

Serological IgG Testing to Diagnose Alimentary Induced Diseases and Monitoring Efficacy of an Individual Defined Diet in Dogs

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I. INTRODUCTION

Abstract—Background. Food-related allergies and intolerances are frequently occurring in dogs. Diagnosis and monitoring according ‘Golden Standard’ of elimination efficiency is, however, time consuming, expensive, and requires expert clinical setting. In order to facilitate rapid and robust, quantitative testing of intolerance, and determining the individual offending foods, a serological test is implicated for Alimentary Induced Diseases and manifestations. **Method.** As we developed Medisynx IgG Human Screening Test ELISA before and the dog’ immune system is most similar to humans, we were able to develop Medisynx IgG Dog Screening Test ELISA as well. In this randomized, double-blind, split-sample, retro perspective study 47 dogs suffering from Canine Atopic Dermatitis (CAD) and several secondary induced reactions were included to participate in serological Medisynx IgG Dog Screening Test ELISA (within $< 0,02$ % SD). Results were expressed as titers relative to the standard OD readings to diagnose alimentary induced diseases and monitoring efficacy of an individual eliminating diet in dogs. Split sample analysis was performed by independently sending 2 times 3 ml serum under two unique codes. **Results.** The veterinarian monitored these dogs to check dog’ results at least at 3, 7, 21, 49, 70 days and after period of 6 and 12 months on an individual negative diet and a positive challenge (retrospectively) at 6 months. Data of each dog were recorded in a screening form and reported that a complete recovery of all clinical manifestations was observed at or less than 70 days (between 50 and 70 days) in the majority of dogs (44 out of 47 dogs =93.6%). **Conclusion.** Challenge results showed a significant result of 100% in specificity as well as 100% positive predicted value. On the other hand, sensitivity was 95,7% and negative predictive value was 95,7%. In conclusion, an individual diet based on IgG ELISA in dogs provides a significant improvement of atopic dermatitis and pruritus including all other non-specific defined allergic skin reactions as erythema, itching, biting and gnawing at toes, as well as to several secondary manifestations like chronic diarrhoea, chronic constipation, otitis media, obesity, laziness or inactive behaviour, pain and muscular stiffness causing a movement disorders, excessive lacrimation, hyper behaviour, nervous behaviour and not possible to stay alone at home, anxiety, biting and aggressive behaviour and disobedience behaviour. Furthermore, we conclude that a relatively more severe systemic candidiasis, as shown by relatively higher titer (class 3 and 4 IgG reactions to *Candida albicans*), influence the duration of recovery from clinical manifestations in affected dogs. These findings are consistent with our preliminary human clinical studies.

Keywords—Allergy, canine atopic dermatitis (CAD), food allergens, IgG-ELISA, food-incompatibility.

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FOOD-RELATED allergies and intolerances are frequently occurring in dogs and despite contradiction in certain breeds tend to be more susceptible than others.[1] In addition, a minority of dogs are suffering from typical IgE-mediated food allergy directed against dietary components. The gold standard of diagnosis of food intolerance is based on elimination and subsequent challenge to determine the offending foods and determine the relative threshold. [2] From this, the individually optimized food can be derived. Diagnosis and monitoring of elimination efficiency is, however, time consuming, expensive, and requires expert clinical setting. In order to facilitate rapid and robust, quantitative testing of intolerance, and determining the individual offending foods, a serological test is implicated. Similar to the situation in human food intolerance, IgG testing is widely used.[3]

Hypersensitivity reactions to foods can be classified as IgE-mediated, non IgE-mediated or a mixture of both. [4], [5] The majority of such food hypersensitivities are associated with non IgE-mediated reactions. Increased allergen uptake by a disturbed epithelium due to gut dysbiosis, mucus damage, decreased epithelial integrity (due to loss of tight junctions) can be the result of food components in combination with pathogens or altered stress levels. [6], [7] Local reactions in the gut epithelium and lamina propria are linked to IgE formation and subsequent histamine release, and are widely considered to be based on a disturbed T-cell equilibrium between regulatory T-cell induced tolerance (due to the cytokines IL-10 and TGF- β) and Th2 induced activation (due to the cytokines IL4, IL-5 and IL-13), resulting in clinical manifestations of allergy. [8] When the immune activation and its associated Th2 cells and cytokines, and food component-specific B-cells reach the draining mesenteric lymph node, the reaction can spread systemically to other preferable mucosal tissues, including airways and skin. The altered mucosal and skin tissues based on T-cells and cytokines can subsequently induce local production of different IgG subclasses thereby contributing to the development of systemic intolerances. [9] Especially slower onset food reactions may involve type III immune complex reactions. For this IgG antibodies are required, but this is difficult as IgG anti-food antibodies are not uncommon in healthy individuals. [10]

