IgAN (24) and 3)– treatment with corticosteroids targeting the gut mucosa protect renal function among IgAN patients (25).

It is known that not just the intestinal immune system, but also systemic immunity, is largely influenced by antigenic stimulation from the commensal microflora and, more specifically, the intestinal microbiota (26). Mice overexpressing the B cell activation factor of the TNF family (BAFF) exhibit an elevation in serum IgA in association with a renal disease that has features similar to IgAN (27). Under germ-free conditions these transgenic mice have decreased serum IgA as well as an abolition of glomerular IgA deposits. Similarly, glomerular human IgA1 deposits are reduced under germ-free conditions, along with a significant reduction in serum IgA1, in mice expressing the human $\alpha 1$ heavy chain instead of the murine μ chain (28). In both of these models mice were deprived of stimulation by commensal flora from birth, which prevents normal maturation of the immune system (29).

SUMMARY OF THE INVENTION:

The present invention relates to use of antibiotics for the treatment of immunoglobulin A nephropathy. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

Immunoglobulin A nephropathy (IgAN) is the most common primary glomerulonephritis worldwide. IgA is mainly produced by the gut associated lymphoid tissue (GALT). Both experimental and clinical data suggest a role of the gut microbiota in this disease. The inventors aimed to determine if an intervention targeting the gut microbiota could impact disease development in a humanized mouse model of IgAN, the $\alpha 1^{KI}$ -CD89^{Tg} mice. Four-week old mice were divided into two groups to receive either antibiotics or vehicle-control by oral gavage twice a week for eight weeks. Faecal bacterial load and proteinuria were quantified both at the beginning and the end of the experiment where blood, kidneys and intestinal tissue were collected. Serum mouse IgG (mIgG) and human IgA1 (hIgA1) containing complexes were quantified. Renal and intestinal tissue were analysed by optical microscopy after hematoxylin and eosin coloration and immunohistochemistry with anti-human IgA and anti-mouse CD11b antibodies. Antibiotic treatment efficiently depleted the faecal microbiota, impaired GALT architecture and impacted mouse IgA production. However, while hIgA1 and mIgG serum levels were unchanged, the antibiotic treatment markedly prevented hIgA1 mesangial

deposition, glomerular inflammation and the development of proteinuria. This was associated with a significant decrease in circulating hIgA1-mIgG complexes. Notably, final faecal bacterial load strongly correlated with critical clinical and patho-physiological features of IgAN such as proteinuria and IgA1-IgG complexes. These data support an essential role of the gut microbiota in the generation of mucosa-derived nephrotoxic IgA1 and in IgAN development opening new avenues for therapeutic approaches in this disease.

Accordingly, the first object of the present invention relates to a method of treating immunoglobulin A nephropathy (IgAN) in a patient in need thereof comprising administering to the patient a therapeutically effective amount of at least one antibiotic.

As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of patient at risk of contracting the disease or suspected to have contracted the disease as well as patients who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a patient during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a patient during treatment of an illness, e.g., to keep the patient in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., disease manifestation, etc.]).

In some embodiments, the antibiotic is selected from the group consisting of nitroimidazoles, glycopeptide antibiotics, aminoglycosides, beta lactams, quinolones or fluoroquinolones, macrolides, sulfonamides, sulfamethaxozoles, tetracyclines, streptogramins, oxazolidinones (such as linezolid), rifamycins, glycopeptides, polymixins, lipo-peptide antibiotics.

Nitroimidazoles are an organic compound with the formula $O_2NC_3H_2N_2H$. The nitro group at position 5 on the imidazole ring is the most common positional isomer. Examples of Nitroimidazoles include metronidazole, tinidazole, nimorazole, dimetridazole, pretomanid, ornidazole, megazol, and azanidazole.

Glycopeptide antibiotics are a class of drugs of microbial origin that are composed of glycosylated cyclic or polycyclic nonribosomal peptides. Significant glycopeptide antibiotics include the anti-infective antibiotics vancomycin, teicoplanin, telavancin, ramoplanin and decaplanin, and the antitumor antibiotic bleomycin.

Tetracyclines belong to a class that shares a four-membered ring structure composed of four fused 6-membered (hexacyclic) rings. The tetracyclines exhibit their activity by inhibiting the binding of the aminoacyl tRNA to the 30S ribosomal subunit in susceptible bacteria. Tetracyclines for use in the invention include chlortetracycline, demeclocycline, doxycycline, minocycline, oxytetracycline, chlortetracycline, methacycline, mecocycline, tigecycline, limecycline, and tetracycline.

Aminoglycosides are compounds derived from species of *Streptomyces* or *Micomonospora* bacteria and are primarily used to treat infections caused by gram-negative bacteria. Drugs belonging to this class all possess the same basic chemical structure, i.e., a central hexose or diaminohexose molecule to which two or more amino sugars are attached by a glycosidic bond. The aminoglycosides are bactericidal antibiotics that bind to the 30S ribosome and inhibit bacterial protein synthesis. They are active primarily against aerobic gram-negative bacilli and staphylococci. Aminoglycoside antibiotics for use in the invention include amikacin (Amikin®), gentamicin (Garamycin®), kanamycin (Kantrex®), neomycin (Mycifradin®), netilmicin (Netromycin®), paromomycin (Humatin®), streptomycin, and tobramycin (TOBI Solution®, TobraDex®).

Macrolides are a group of polyketide antibiotic drugs whose activity stems from the presence of a macrolide ring (a large 14-, 15-, or 16-membered lactone ring) to which one or more deoxy sugars, usually cladinose and desosamine, are attached. Macrolides are primarily bacteriostatic and bind to the 50S subunit of the ribosome, thereby inhibiting bacterial synthesis. Macrolides are active against aerobic and anaerobic gram positive cocci (with the exception of

enterococci) and against gram-negative anaerobes. Macrolides for use in the invention include azithromycin (Zithromax®), clarithromycin (Biaxin®), dirithromycin (Dynabac®), erythromycin, clindamycin, josamycin, roxithromycin and lincomycin.

