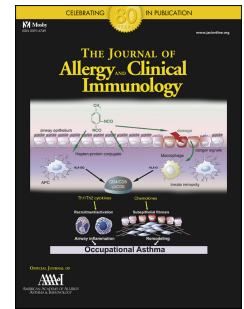


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John W. Steinke, Ph.D., Shawna L. Pochan, C.N.M., M.P.H., Hayley R. James, B.S., Thomas A.E. Platts-Mills, M.D., Ph.D, F.R.S, Scott P. Commins, M.D., Ph.D.



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Altered metabolic profile in patients with IgE to galactose-alpha-1,3-galactose following *in vivo*
food challenge

John W Steinke, Ph.D.^{1, 2}

Shawna L Pochan, C.N.M., M.P.H.¹

Hayley R James, B.S.¹

Thomas A.E. Platts-Mills, M.D., Ph.D, F.R.S.^{1, 2}

Scott P Commins, M.D., Ph.D.^{3**}

¹Department of Medicine and ²Carter Immunology Center
University of Virginia Health System, Charlottesville, VA 22908 USA; and ³Department of
Medicine, University of North Carolina School of Medicine, Chapel Hill, NC 27516 USA

Running Title: Metabolic profile after food challenge of meat allergic patients

**To whom correspondence should be addressed at: Thurston Arthritis Research Center,
3300 Thurston Bldg., CB# 7280, Chapel Hill, NC 27599-7280 United States of America; 1-
866-827-2862 (ph); (919) 966-1739 (fax); email: scommins@email.unc.edu

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AI-20565 (to T.A.E.P.-M.), and R21 AI087985 (to S.P.C. and T.A.E.P.-M.).

24 **Abbreviations**

25	Alpha-gal	galactose-alpha-1,3-galactose
26	GC/MS	gas chromatography/mass spectrometry
27	IDO	indole 2,3-dioxygenase
28	IFN	interferon
29	TNF	tumor necrosis factor

30

31 **Keywords**

32 Red meat allergy, alpha-gal, metabolomics, food challenge, biomarker, lipid and fatty acid
33 metabolism

34

35 Capsule summary: Determination of metabolic changes that occur following the development of
36 and after red meat challenge in alpha-gal allergic individuals will aid in biomarkers for patient
37 identification and targeted therapeutic development.

To the editor,
Over the past few years, a large number of patients have been identified who experience delayed anaphylaxis or urticaria following eating red meat and who have IgE antibodies to the oligosaccharide galactose alpha-1,3-galactose (alpha-gal)¹⁻³. Patients with this alpha-gal syndrome (AS) had tolerated mammalian products prior to onset of the disease and in many cases recall tick bites prior to sensitization^{2,4}. Many anecdotal reports suggest that glycolipids may be as important as glycoproteins in the clinical episodes. Furthermore, delayed absorption and processing of glycolipids could provide a rational explanation for the delay in onset of symptoms.

To investigate the cause of the delay, we carried out metabolomic analysis, the study of the unique chemical fingerprints of cellular process, of 449 biochemicals, which are the chemical compounds that make up a living cell, on blood samples obtained before and after challenge with pork in 8 allergic subjects and 7 controls⁵. We report the results of the analysis at baseline as well as at 2, 4, and 6 hours after challenge (see Table E1 for patient characteristics and online supplement for methods). There were no differences in the ages of the groups, however a significant difference was noted in total IgE (meat allergic 179±30 vs control 63.3±29.8: $p<0.02$). When individual components of IgE were measured, only the meat allergic group had measureable levels of IgE to alpha-gal, beef, pork and cow's milk.

Serum was collected from subjects who underwent meat challenge at 0, 2, 4 and 6 hr time points and metabolomic analysis of 449 biochemicals was performed. Table E2 shows the number of biochemicals significantly different ($p<0.05$) or those approaching significance ($0.05<p<0.10$) after correction for multiple comparisons using False Discovery Rate in the control and meat allergic subjects. The overall changes were greater in the control group with 84

and 108 significant changes at the 4 and 6 hr time points compared to 63 and 89 changes in the meat allergic group. Comparison of the groups at baseline revealed 23 biochemicals that were significantly different. Further biochemical differences were observed between groups during the challenge with 21, 16 and 25 differences detected at the 2, 4 and 6 hr time points, respectively.