Currently, a number of clinical laboratories have set up ELISA/EIA (Enzyme Immunoassays) panels to test the presence of IgG antibodies in patients to numerous food allergens. This is based on the findings that certain subclasses

of IgG have been associated with the *in vitro* degranulation of basophils and mast cells, the activation of the complement cascade (both of which are important mechanisms in allergy and anaphylaxis), and the observation that high circulating serum concentrations of some IgG subtypes have been measured in certain atopic individuals. [5], [11] The premise behind this testing is that high circulating levels of IgG antibodies are correlated with clinical food allergy signs and symptoms. These tests would help the physician pinpoint food allergies in their patients so that patients might avoid these foods and their associated signs and symptoms. [12]

Canine atopic dermatitis (CAD) is a common cause of hypersensitive skin diseases in dogs, affecting 10-15% of dogs. [13], [14] Canine Atopic dermatitis is an IgE-mediated genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features, like chronicity, pruritus, typical lesion distribution and a positive familial history. The involved allergens are dust mites, pollens, mould spores, animal dander, insects, and other miscellaneous allergens. [11] The exact pathogenesis of CAD is not yet completely established, but it is thought to involve immunoglobulin (Ig)E-mediated immediate and late-phase hypersensitivity reactions to environmental allergens. Diagnosis is only based on clinical evaluation: the skin in affected body sites is typically erythematous. With chronicity, widespread erythema, lichenification, and hyperpigmentation are noted. Excoriations are notable in some dogs. Alopecia in CAD is the result of pruritic behaviour of licking, biting, scratching, chewing, rubbing, etc. Light coated breeds may also have salivary staining at body sites that are licked and chewed. Some dogs will not have any lesions, and pruritus is the only problem. The distribution of lesions and pruritus in CAD is characteristic. Lesions will be present on the face, ears, paws, limbs, and ventral aspects of the body. The perineum is a commonly affected site. [1], [15]-[19] The gold standard for the diagnosis of such a condition is the restricted dietary trial and the subsequent provocation challenge. Some attempts have been made to develop serological tests but none of these tests accurately predicted canine food sensitivity. [20] The aim of the present study was to determine the incidence of food hypersensitivity dermatitis and to evaluate a newly developed serological test for the diagnosis of food allergy in dogs.

II. MATERIALS AND METHODS

A. Patients

Included in the randomized, double-blind, split-sample, retro perspective study were dogs suffering from atopic dermatitis and/or pruritus and/or secondary manifestations including all other nonspecific defined allergy skin reactions shown as erythema, itching, licking, biting and gnawing at feet or toes, as well as presents of manifestations like GI-tact symptoms as gagging, vomiting, chronic diarrhoea or chronic constipation, otitis media, obesity, laziness or inactive behaviour, pain and muscular stiffness causing movement disorder, excessive lacrimation, hyper or hypo / absence

behaviour, nervous behaviour and not possible to stay alone at home, anxiety, biting or aggressive behaviour and disobedience behaviour, focusing on the last 3 years. The independent veterinarian included 47 dogs with main manifestation CAD and possible other secondary manifestations.

From the dogs serum samples were obtained by vena puncture of 6 ml peripheral blood using EDTA Vacutainer tubes (BD Biosciences). Serum samples (3 ml) were obtained after centrifugation and stored at -20°C until use.