Ketolides belong to a class of semi-synthetic 14-membered ring macrolides in which the erythromycin macrolactone ring structure and the D-desosamine sugar attached at position 5 are retained, however, replacing the L-cladinose moiety and hydroxyl group at position 3 is a 3-keto functional group. The ketolides bind to the 23S rRNA, and their mechanism of action is similar to that of macrolides (Zhanel, G. G., et al., *Drugs*, 2001; 61(4):443-98).

Structurally, the quinolones possess a 1,4 dihydro-4-oxo-quinolinyl moiety bearing an essential carboxyl group at position 3. Functionally, the quinolones inhibit prokaryotic type II topoisomerases, namely DNA gyrase and, in a few cases, topoisomerase IV, through direct binding to the bacterial chromosome. Quinolones for use in the invention span first, second, third and fourth generation quinolones, including fluoroquinolones. Such compounds include nalidixic acid, cinoxacin, oxolinic acid, flumequine, pipemidic acid, rosoxacin, norfloxacin, lomefloxacin, ofloxacin, enrofloxacin, ciprofloxacin, enoxacin, amifloxacin, fleroxacin, gatifloxacin, gemifloxacin, clinafloxacin, sitafloxacin, pefloxacin, rufloxacin, sparfloxacin, temafloxacin, tosufloxacin, grepafloxacin, levofloxacin, moxifloxacin, and trovafloxacin. Additional quinolones suitable for use in the invention include those described in Hooper, D., and Rubinstein, E., *"Quinolone Antimicrobial Agents, 4th Edition"*, American Society of Microbiology Press, Washington D.C. (2004).

Drugs belonging to the sulfonamide class all possess a sulfonamide moiety, $-\text{SO}_2\text{NH}_2$, or a substituted sulfonamide moiety, where one of the hydrogens on the nitrogen is replaced by an organic substituent. Illustrative N-substituents include substituted or unsubstituted thiazole, pyrimidine, isoxazole, and other functional groups. Sulfonamide antibiotics all share a common structural feature, i.e., they are all benzene sulfonamides, meaning that the sulfonamide functionality is directly attached to a benzene ring. The structure of sulfonamide antibiotics is similar to p-aminobenzoic acid (PABA), a compound that is needed in bacteria as a substrate for the enzyme, dihydropteroate synthetase, for the synthesis of tetrahydro-folic acid. The sulfonamides function as antibiotics by interfering with the metabolic processes in bacteria that require PABA, thereby inhibiting bacterial growth and activity. Sulfonamide antibiotics for use in the invention include the following: mafenide, phtalylsulfathiazole, succinylsulfathiazole, sulfacetamide, sulfadiazine, sulfadoxine, sulfamazone, sulfamethazine, sulfamethoxazole, sulfametopirazine, sulfametoxypiridazine, sulfametrol,

sulfamonomethoxine, sulfamylon, sulfanilamide, sulfaquinoxaline, sulfasalazine, sulfathiazole, sulfisoxazole, sulfisoxazole diolamine, and sulfaguanidine.

All members of beta-lactams possess a beta-lactam ring and a carboxyl group, resulting in 55 similarities in both their pharmacokinetics and mechanism of action. The majority of clinically useful beta-lactams belong to either the penicillin group or the cephalosporin group, including cefamycins and oxacephems. The beta-lactams also include the carbapenems and monobactams. Generally speaking, beta-lactams inhibit bacterial cell wall synthesis. More specifically, these antibiotics cause 'nicks' in the peptidoglycan net of the cell wall that allow the bacterial protoplasm to flow from its protective net into the surrounding hypotonic medium.

Fluid then accumulates in the naked protoplast (a cell devoid of its wall), and it eventually bursts, leading to death of the organism. Mechanistically, beta-lactams act by inhibiting D-alanyl-D-alanine transpeptidase activity by forming stable esters with the carboxyl of the open lactam ring attached to the hydroxyl group of the enzyme target site. Beta-lactams are extremely effective and typically are of low toxicity. As a group, these drugs are active against many gram-positive, gram-negative and anaerobic organisms. Drugs falling into this category include 2-(3-alanyl)clavam, 2-hydroxymethylclavam, 7-methoxycephalosporin, epi-thienamycin, acetyl-thienamycin, amoxicillin, apalcillin, aspoxicillin, azidocillin, azlocillin, aztreonam, bacampicillin, blapenem, carbenicillin, carfecillin, carindacillin, carpetimycin A and B, cefacetil, cefaclor, cefadroxil, cefalexin, cefaloglycin, cefaloridine, cefalotin, cefamandole, cefapirin, cefatrizine, cefazedone, cefazolin, cefbuperazone, cefcapene, cefdinir, cefditoren, cefepime, cefetamet, cefixime, cefinenoxime, cefinetazole, cefminox, cefmolexin, cefodizime, cefonicid, cefoperazone, ceforamide, cefoselis, cefotaxime, cefotetan, cefotiam, cefoxitin, cefozopran, cefpiramide, cefpirome, cefpodoxime, cefprozil, cefquinome, cefradine, cefroxadine, cefsulodin, ceftazidime, cefteram, ceftezole, ceftibuten, ceftizoxime, ceftriaxone, cefuroxime, cephalosporin C, cephamycin A, cephamycin C, cephalothin, chitinovorin A, chitinovorin B, chitinovorin C, ciclacillin, clometocillin, cloxacillin, cycloserine, deoxy pluracidomycin B and C, dicloxacillin, dihydro pluracidomycin C, epicillin, epithienamycin D, E, and F, ertapenem, faropenem, flomoxef, flucloxacillin, hetacillin, imipenem, lenampicillin, loracarbef, mecillinam, meropenem, metampicillin, meticillin (also referred to as methicillin), mezlocillin, moxalactam, nafcillin, northienamycin, oxacillin, panipenem, penamecillin, penicillin G, N, and V, phenethicillin, piperacillin, pivampicillin, pivcefalexin, pivmecillinam, pivmecillinam, pluracidomycin B, C, and D, propicillin, sarmoxicillin, sulbactam, sultamicillin, talampicillin, temocillin, terconazole, thienamycin, and ticarcillin.