To identify biomarkers related to AS meat allergy, a random forest analysis was performed. A predictive accuracy of 56.3% at baseline that rose to 62.5% by 6 hours was observed (Figure E1A). These values are greater than random chance (50%) suggesting they may be useful in stratifying patient groups. Figure E1B lists the top 30 metabolites based on their importance in separating these populations at 6 hours. Principle component analysis (Figure E2) revealed considerable overlap between groups and time points, suggesting minor metabolic differences in amino acids, lipids, glucose metabolism and bile acids may contribute to the meat allergic phenotype. Some components used for analysis were not statistically significant, but reflect consistent changes in pathways. To test for interaction, two-way ANOVA with repeated measures was performed (Table E3) and 20 biochemicals (Table E4) were significantly different ($p < 0.05$) for meat allergic status and 32 biochemicals (Table E5) for meat allergic status and time post-feeding.

We posited that basal differences in metabolism could be detected between meat allergic and healthy control groups. Of the 449 metabolites analyzed, 20 were significantly different between the groups. Figure 1A illustrates the distribution of metabolites in the biochemical pathways with the biggest differences observed in amino acid/peptide and lipid metabolism. Two examples for each of the amino acid/peptide (Fig 1B) and lipid (Fig 1C) pathways are shown depicting the differences in metabolite levels with increases found in the meat allergic

group. Two-way ANOVA identified potential interactions between variables. Of the 449 metabolites, 32 showed significant interaction between meat allergy status and time. Figure 2A illustrates the distribution of metabolites, again with the biggest differences being observed in amino acid/peptide and lipid metabolism pathways. In Figures 2B and 2C, the metabolite levels for two compounds are presented for the time course of the food challenge in healthy control and meat allergic subjects.

The most dysregulated pathways at baseline and following meat challenge were in lipid and fatty acid metabolism (Figures 1 and 2). Multiple fatty acids including caprylate and stearate accumulated in control serum, but not meat allergic, following meat consumption and were over represented by 6 hours. These trends could signal alterations in fat hydrolysis, absorption and/or lipid oxidation that lead to the delay in symptoms observed in the meat allergic group. Carnitine conjugated lipids were also lower in allergic compared to control individuals and may reflect reduced fatty acid transport into the mitochondria. Bile acids facilitate the secretion, absorption and transport of lipids. Higher levels of 7-alpha-hydroxycholesterol and reduced lathosterol were found following challenge in the allergic group and suggests cholesterol availability for hormone synthesis would be diminished. This observation is supported by reduced levels of multiple steroids including cortisol and cortisone by 6 hours. Alpha-gal is present in mammals on both glycoproteins and glycolipids, including chylomicrons. We have speculated that the delay in symptoms is due to a delay in the appearance of small lipid bilayer vesicles carrying multiple alpha-gal epitopes with LDL in circulation. Chylomicrons enter circulation via the thoracic duct through a process that takes several hours. Alterations in lipid and fatty acid metabolism observed in this study are consistent with the clinical delay.

A potential limitation of this study is the small sample size included in each group. Inclusion of more subjects would increase the number of metabolites that are different not only at baseline, but also following meat challenge. It seems that the important systems would be identified in the initial screen and other metabolites identified by increased screening would be downstream products of these dysregulated pathways. Along these lines, larger metabolic panels might have identified other important compounds and pathways. An additional concern is that subjects selected for meat challenge had mild symptoms and those with previous anaphylactic episodes were excluded. For safety reasons these subjects could not be challenged. Other control groups might be useful such as food, but not meat allergic individuals challenged with meat and food, but not meat allergic individuals challenged with the food they are allergic.