Inclusion criteria were: dogs suffering from main manifestation atopic dermatitis; and possible other secondary manifestations as pruritus and all other nonspecific defined allergy skin reactions shown as erythema; itching, licking, biting and gnawing at feet or toes; as well as presents of manifestations / features like GI-tact symptoms as gagging, vomiting, chronic diarrhoea or chronic constipation, Otitis Media, obesity, laziness or inactive behaviour, pain and muscular stiffness causing movement disorder, excessive lacrimation, hyper or hypo / absence behaviour, nervous behaviour and not possible to stay alone at home, anxiety, biting or aggressive behaviour and disobedience behaviour, focusing on the last 3 years; free of parasites (fleas, ticks, amoebas); willing to place dog to an individual negative diet and willing to carry out a final positive challenge at 6 months. Exclusion criteria were dogs aged < 7 months and > 13 years, and those showing kidney or liver value deviations upon routine serological testing. The veterinarian van Tilburg monitored these dogs and to check dog's results at least at: 3, 7, 21, 49, 70 days and after period of 6 months and 12 months on an individual negative diet and was asked to record this data of each dog in a screening form.

B. Food IgG Dog Screening ELISA

The Medisynx IgG Dog Screening Test ELISA was used (www.medisynx.com). Medisynx IgG Dog Screening Test ELISA was carried out in split-sample analysis by sending in two separate blood-samples under two unique codes of the same dog, to under determine the reproducibility of this assay. Microtiter plates were coated with 42 individual food antigens in duplo in addition to 2 times 6 reference food allergen substances. The purified samples were prepared from biologically grown food products supplied by selected long-standing vendors and heated up to 70°C shortly and rapidly cooled down in a sterile bottle. Thereby, these preparations are free from pesticides, antimicrobials and contaminations. Diluted dog serum or prepared control standards were added and incubated for 48 hrs at 4°C. After washing and blocking with irrelevant protein, a polyclonal goat anti-dog-IgG peroxidase-conjugate will be added and incubated during 60 minutes at 37°C. After repeated washing a substrate solution of tetramethylbenzidine (TMB) was added and incubated during 20 minutes at room temperature. This colouring will be stopped by adding sulphuric acid. The resulting colour change into yellow will be measured as optical density in a spectrophotometer at 450 nm.

The standard concentrations ranged from 0.25 (class 1), 0.40 (class 2), 3.50 (class 3), 12.50 (class 4), 100.00 (class 5) µg/ml (U/ml). Constant and conditioned circumstances are crucial to obtain precise outcomes within <0,02 % SD. Results were expressed as titers relative to the standard OD readings.

As we developed and studied Medisynx Food IgG Human Screening Test ELISA before and the dog's immune system is the most similar to humans [25], we were able to develop Medisynx Food IgG Dog Screening Test ELISA as well.

C. *Candida* IgG ELISA

Similar to the food-specific IgG ELISA, also a *Candida*-specific IgG ELISA was developed and used for testing of dog serum samples.[21] The same titer determination relative to known *Candida* standard samples was performed.

III. RESULTS

A. Food Incompatibility and Individual

1) Defined Diet

The consulting veterinarian had included 47 dogs in the study based on the inclusion criteria and IgG ELISA tests were performed on 42 food substances in the period March 2012 to January 2013.

TABLE I
FREQUENCY DISTRIBUTION OF POSITIVE IGG TESTS ON MAXIMUM 42 FOODS OF DOGS INCLUDED IN THE STUDY (N=47)

# pos IgG	Class 0+1	Class 2+3+4	Total
0-7	6	2	8
8-14	7	8	15
15-21	9	11	20
22-28	10	8	18
29-35	10	9	19
36-42	0	5	5
	42	42	

#pos IgG refers to the number of IgG test positive for the 42 tested food components. For definition of titer classes refer to Table II.

The frequency distribution resulted in a normal distribution with few dogs showing positive IgG titers to 1-7 foods depending on the titer classes in which the responses were segregated (n=6 vs n=2). Also few dogs showed IgG titers to nearly all 42 foods (36-42 foods; n=5). Most dogs showing positive IgG tests responded to 8 up to 35 foods (n=36 vs n=36 for the two titer groups). The IgG test thus segregates broad weak and broad high responder dogs and even identified the few dogs that responded highly to only very few foods (n=6) from dogs that responded strongly to a broad array of food components (n=5).

Subsequently, the veterinarian monitored these dogs and their course of recovery during a period of two times 6 months, in which the dogs received an individual diet based on only negative reacting foods in the IgG ELISA, and executing an individually defined diet based on the positive reacting foods in the IgG ELISA, at 3, 7, 21, 49, 70 days and after a period of 6 months and 12 months on the individual diet.