In some embodiments, a mix of antibiotics comprising 2, 3, 4, or 5 antibiotics is administered to the patient.

In some embodiments, the antibiotic of the present invention is preferably administered to the patient via the oral or rectal route.

5 By a "therapeutically effective amount" of the antibiotic as above described is meant a sufficient amount to provide a therapeutic effect. It will be understood, however, that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including
10 the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical
15 arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Typically, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the
20 active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

25 According to the invention, the antibiotic is administered to the subject in the form of a pharmaceutical composition. Typically, the antibiotic may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions. "Pharmaceutically" or "pharmaceutically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic
30 or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Oral compositions generally include an inert diluent or an edible carrier. Typically, for the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and

used in the form of oral solution (e.g. for pediatric purpose) tablets, troches, or capsules, e.g., gelatin capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain
5 any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or
10 orange flavoring. Formulations for oral delivery may advantageously incorporate agents to improve stability within the gastrointestinal tract and/or to enhance absorption.

In some embodiments, the method of the present invention further comprises a step consisting of performing a fecal microbiota transplantation. As used herein, the term “fecal microbiota transplantation” or FMT has its general meaning in the art and refers to a procedure
15 in which fecal matter, or stool, is collected from a donor, and then placed into the intestinal tract of a recipient in order to directly change the recipient’s gut microbial composition and confer a health benefit. FMT represents the one therapeutic protocol that allows the fastest reconstitution of a normal composition of colon microbial communities. The process involves first selecting a donor without a family history of IgAN and optionally other disease (e.g. metabolic, and
20 malignant diseases) and screening for any potential pathogens. The feces are then prepared by mixing with water or normal saline, followed by a filtration step to remove any particulate matter. The mixture can be administered through a nasogastric tube, nasojejunal tube, esophagogastroduodenoscopy, colonoscopy, or retention enema. The transplantation may also be carried out by mouth in the form of a capsule containing freeze-dried fecal material.

25 The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: Antibiotic treatment prevents IgAN in α KI-CD89Tg mice. (A)
30 **Quantification of immunostaining for human IgA1 in frozen kidney sections of mice that received antibiotics or vehicle (percentage of positive glomerular area) as measured by ImageJ. (B) Representative sections of kidney sections after immunostaining for hIgA1 in mice receiving antibiotics or vehicle (original magnification x50, insert x200). (C) Urinary protein-to-creatinine ratio at the beginning and the end of the experiment (g/mmol) in mice that received**

antibiotics or vehicle. Effect of the treatment on the development of proteinuria was analysed with a linear mixed model. **(D)** Quantification of immunostaining for murine CD11b in frozen kidney sections from mice that received antibiotic mix or vehicle (percentage of glomeruli with CD11b positive cells) as measured by ImageJ (left panel). Representative sections of kidney sections after immunostaining for murine CD11b from mice that received antibiotics or vehicle are shown (right panels, original magnification x400). **(E)** Nuclei were counted in 20 randomly chosen glomeruli for each mouse that received either antibiotics or vehicle (nuclei/glomerulus) and compared using Wilcoxon's test (left panels). Representative glomeruli of mice that received antibiotics or vehicle are shown (right panels, original magnification x400)

Figure 2: Effect of individual antibiotic treatment. (A) Glomerular IgA deposition in individual antibiotic treatment groups (immunofluorescence intensity quantified by Imaris software). **(B)** Effect of antibiotics on renal function as measured by serum Cystatin C levels.

EXAMPLE:

Material & Methods

***In vivo* experiments**

Mice were raised and maintained in a specific pathogen free (SPF) mouse facility at the Centre for Research on Inflammation, Paris, France. All experiments were performed in accordance with national ethics guidelines and with approval of the local ethics committee (Comité d'Éthique Expérimentation Animale Bichat-Debré). Mice remained with their mother for suckling until 4-weeks old and were weaned after collection of urine and faeces. Then, littermates were randomly assigned to receive either 40µl/g body-weight of either a broad-spectrum antibiotic-mix (metronidazole (5mg/ml), neomycin (5mg/ml), vancomycin (2.5mg/ml) and amoxicillin (5mg/ml)) or vehicle-control (Na₂HPO₄·12H₂O: 1.5g/l and NaCl 7.4g/l), twice a week for eight weeks by oral gavage. After eight weeks of treatment, urine and faeces were again collected, immediately before the 12-week-old mice were anaesthetised. Blood was collected by cardiac puncture and mice were sacrificed by cervical dislocation. Kidneys and small intestine containing Peyer's Patches (PP) were collected. Organs were conserved in either OCT (CML, Nemours, France) or formalin 10% (Sigma-Aldrich, Saint-Quentin Fallavier, France).

Faecal bacterial load quantification

Mouse stool-samples were collected in a sterile tube, aliquoted (150mg), and DNA was extracted using both mechanical and chemical lysis as described (30). Quantitative PCR targeting the Bacteria domain (Eubacteria Eub338 probe) was performed to analyse bacterial loads.