In summary, metabolomic analysis of subjects with red meat allergy related to high levels of IgE to alpha-gal identified basal and food-induced differences following challenge with red meat that were not observed in healthy subjects. These observations suggest that meat allergy is accompanied by disruptions in amino acid catabolism, lipid and carbohydrate metabolism and synthesis of bile acids that may reflect differences in the microbiome and nutrient absorption. These metabolic changes are likely to contribute to symptoms through alterations of components of the allergic response.

John W Steinke, Ph.D.^{a,b}

Shawna L Pochan, C.N.M., M.P.H.^a

Hayley R James, B.S.^a

Thomas A.E. Platts-Mills, M.D., Ph.D, F.R.S^{a,b}

Scott P Commins, M.D., Ph.D.^c

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130 ^aDepartment of Medicine and ^bCarter Immunology Center

131 University of Virginia Health System, Charlottesville, VA USA; ^cDepartment of Medicine,

132 University of North Carolina School of Medicine, Chapel Hill, NC USA

133 email: scommins@email.unc.edu

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136 Disclosure of potential conflict of interest: The authors declare that they have no relevant

137 conflicts of interest.

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Figure Legends

Figure 1 Baseline metabolomic differences between meat allergic and control subjects.

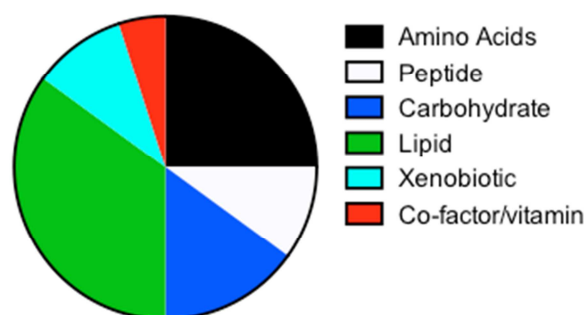
A. Differentially expressed metabolites between control and meat allergic subjects displaying the major metabolic pathways and proportion altered. B. Representative box plots showing basal expression levels of two peptides aspartylphenylalanine and leucylglycine that were significantly different ($p < 0.05$). C. Representative box plots showing the basal expression levels of two lipids taurocholate and 10-undecenoate that were significantly different ($p < 0.05$). The blue box is control and red box meat allergic with the box representing the upper and lower quartiles, the black bar being the median and the whiskers representing the maximum and minimum of distribution.

Figure 2 Time-dependent differences between meat allergic and control subjects

following meat challenge. A. Metabolites differentially expressed at baseline showing interaction between meat allergic status and time after challenge with major metabolic pathways and proportion altered displayed. B. Representative line plot showing the time dependent change in expression of the peptide alpha-glutamyltryptophan that showed significant interaction ($p < 0.05$). C. Representative line plot showing the time dependent change in expression of the lipid dodecanedioate that showed significant interaction ($p < 0.05$). The blue line is control and red line meat allergic with the circle representing the mean value and the whiskers representing $\pm$ one standard deviation.

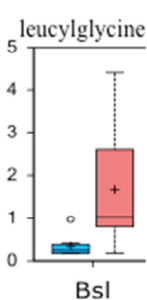
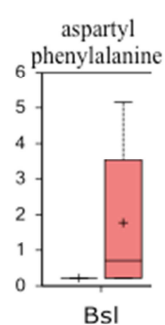
A

Meat Status-basal differences

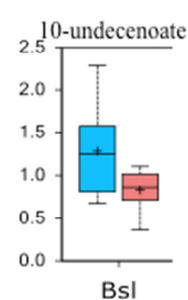
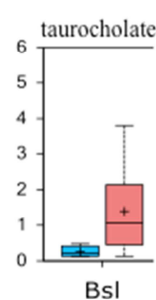