The majority of dogs (34 out of 47 dogs = 72,3%), showed an improvement of at least one of the clinical manifestations

of food incompatibility within 3 days after starting their individual defined diet. All dogs showed an improvement of at least one manifestation by 7 days of diet. The majority of dogs (36 of 47 dogs, 76,6%) showed at least a decrease in two or more clinical manifestations within 21 days.

Within 7 weeks of individual defined diet, 18 out of 47 dogs (38.3%) had three or more negative reactions of their initial clinical manifestations. On the other hand, 29 out of 47 dogs (61.7%) had 3 or more negative reactions of their manifestations after 50 days but within 70 days of their diet, shown in Table I.

2) *Candida*-Specific IgG Titers

In all serum samples also the titer of *Candida albicans* specific IgG was determined. The results were expressed as negative results in the food-specific IgG ELISA graded to the positivity in the *Candida* IgG ELISA for serum samples selected for days on the individual defined diet without clinical manifestations of atopic dermatitis.

TABLE II
NEGATIVE REACTION(S) TO IN NUMBER OF DAYS
SERUM CANDIDA-SPECIFIC IGG TITER CLASSES OF N=47 DOGS IN THE STUDY

Class 0 = < 0,25 U/ml (µg/ml)	no Systemic Candidiasis found in 11 dogs
Class 1 = 0,25 – 0,40 U/ml (µg/ml)	low grade Systemic Candidiasis found in 1 dog
Class 2 = 0,40 - 3,50 U/ml (µg/ml)	Systemic Candidiasis determined in 8 dogs
Class 3 = 3,50 – 12,50 U/ml (µg/ml)	Systemic Candidiasis determined in 3 dogs
Class 4 = 12,50 – 100,00 U/ml (µg/ml)	Systemic Candidiasis determined in 24 dogs

Almost manifestation-free stands for a dog that is free of at least 90% of all clinical manifestations listed in the inclusion criteria.

The percentage of dogs in this study in which *Candida albicans* - specific IgG titers were found are expressed in Fig. 1. The titers were expressed in classes as described in Table II.

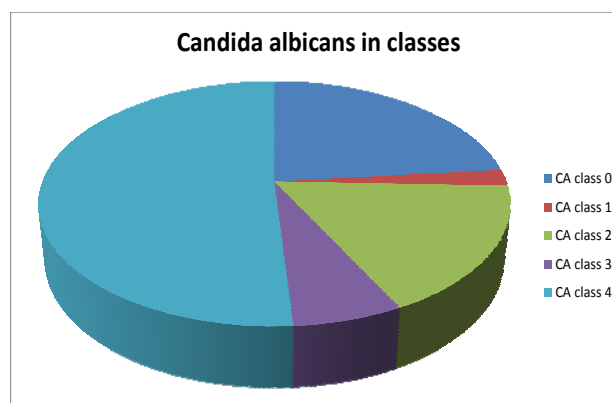


Fig. 1 Graphic representation of classes to *Candida albicans* of test results in percentages at start study

The relation between improved clinical manifestations of food allergy and food intolerance when being put on an optimized individual defined diet based on individual positivity in the food-specific IgG ELISA, for an extensive

period of time, relative to positivity in the *Candida albicans* specific IgG ELISA is also presented in Fig. 2.

Negative clinical reactions (improvement of manifestations) in accordance to a positive titer for *Candida albicans* specific IgG for dogs on negative individually determined diet based on positivity for food specific IgG, during first 6 months, followed up by a positive challenge and continuing on negative diet for another 6 months.

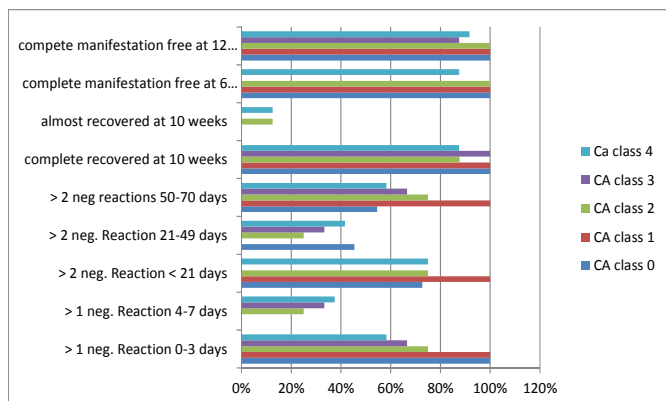


Fig. 2 Clinical manifestations in dogs after receiving an optimized individual defined diet according to negativity in IgG test