Histological procedures

Paraffin-embedded, 4 μ m sections of intestine and kidney were stained with hematoxylin-eosin for morphological analysis. For immunohistochemistry, 4 μ m sections of cryostat frozen intestine or kidney were fixed in acetone, incubated for 1 h in 5% bovine serum albumin (Euromedex, Souffelweyersheim, France), followed by 1 h 30 min at room temperature with biotinylated mouse anti-human IgA (Catalog number 555884) for gut and kidney or anti-mouse CD11b (Catalog number 553309) for kidney only (both from BD Biosciences, Le Pont de Claix, France). Detection was performed with vectastain elite ABCkit (Vector, Burlingame, CA, USA). Slides were mounted with Immuno-mount (Thermo Fisher scientific) and read with an upright microscope, DM2000 (Leica, Wetzlar, Germany) at 400x magnification using IM50 software (Leica). Quantification of hIgA1-positive intestinal gut was performed by counting the number of hIgA1-positive cells in the intestinal mucosa for each section at 400x magnification and dividing by the total number of individual fields required to cover the entire section. Quantification of glomerular cellularity and CD11b positive cells was performed by counting the number of nuclei/CD11b positive cells in 20 randomly chosen glomeruli for each mouse. Glomerular area positive for human IgA1 was reported as the area positive for hIgA1 as a percentage of the total area of the glomerulus as measured using Image J as previously described (22).

ELISA

Serum levels of human IgA, and mouse IgG were assessed with the corresponding Bethyl quantification (E88-102 and E90-131 respectively) set according to the manufacturer's instructions. Regarding hIgA1 complexes, serum complexes were isolated using polyethylene glycol precipitation. Measurement of IgA-IgG and IgA-sCD89 complexes were determined with a sandwich enzyme-linked immunosorbent assay (31). Anti-human IgA (Bethyl Laboratories, 10 μ g/ml) or A3 mAb anti-human CD89 (10 μ g/ml) was used for coating. Precipitated sera (1:5 diluted) were then added and revealed with anti-mouse IgG (1:5000 dilution) or anti-human IgA (1:2000 dilution) coupled with alkaline phosphatase (Southern Biotech, Birmingham, AL, USA). The OD at 405 nm was measured after the addition of alkaline phosphatase substrate (Sigma-Aldrich). The complex levels were expressed as OD.

Statistical analysis

Statistical analyses were performed using RStudio integrated development environment (IDE) for R (RStudio, Inc., Boston, MA). Comparisons between numerical variables were performed using Wilcoxon's sum-rank test. Categorical variables were compared using Fisher's exact test. Correlations were analyzed with Spearman's test. Effect of treatment on mice weight

gain was analysed with a likelihood ratio test. Effect of treatment on faecal bacterial load reduction and proteinuria was analysed using a mixed-effect model with random-effect attributed to mice and fixed-effect to treatment group and time.

Individual antibiotic treatment

Mice were treated with vancomycin (n=2), amoxicillin (n=2), gentamicin(n=3) or metronidazole (n=2) from the age of 12 weeks until 20 weeks old. Immunofluorescent staining of human IgA deposits in glomeruli was performed on 4µm frozen sections; images of 10 glomeruli were taken from each mouse by confocal microscopy (Zeiss) and analysed using Imaris software.

Renal function was determined by clearance of cystatin c from mouse serum by ELISA (R+D systems) with lower levels of cystatin C indicating improved renal function.

Results

Antibiotic treatment depletes gut microbiota without liver and renal toxicity

In order to deplete the microbiota, we subjected four week-old α1KI-CD89Tg mice to receive either vehicle-control (VC) (n=15) or broad-spectrum antibiotics (ATB) (n = 16) by oral gavage for eight weeks (**data not shown**). Five mice died during the experiment (2 in the ATB group and 3 in the VC group, p>0.1, data not shown). ATB treatment resulted in an effective depletion (p < 0.001) of the mouse gut microbiota as compared to VC (**data not shown**). There was one mouse in the ATB group in which microbiota depletion was not achieved, so it was subsequently excluded from analyses regarding disease phenotype. Compared to samples collected at 4-weeks old, before initiation of the protocol, faecal bacterial load was reduced 613.0 ± 236.7-fold at the end of the experiment in the ATB group as compared to 1.5 ± 1.1-fold in the VC group. Antibiotic treatment resulted in significantly less weight-gain (p < 0.001) with a final weight (mean± SD) of 23.2 ± 2.6 g in the ATB group and 26.5 ± 3.5 g in the VC group (**data not shown**). No evidence of liver or renal toxicity was detected: there were no significant difference between groups in bilirubin, alkaline phosphatase, aspartate aminotransferase (AST) and serum creatinine (**data not shown**, p > 0.1)

Microbiota depletion abolishes the IgA nephropathy phenotype

Twelve-week old α1KI-CD89Tg spontaneously develop mesangial IgA1 deposition, along with proteinuria, mimicking human IgA nephropathy as described previously (14). Anti-human IgA immunostaining of mouse kidney revealed that hIgA deposition was significantly reduced (p = 0.008) in ATB mice compared to VC mice (**Figure 1A and C**). At twelve-weeks old, mice in the VC group α1KI-CD89Tg VC mice developed proteinuria (initial and final

urinary protein-to-creatinine ratio (UPCR) 0.7 ± 0.2 and 3.7 ± 0.4 g/mmol respectively), in keeping with our previous report of this model (14). Conversely, antibiotic treated mice were protected from renal disease (initial and final UPCR 0.8 ± 0.2 and 0.8 ± 0.1 g/mmol). Antibiotics significantly affected the development of proteinuria over time ($p < 0.001$, **Figure 1B**). These results were confirmed in an additional experiment using a different protocol where the mice's drinking water was supplemented with broad-spectrum antibiotics for 12 weeks (**data not shown**). Additionally, antibiotics prevented the development of glomerular inflammation as illustrated by fewer glomeruli with infiltrating CD11b positive cells ($p = 0.008$, **Figures 1D**) despite a similar number of cells in the glomeruli of both groups ($p = 0.157$, **Figures 1E**). It is noteworthy that antibiotic treatment reduced the formation of human IgA1–mouse IgG complexes ($p = 0.002$, **data not shown**), one of the major hallmarks of the disease, independently of hIgA1 ($\rho = -0.232$, $p = 0.352$, **data not shown**) or mouse IgG serum levels ($\rho = 0.007$, $p = 0.980$, **data not shown**). Conversely, serum levels of hIgA-sCD89 complexes were similar in the ATB and the VC group ($p = 0.564$, **data not shown**).