Total=20 metabolites

B

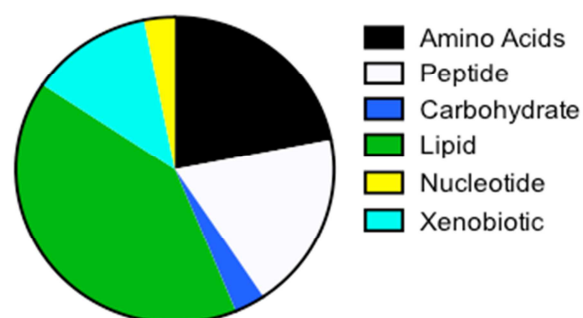


C



A

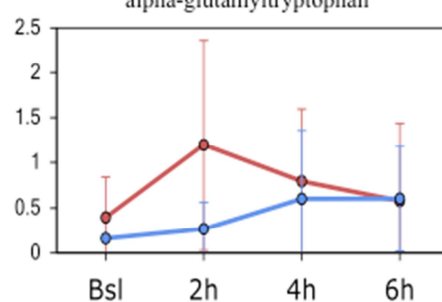
Meat Status-time



Total=32 metabolites

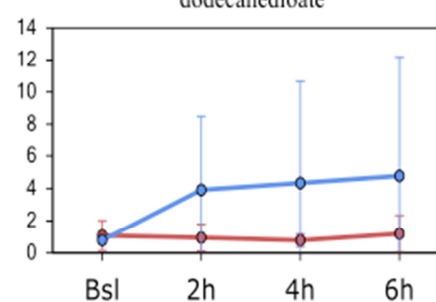
B

alpha-glutamyltryptophan



C

dodecanedioate



Online supplement

Methods

Subjects. Many of the individuals participating in the study have previously been described as part of another study examining the clinical response following oral food challenge^{E1}. Briefly, patients who presented to the Charlottesville allergy clinic with delayed urticarial reactions to mammalian meat and positive titers of IgE antibody to alpha-gal were enrolled, as well as control subjects with negative titers of IgE antibody to alpha-gal and no history of reactions to mammalian meat. From the original study, 8 samples from the alpha-gal positive group and 7 samples from the control group were used for metabolomic analysis. The alpha-gal positive subjects were all on a diet avoiding red meat prior to challenges. All subjects signed informed consent using protocols approved by the Human Investigation Committee at the University of Virginia (IRB # 15098).

Immunoassays. Total and specific IgE antibodies were measured by using the commercially available ImmunoCAP system (Phadia US, Portage, Mich). The assays were performed with the ImmunoCAP 250 instrument, and results were expressed as international units per milliliter, where the international unit both for specific and total IgE is approximately 2.4 ng. For specific assays, the standard cutoff point for a positive reaction was 0.35 IU/mL. The streptavidin CAP technique was used to measure IgE levels to alpha-gal, in which approximately 1 µg of biotinylated antigen was added to each CAP^{E2}.

Mammalian meat food challenge. The food challenges have previously been described as part of another study examining symptomatic and immunological responses following oral food challenge^{E1}. Briefly, mammalian meat challenges were performed in the allergy clinic of the University of Virginia Hospital. Participants were invited to return for a meat challenge based on the results of serum screening (sIgE to alpha-gal >1.0), severity of symptoms at prior reaction,

and appropriate medical history (eg, not currently taking β -adrenergic receptor blocker medication and no medical contraindication to epinephrine). Subjects were asked to avoid oral antihistamines and leukotriene modifier therapy for 7 days before the challenge. Vital signs and intravenous access were obtained, and subjects consumed 150 g of mammalian meat under observation (roughly equivalent to 2-3 sausage patties). Subjects were monitored for reactions, including a change in vital signs, after the food challenge. Blood was sampled immediately before and then every two hours after the dose of meat was eaten. Blood was allowed to clot for 30 min and spun for separation of serum and cellular components and the serum fraction was frozen and stored at -80°C until metabolomic profiling was performed. All subjects were monitored for 2 hours after the treatment of clinical symptoms and were released when vital signs were stable. Subjects were allowed to consume water, but no other food or vigorous exercise was permitted during the duration of the food challenge.