A complete recovery of all clinical manifestations was observed at or less than 70 days (between 50 and 70 days) in the majority of dogs, as 44 of 47 dogs (93.6%) showed no more manifestations by 70 days of diet. The remaining 3 dogs were almost manifestation-free by 70 days, probably due to the fact that their diet still consisted some 'forbidden' class 3 and class 4 food substances as these individual dogs had to be placed on a well-balanced diet, to prevent deficiencies. I.e. two dogs showed class 2, 3 and 4 results to all kinds of meat, but they could not be placed on an only fish-diet as that will possible lead to deficiencies.

Re-analysis after 6 months of diet showed for class 0 and class 1 and class 2 *Candida albicans* results a significant remaining recovery in these dogs, whereas dogs with a class 3 demonstrated that 2 of 3 dogs and in class 4 21 of 24 dogs to *Candida albicans* exhibit a remaining manifestation free result.

Re-analysis after 12 months showed that all dogs in all classes remained without clinical manifestations, except for 3 dogs, 1 dog of class 3 *Candida albicans* and 2 dogs of class 4 *Candida albicans* they were almost at manifestations free result as their diet needs to contain some forbidden food substances in rotation.

The veterinarian reported that some dog-owners noticed immediate reactions to their dog in case of a food mistake, by taking in a *not-permitted food substance*, during first 6 months. This unforeseen action can be regarded as a positive re-challenge to food reactions.

All dogs without systemic candidiasis and a titer class 0 to *Candida albicans*, reduced at least one of their manifestations in 0-3 days and two or more improvements within 21 days of diet. Whereas dogs with systemic candidiasis, dogs with a titer

class 3 and class 4 to *Candida albicans* showed only 14 of 24 dogs improvement of one or more manifestations in 4-7 days. This indicated that a relatively more severe systemic candidiasis, class 3 or 4 to *Candida albicans*, is of main influence to delay recovery in dogs.

IV. CHALLENGE RESULTS

During 6 months all dogs were placed on their individual diet based on *only* negative results in the food-specific IgG titers, and this 6 months period was selected to reset immunological reactions by feed-back and to re-establish homeostasis in affected dogs. At 6 months dog-owners carried out a positive food challenge, by taking in a diet consisting of *all forbidden*, class 3 and class 4 food substances until positive reactions appeared and they could stop challenge and returned to their diet based on negative results again (Table III)

The veterinarian reported that within 3 days all dogs to *Candida albicans* class 0, class 1, class 2 and class 3 suffered from diarrhoea and onset of erythema, whereas all dogs in class 4, showed next to diarrhoea and onset of erythema even more severe manifestations as urticaria, itching, biting and gnawing at their feet and toes, nervous, hyper and irritated behaviour within 3 days. Two dogs did not participate at positive challenge as they were not complete manifestation free at 6 months.

TABLE III
 RESULTS OF CHALLENGE TESTS

Reactions Dog	Pos. diet	Neg. diet	Total
Pos. Reactions	45	2	47
Neg. Reactions	0	45	45
Total	45	47	92

Results challenge and predictable values

Sensitivity: $a/a+b = 45/47 = 95,7\%$
 Specificity: $c/c+d = 45/45 = 100\%$
 pos. predictable value: $a/a+c = 45/45 = 100\%$
 neg. predictable value: $d/b+d = 45/47 = 95,7\%$

A positive diet refers to a diet with non-permitted and forbidden food substances according the food allergen specific IgG ELISA test result of the individual dog.

A negative diet refers to a diet with only permitted food substances according to the individual IgG ELSIA test result of the individual dog.

A positive reaction implies that one or more clinical manifestation occurred after taking in a negative or positive food product and exacerbation of manifestation(s) was observed and reported by the veterinarian.

A negative reaction implies that one or more manifestations were reduced or diminished after taking in negative or positive food product and this result was observed and reported by the veterinarian.