Microbiota depletion alters GALT structure, but not serum human IgA1 production

As germ-free mice display impaired adaptive immune functions, including decreases in circulating immunoglobulin levels, we evaluated the effect of an antibiotic/microbiota-depletion protocol on circulating IgA1 levels and GALT. For this purpose, we first collected all thickened portions of the small intestine suspected to contain Peyer's patches. Light microscopy visualisation of small intestine revealed the presence of lymphoid structures in eight (67 %) VC mice and three (23 %) ATB mice ($p = 0.047$, **data not shown**). In addition, lymphoid structures identified in ATB mice had altered architecture (**data not shown**). Immunostaining of intestinal tissue with anti-human IgA did not reveal any differences in the number of hIgA1⁺ cells ($p = 0.784$, **data not shown**) nor in the staining pattern (**data not shown**) suggesting that homing of hIgA1 producing plasma cells into the submucosa was not impaired in our model. In agreement with these results, mice given either ATB or VC displayed similar serum levels of hIgA1 (401 ± 37 and 359 ± 54 mg/L respectively, $p = 0.316$, **data not shown**). Circulating murine IgG levels were the same in both groups ($ART130 \pm 17$ and $VC 122 \pm 14$ mg/L, $p = 0.979$, **data not shown**).

Faecal bacterial load is associated with clinical and pathophysiological features of IgA nephropathy

To demonstrate a relationship between microbiota depletion and the prevention of IgAN, rather than a direct effect of antibiotics on glomerular injury, we analysed the

relationship between faecal bacterial-load and serum immunoglobulin levels, circulating IgA-immune complexes and proteinuria. Final faecal bacterial load did not correlate with human IgA1 ($\rho = -0.123$, $p = 0.556$, **data not shown**) or mouse IgG serum levels ($\rho = -0.054$, $p = 0.798$, **data not shown**). Final faecal bacterial load did correlate with urinary protein-to-creatinine ratio ($\rho = 0.644$, $p = 0.001$, **data not shown**) and hIgA1-mIgG complexes ($\rho = 0.581$, $p = 0.016$ **data not shown**), but not with hIgA1-sCD89 complexes ($\rho = 0.262$, $p = 0.308$, **data not shown**). However, hIgA1-sCD89 complexes correlated with urinary protein-to-creatinine ratio ($\rho = 0.571$, $p = 0.023$, **data not shown**).

Individual antibiotic treatment

Significant variation in IgA deposition was seen to be induced by individual antibiotic therapies. The aminoglycoside antibiotic, genetecin (G418), appeared to both decrease glomerular IgA deposition and improve renal function (**Figure 2A and 2B**) in alpha 1 KI-CD89tg mice compared to other antibiotic treatments and untreated mice. Amoxicillin alone appeared to aggravate glomerular IgA deposition and renal function in one mouse, but not in another.

Discussion:

Here, we present data which demonstrate an important role of the gut-microbiota in disease development in the $\alpha 1$ KI-CD89tg mouse model of IgAN, which is consistent with the findings of recent clinical studies. The advantage of our model over previously described axenic models is that we were able to study the effect of microbiota modulation in mice with a mature immune system. Our results implicate the intestinal microbiota in the formation of IgA1-IgG immune complexes, a crucial determinant of IgAN pathogenesis. Given the current lack of specific therapies for IgAN, these results open up promising new avenues of investigation for treatments that target the microbiota and provide a new, easily workable, model that can be used to experiment further with the role of the microbiota in IgAN.

IgAN pathogenesis, as the name implies, is dependent on the formation of aberrant IgA1 and IgA1 immune complexes (32). Our data suggest that these pathogenic forms of IgA1 are being generated at the mucosa where MALT, the principal producer of IgA, is found, and where IgA plays its principal immunological role. Early development of the immune system, and more particularly of MALT, is largely dependent on antigenic stimulation by antigens from commensal bacteria and reciprocally, the composition of the commensal microbiota depends on MALT function (26, 29). This results in dynamic cross-talk and equilibrium is achieved when there is a healthy symbiosis between flora and host. However, a problem with either the

immune or the microbiological component of this system may occur, leading to a disease-associated dysbiosis (33), as has been reported in IgAN (24). However, the “chicken and egg” question remains, as to whether the altered microbiota is a cause or effect of the disease. Our results, showing the prevention of IgAN by depletion of the microbiota in α 1KI-CD89Tg mice, support a causal role of an intestinal-microbiota dysbiosis in IgAN. Indeed, microbiota depletion in these mice completely abrogated hIgA1 deposition and prevented the development of proteinuria, as compared to mice that were given vehicle control. If alterations in the microbiota, as reported from clinical studies, were an effect of the disease rather than a cause of the disease, we would not anticipate these dramatic effects on glomerular IgA1 deposition when the microbiota is depleted by antibiotic therapy.