Metabolomic profiling platform. Processing, analysis and identification of compounds was performed by Metabolon, Inc. (Durham, NC). Samples were extracted/depleted using an automated MicroLab STAR system (Hamilton Company, Salt Lake City, UT) to remove residual protein. Extracted samples were analyzed using a combination of three independent instrumentation methods: ultrahigh performance liquid chromatography/tandem mass spectrometry (UHLC/MS/MS²) optimized for basic compounds, UHLC/MS/MS² optimized for acidic species and gas chromatography/mass spectrometry (GC/MS) for non polar metabolites^{E3}. For quality control, aliquots of pooled samples were included in separate runs serving as technical replicates to monitor potential variability in the quantitation of detected biochemicals. Median relative standard deviation was 13% for technical replicates and 9% for internal standards.

Metabolite Identification. Metabolites were identified by automated comparison of ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight, preferred adducts, in-source fragments as well as their associated MS/MS spectra^{E3}. The library allows rapid identification of metabolites in the experimental samples with a high degree of confidence.

Statistical analyses. If any values were missing, values were assumed to be below the level of detection. Initial classification utilized random forest analyses, providing an estimate of the ability to classify individuals in a new data set. Random forest is an unbiased prediction algorithm that uses supervised classification based on an ensemble of decision trees. For a given decision tree, a random subset of the data with identifying true class information is selected to build the tree (“bootstrap sample” or “training set”), and then the remaining data, the “out-of-bag” (OOB) variables, are passed down the tree to obtain a class prediction for each sample. This process is repeated thousands of times to produce the forest. Final classification of each sample is determined by computing the class prediction frequency (“votes”) for the OOB variables over the whole forest. When the full forest is grown, the class predictions are compared to the true classes, generating the “OOB error rate” as a measure of prediction accuracy. Thus, the prediction accuracy is an unbiased estimate of how well one can predict sample class in a new data set. Given the multiple comparisons inherent in analysis of metabolites corrections were made using false discovery rate analysis (q-value). Between-group relative differences were assessed using two-way ANOVA with repeated measures (p-value). Pathways were assigned for each metabolite, also allowing examination of overrepresented pathways. After multiple correction analysis, a p value of <0.05 was considered significant.

ACCEPTED MANUSCRIPT

Table E1**Patient characteristics**

Subject	Age $\pm$ SEM	M/F	IgE Total (IU/ml)	IgE alpha-gal (IU/ml)	IgE beef (IU/ml)	IgE pork (IU/ml)	IgE cow's milk (IU/ml)
Meat allergic	41.0 $\pm$ 6.0	6/2	179 $\pm$ 30*	26.2 $\pm$ 3.5	6.8 $\pm$ 1.0	6.6 $\pm$ 1.1	2.3 $\pm$ 0.5
Healthy control	33.6 $\pm$ 1.5	5/2	63.3 $\pm$ 29.8	<0.35	<0.35	<0.35	<0.35

*p<0.02

Table E2

Between group biochemical differences

ANOVA Contrasts		Total biochemicals $p \leq 0.05$	Biochemicals (↑↓)	Total biochemicals $0.05 < p < 0.10$	Biochemicals (↑↓)
<u>Meat Allergic</u> Control	Baseline	23	17 6	19	13 6
	2h post	21	9 12	12	8 4
	4h post	16	11 5	12	8 4
	6h post	25	10 15	17	5 12
Control	<u>2h post</u> Baseline	48	39 9	25	21 4
	<u>4h post</u> Baseline	84	56 28	29	18 11
	<u>6h post</u> Baseline	108	67 41	42	19 23
Meat Allergic	<u>2h post</u> Baseline	37	28 9	38	19 19
	<u>4h post</u> Baseline	63	28 35	37	18 19
	<u>6h post</u> Baseline	89	27 62	36	14 22

Table E3**Biochemical interaction of meat allergic status and time post challenge**