These challenge results showed a significant result of 100% in specificity as well as 100% positive predicted value. On the other hand, sensitivity was 95,7% and negative predictive value was 95,7%.

V. DISCUSSION

Food-related allergy or intolerance is a common differential diagnosis for pruritic dogs. The only way to diagnose canine atopic dermatitis is an elimination diet of 6-8 weeks with a protein and a carbohydrate source not previously fed. In humans, patch testing has been shown to be a useful tool to diagnose food allergies. In veterinary medicine, serum food allergen-specific antibody testing is widely offered to identify suitable ingredients for such diets. [2] A positive reaction of a dog on these tests was shown not to be very helpful, but a negative result indicated that this food was tolerated well. It was therefore concluded that serum IgG testing could be helpful in choosing ingredients for an elimination diet in a dog with suspected adverse food reaction. Such results, however, are contradicted by others who showed no significant difference in pre- and post-diet levels for any neither of the individual allergens nor for the total IgE and IgG concentrations of all antigens after an elimination diet of 6-8 weeks. [2], [18]

In this study, the results of an elimination/challenge test were used as indicators of true food reactions in order to calculate the sensitivity, specificity, and positive predictive value (PPV) of each test. Sensitivity, however, is generally lower than specificity, likely because different mechanisms can underlie food sensitivities. A study using a number of tests showed that some tests may be useful in identifying foods to which an individual is reactive; however, no one test was likely to identify all reactive foods. [4]

It is widely accepted that there is no credible evidence that measuring IgG antibodies is useful for diagnosing food allergy or intolerance, nor that IgG antibodies cause symptoms. In fact, IgG antibodies reflect exposure to allergen but not the presence of disease. A number of studies, however, indicate that IgG testing can indeed be helpful in diagnosing food related atopic diseases. [2], [3], [10], [12], [18] Increase in IgG to a certain food is considered to indicate an intolerance to that food. Similar to humans, also in dogs IgG antibodies against food allergens were found in sera of 62% healthy persons with levels higher than 10.0 µg/ml. [10], [11], [22] In a small proportion of individuals, high serum levels of IgE against the same food allergens were found. Common occurrence of IgG against food allergens in healthy individuals (without any symptoms of allergy or food intolerance) argues against the possible participation of these antibodies in the pathogenesis of food allergy. [10] Before engaging in IgG testing of food antigens it is important to realize that many food products, including those for testing contain micro-organisms and many individuals containing IgG antibodies specific for microorganisms. For this reason, it is essential to control for sterility and reliability of the food preparations for testing. [23], [24] This study adds to the increasing relevance of the use of food-specific IgG testing for canine atopic dermatitis diagnosis and monitoring of efficacy of treatment, e.g. clinical improvement based on optimized diets.

An unbalanced immune system, as in the case of food allergy or food intolerance, is most often reflected in gut dysbiosis and can result in candida overgrowth. [21] In

addition, a diet rich in sugars or processed carbohydrates can contribute further to candidiasis, itchy skin, leaky gut, and irritable bowel syndrome-like symptoms. [6], [20] Dietary treatments with reduced sugar contents and anti-fungal medication (e.g. antimycotica Nystatin or Miconazole) can help to restore gut integrity and relieve the symptoms of food intolerance or allergy.

A randomized, double-blind, split-sample and retro perspective study with a long term follow up will be performed as a follow up to the current study in dogs and humans. The goal of this study is to obtain a high quality data set to compare different study designs and their outcome in relation to sensitivity, specificity and negative and positive predictable value rates.

In conclusion, an individual defined diet based on IgG ELISA in dogs provides a significant result to atopic dermatitis and pruritus including all other non-specific defined allergy skin reactions shown as erythema, itching, biting and gnawing at toes, as well as to several secondary manifestations like chronic diarrhoea, chronic constipation, otitis media, obesity, laziness or inactive behaviour, pain and muscular stiffness causing a movement disorders, excessive lacrimation, hyper behaviour, nervous behaviour and not possible to stay alone at home, anxiety, biting and aggressive behaviour and disobedience behaviour. Furthermore, we conclude that a relatively more severe systemic candidiasis, as shown by relatively higher titer (class 3 and 4 IgG reactions to *Candida albicans*), influence the duration of recovery from clinical manifestations in affected dogs. These figures are in correspondence to our preliminary human clinical studies.

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