Two previous studies have analysed the relationship between bacterial environment and disease development in IgAN mouse models. In both instances, breeding the mice under germ-free conditions prevented the disease. However, this axenic approach was also associated with an impairment of immune system maturation and, notably, an important decrease in circulating levels of immunoglobulins that are found deposited in the glomeruli of the respective models (mouse IgA in the BAFF model (27) and hIgA1 the α 1KI model (28)). By contrast, here, mice given antibiotics after weaning displayed hIgA1 serum levels similar to their vehicle-treated littermates, and there was no difference between groups in circulating IgG levels, suggesting that systemic immune system maturation was not impaired by microbiota depletion. Moreover, although microbiota depletion altered GALT architecture, it did not affect hIgA1 producing cells.

Our treatment protocols involved antibiotic administration either twice per-week by gavage or continuously in drinking water, with high-doses of broad-spectrum antibiotics. The antibiotic treatments included compounds that are absorbed (amoxicillin and metronidazole) or poorly absorbed (vancomycin and aminoglycosides) from the gut. The systemically absorbed antibiotics would have also affected the extra-digestive commensal microbiota (e. g. lung), although in the individual antibiotic experiment it was an aminoglycoside with poor oral bioavailability that showed the strongest effect.. The composition of salivary microbiota from European IgAN patients was shown to differ from that of healthy controls (34), although no significant difference was found between the tonsillar microbiota of Asian adult with IgAN patients and those with recurrent tonsillitis (35). Due to the invasive nature of sample collection for the Asian tonsillar study, a healthy adult control group was not possible, making the results ambiguous, as the possibility of a tonsillar dysbiosis common to patients with IgAN and recurrent tonsillitis is not excluded. Interestingly, in our study, faecal bacterial load correlated

with proteinuria, supporting the role of bacterial stimulation of mucosal immunity in IgAN pathogenesis, and consistent with the clinical observation of IgAN exacerbated by ear and throat infections. Moreover, final faecal bacterial load correlated with hIgA1-mIgG complex serum levels, but not with hIgA1 or mIgG levels. This demonstrates that: 1) the disease phenotype of α 1KI-CD89Tg mice depends on the formation of IgA1-IgG complexes, a key pathophysiological feature of human IgAN and 2) the formation of these complexes is promoted by the presence of intestinal bacteria, independently of the circulating levels of any of their components.

The variable effects of individual antibiotics on disease phenotype is likely due to differences in baseline microbiota structure of the mice, and variations in drug-susceptibility between different species and strains. This indicates that some specific species could play a more important role than others. Apart from a broad-spectrum cocktail of four antibiotics, an aminoglycoside geneticin had the most consistent beneficial effects.

Given that circulating levels of hIgA1 and the number of hIgA1 positive B-cells in the lamina propria did not differ between groups, the protective effect of microbiota depletion may be mediated through a reduction in the production of anti-Tn antigen specific IgG, an effect on IgA glycosylation or by reducing an effect of bacterial antigens on immune complex formation. Deposition of streptococcal M protein in the glomeruli of IgAN patients has been described (36); together with our results this suggests that bacterial antigens might promote the formation of these nephrotoxic immune complexes.

In conclusion, the data presented here strongly support a causative role of commensal bacteria in the pathophysiology of IgAN and suggest that the microbiota is a candidate therapeutic target.