Two-way ANOVA with repeated measures	MA status Main effect	Time Main effect	MA status:Time Interaction
Total biochemicals p<0.05	20	103	32
Total biochemicals 0.05<p<0.10	21	41	31

MA = meat allergic

Table E4
Metabolite differences due to meat status

Metabolite	Pathway	Expression meat allergic vs control
3-hydroxyisobutyrate	Amino acid	↓
3-hydroxy-2-ethylpropionate	Amino acid	↓
Arginine	Amino acid	↑
Creatine	Amino acid	↑
Creatinine	Amino acid	↓
Aspartylphenylalanine	Peptide	↑
Leucylglycine	Peptide	↑
Fucose	Carbohydrate	↑
Sucrose	Carbohydrate	↑
Lactate	Carbohydrate	↑
pelargonate	Lipid	↓
10-undecenoate	Lipid	↓
2-aminoheptanoic acid	Lipid	↑
2-hydroxydecanoic acid	Lipid	↓
Dodecanedioate	Lipid	↓
15-methylpalmitate	Lipid	↓
Taurocholate	Lipid	↑
L-uroglobin	Co-factor/vitamin	↑
o-sulfo-L-tyrosine	Xenobiotic	↓
Caffeine	Xenobiotic	↑

Table E5
Metabolite differences due to meat status and time following challenge

Metabolite	Pathway	Expression meat allergic vs control
N-acetylglycine	Amino acid	↓
Beta-hydroxypyruvate	Amino acid	↑
p-cresol sulfate	Amino acid	↓
3-phenylpropionate	Amino acid	↑
Isobutyrylcarnitine	Amino acid	↑
Citrulline	Amino acid	↑
5-oxoproline	Amino acid	↑
Pro-hydroxy-pro	Peptide	↓
Leucylalanine	Peptide	↑
Leucylglycine	Peptide	↑
Alpha-glutamyltryptophan	Peptide	↑
Valylaspartate	Peptide	↑
XHWESASXXR	Peptide	↑
Glucose	Carbohydrate	↑
Dihomo-linolenate	Lipid	↓
Docosapentaenoate (DPA)	Lipid	↓
Docosahexaenoate (DHA)	Lipid	↓
Pelargonate	Lipid	↓
Arachidonate	Lipid	↓
2-hydroxypalmitate	Lipid	↑
Dodecanedioate	Lipid	↓
Propionylcarnitine	Lipid	↑
Glycohenodeoxycholate	Lipid	↑
Choline	Lipid	↓
Chiro-inositol	Lipid	↑
1-eicosapentaenoylglycerophosphoethanolamine	Lipid	↓
5-alpha-pregnen-3beta, 20alpha-diol disulfate	Lipid	↓
Inosine	Nucleotide	↑
Hippurate	Xenobiotic	↑
3-hydroxyhippurate	Xenobiotic	↑
Saccharin	Xenobiotic	↓
Paraxanthine	Xenobiotic	↑

Figure E1 Random forest analysis of metabolomics data after meat challenge. A.

Confusion matrix used to determine predictive accuracy in using biochemical differences to stratify patient groups. B. Plot displaying the top 30 metabolites based on their importance to separating the control and meat allergic populations at 6 hours with relevant metabolic pathways indicated.

Figure E2 Principal component analysis of metabolomics data after meat challenge.

Principal component analysis (PCA) revealed considerable overlap between groups, suggesting minor metabolic differences in pathways that may contribute to allergic condition. Samples designated with * represent outliers that are candidates that may be most predictive in distinguishing between groups.

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A

Confusion Matrix for Random Forest Analysis

		Predicted group: baseline		
Actual group		Ctrl	MA	Class Error
	Ctrl	6	2	25%
	MA	5	3	62.5%
Predictive accuracy = 56.25%				

		Predicted group: 6 hour		
Actual group		Ctrl	MA	Class Error
	Ctrl	5	3	37.5%
	MA	3	5	37.5%
Predictive accuracy = 62.5%				

B