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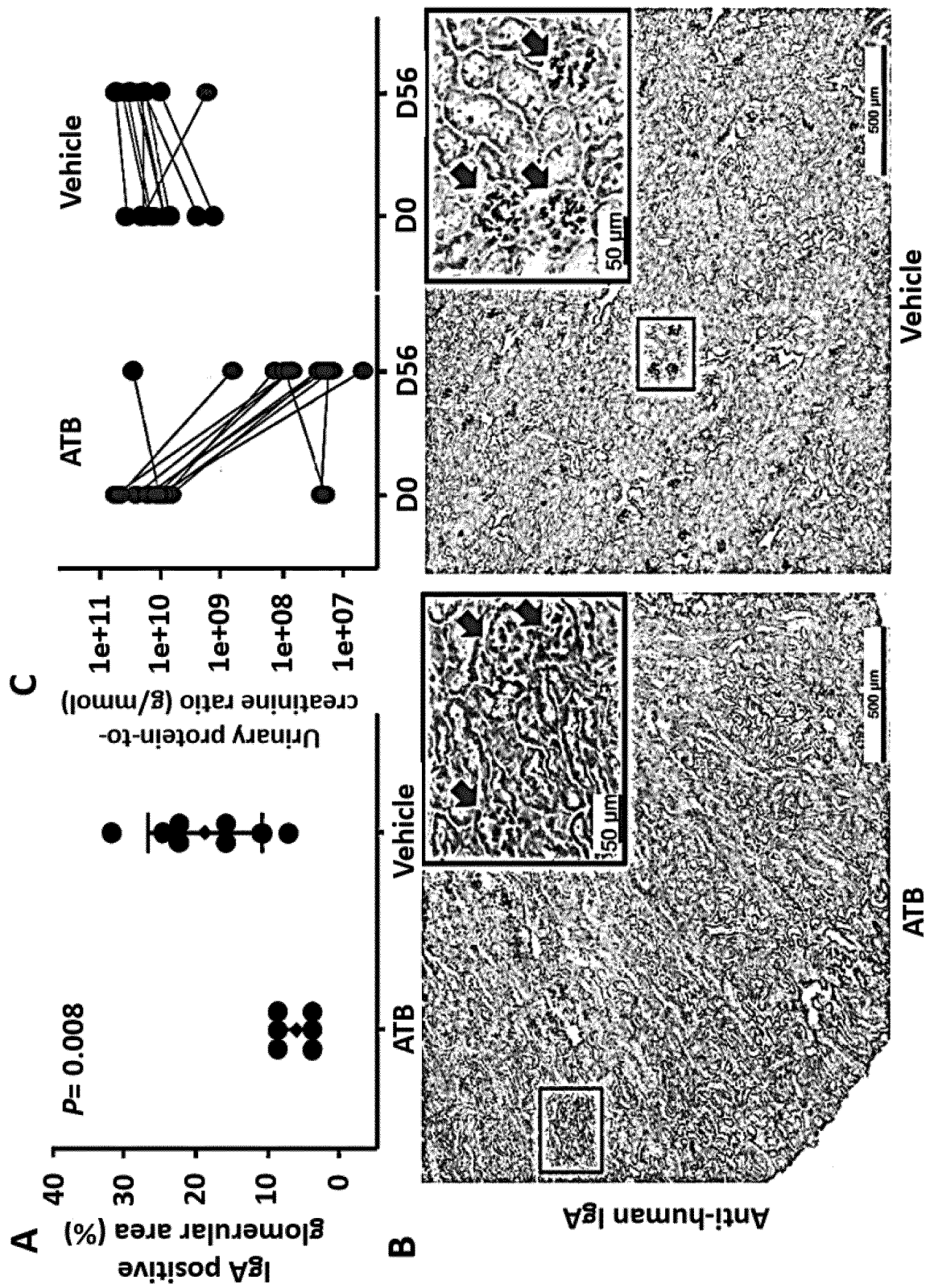
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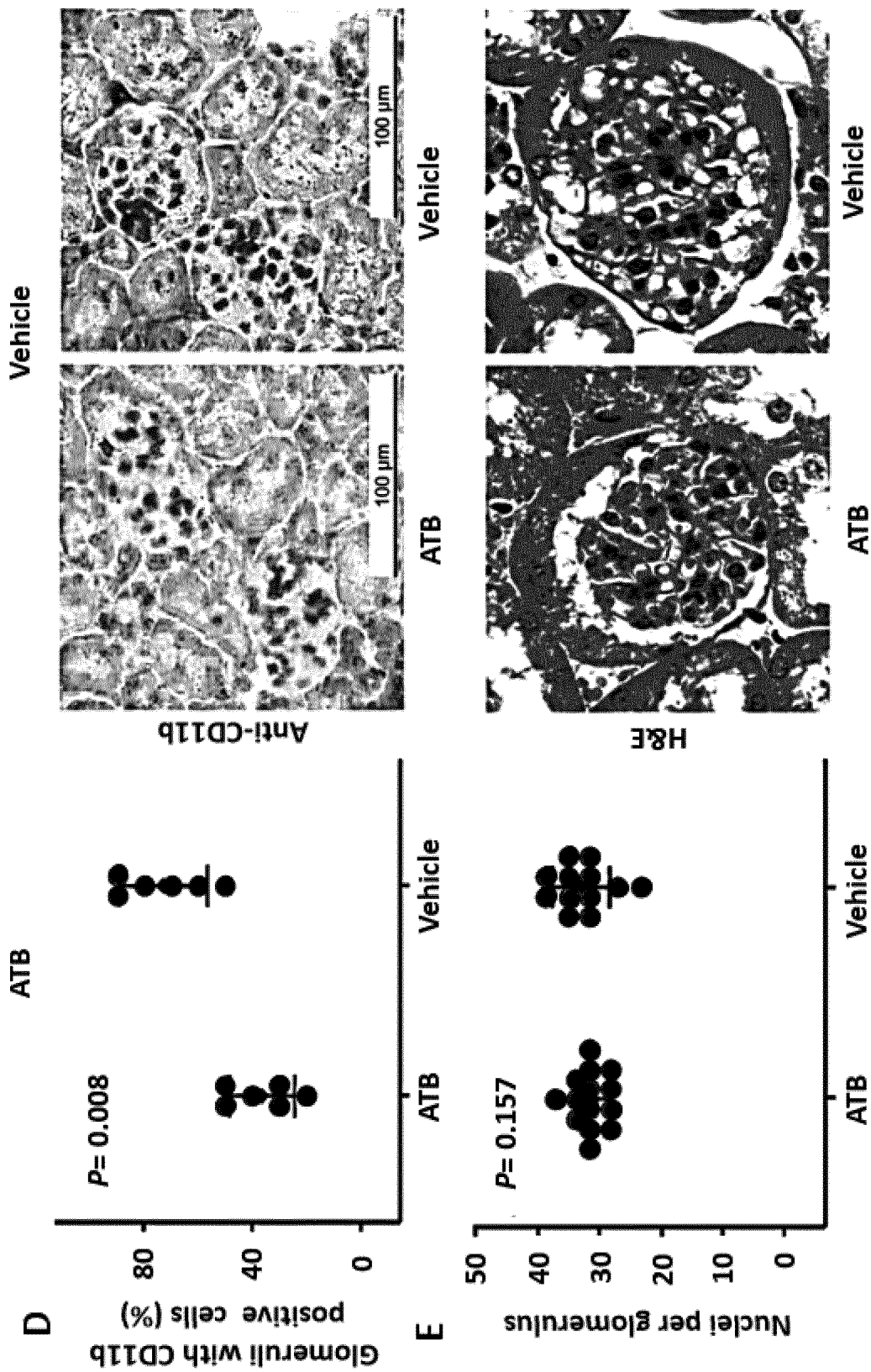
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CLAIMS:

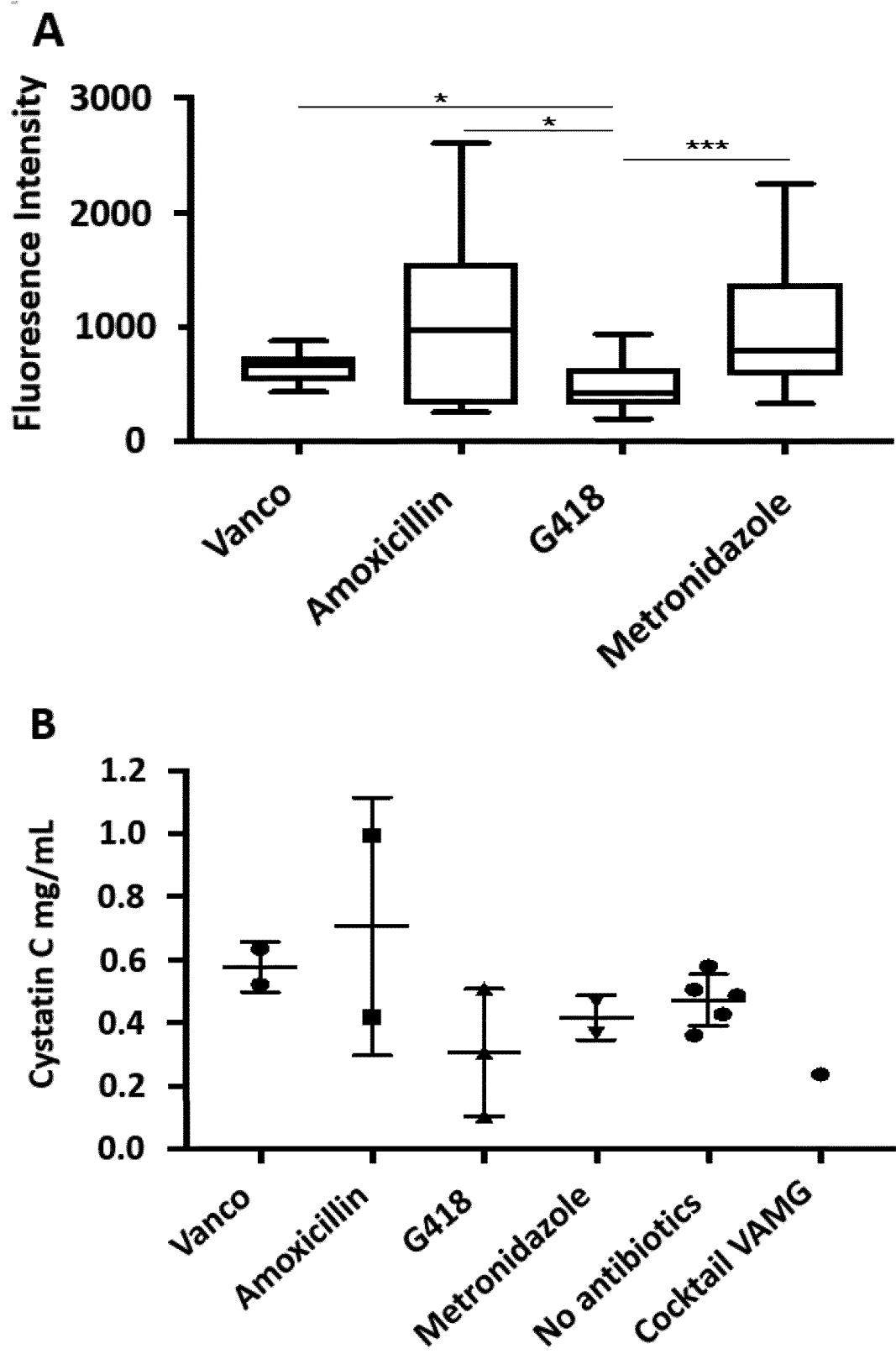
1. A method of treating immunoglobulin A nephropathy (IgAN) in a patient in need thereof comprising administering to the patient a therapeutically effective amount of at least one antibiotic.
- 5 2. The method of claim 1 wherein the antibiotic is selected from the group consisting of nitroimidazoles, glycopeptide antibiotics, aminoglycosides, beta lactams, quinolones or fluoroquinolones, macrolides, sulfonamides, sulfamethaxozoles, tetracyclines, streptogramins, oxazolidinones, rifamycins, glycopeptides, polymixins, lipo-peptide antibiotics.
- 10 3. The method of claim 1 wherein the antibiotic is administered to the patient via oral or rectal route.
4. The method of claim 1, which further comprises a step consisting of performing a fecal microbiota transplantation.



Figures 1A-C



Figures 1D-E



Figures 2A-2B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/070098

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K38/14 A61K38/12 A61K31/65 A61K31/18 A61K31/395
A61P13/12 A61K31/4164 A61K31/43 A61K31/7036

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61P A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, COMPENDEX, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/167327 A1 (CRESSET BIOMOLECULAR DISCOVERY LTD [GB]) 16 October 2014 (2014-10-16)	1-3
Y	page 2, line 24 - page 3, line 11; claims 1,2 page 5, line 9 - page 6, line 5 page 7, line 5 - line 14 ----- -/--	4



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

29 October 2019

Date of mailing of the international search report

13/11/2019

Name and mailing address of the ISA/

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Authorized officer

Bochelen, Damien

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/070098

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LAURIERO GABRIELLA ET AL: "PUTATIVE ROLE OF MICROBIOTA IN THE PROGRESSION OF IGA NEPHROPATHY", NEPHROLOGY DIALYSIS TRANSPLANTATION, OXFORD UNIVERSITY PRESS, GB, vol. 31, no. Suppl. 1, 30 April 2016 (2016-04-30), pages 51-i51, XP009510336, ISSN: 0931-0509, DOI: 10.1093/NDT/GFW139.03 abstract -----	1-4
Y	ROLLINO CRISTIANA ET AL: "IgA nephropathy and infections", JN JOURNAL OF NEPHROLOGY, vol. 29, no. 4, August 2016 (2016-08), pages 463-468, XP009510330, page 464 - page 465 -----	1-4
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2019/070098

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