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Final Report: REDUCING COVID-19 MORTALITY BY CHARACTERIZING THE IMMUNOEPIGENETIC GUT MICROBIOME INTERACTIONS

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The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT

15. SUBJECT TERMS

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Alika Maunakea
UU	UU	UU	UU		19b. TELEPHONE NUMBER: 808-989-1034

RPPR Final Report
as of 16-Nov-2022

Agency Code: 21XD

Proposal Number: 77947BBST1 **Agreement Number: W911NF-21-P-0025**
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DUNS Number: 117522352 EIN:

Report Date: 22-Dec-2021 Date Received: 02-Nov-2022
Final Report for Period Beginning 23-Nov-2020 and Ending 22-Nov-2021
Title: REDUCING COVID-19 MORTALITY BY CHARACTERIZING THE IMMUNOEPIGENETIC GUT MICROBIOME INTERACTIONS
Begin Performance Period: 23-Nov-2020 **End Performance Period:** 22-Nov-2021
Report Term: 0-Other

Submitted By: Ruben Juarez Email: hawaiianalytic@gmail.com
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Distribution Statement:

STEM Degrees: **STEM Participants:**

Major Goals: This proposal's overarching goal is to examine the extent to which gut microbiome composition/diversity, epigenetic regulation of immune function, and dietary factors collectively might reduce or prevent severe clinical outcomes of COVID-19. Substantial progress was made toward understanding these relationships.

Accomplishments: See the uploaded file pictures, charts and figures.

Training Opportunities: Nothing to Report

Results Dissemination: Nothing to Report

Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: Nothing to Report

PARTICIPANTS:

Participant Type: PD/PI
Participant: Alika Maunakea
Person Months Worked: 1.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Co-Investigator
Participant: Ruben Juarez
Person Months Worked: 1.00 **Funding Support:**
Project Contribution:

National Academy Member: N

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Person Months Worked: 4.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Technician
Participant: Andie Conching
Person Months Worked: 2.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Other Professional
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Person Months Worked: 2.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Partners

,

I certify that the information in the report is complete and accurate:
Signature: Ruben Juarez
Signature Date: 11/2/22 4:54AM

FINAL TECHNICAL REPORT

Contract Number: **W911NF21P0025**

Proposal Number: **A20B-T028-0028**

Contractor's Name: **HAWAII INTEGRATED ANALYTICS LLC**

Contractor Address: **2800 Woodlawn Dr Ste 141 Honolulu HI 96822**

Title of the Project: **REDUCING COVID-19 MORTALITY BY CHARACTERIZING THE IMMUNOEPIGENETIC GUT MICROBIOME INTERACTIONS**

Total Contract Amount: **$166,500**

Amount of Funds Paid by DFAS to date: $**138,750**

Total Amount Expended/Invoiced-to-Date: $**138,750**

Number of Employees Working on the Project: **5**

Number of New Employees Placed on Contract This Month: **0**

FINAL TECHNICAL REPORT

This proposal's overarching goal is to examine the extent to which gut microbiome composition/diversity, epigenetic regulation of immune function, and that dietary factors collectively might reduce or prevent severe clinical outcomes of COVID-19. Substantial progress has been made towards understanding these relationships and have achieved the following technical objectives to date, including:

1. **Cohort Study:** We obtained all anthropometric and nutrition/diet data from uninfected and infected study participants recovering from COVID-19, along with the collection of cryopreserved samples from all participants, including blood, plasma and stool biospecimens. In the final phase of the project, we focused on understanding long-COVID in study partipants and how pre-exisitng conditions, in particular obesity, presented an increase risk. Confirmatory analysis revealed the following key findings: (1) Anti-correlation between gut microbial diversity and ability to produce neutralizing antibodies against SARS-CoV-2; (2) Obesity underlies an attenuated antibody response against SARS-CoV-2, lower microbial diversity, and reduced expression of neutralizing antibodies against SARS-COV-2; (3) Significant dysbiosis of the microbiome among obese individuals recovering from COVID-19; (4) Metabolomic differences between obese and normal weight individuals associated with attenuated antibody production, which also corresponded to immune dysregulation and systemic inflammation. We are in the process of preparing a manuscript to summarize these findings and present salient data here.

2. **Key Cohort Characteristics:** To determine the molecular/functional consequence of the obesity-associated attenuated antibody response to SARS-CoV-2 infection we discovered, we examined the temporal ability of individuals in our COVID-19 cohort to neutralize the virus *in vitro* using their convalescent plasma as indicated by the % inhibition of viral antigens (SARS-CoV-2 Surrogate Virus Neutralization Test Kit, GenScript). In general the plasma of those expressing SARS-CoV-2 IgG antibodies (measured using the SeroFlash SARS-CoV-2 IgG/IgM ELISA, Epigentek) could neutralize the virus in vitro. We presented this data in the previous report and extend additional findings here in a subset of the cohort, characterized in **Table 1**. Importantly, we confirmed that there was no significant differences in the duration of post-infection recovery among participants stratified by BMI groups (**Fig. 1**).

Characteristic	Normal, N = 14[1]	Overweight, N = 12[1]	Obese, N = 22[1]	P-value
age_group				0.14
(0,18]	3 (21%)	0 (0%)	5 (26%)	
(18,35]	2 (14%)	2 (18%)	7 (37%)	
(35,50]	4 (29%)	3 (27%)	5 (26%)	
(50,200]	5 (36%)	6 (55%)	2 (11%)	
Unknown	0	1	3	
metadata_sex_m_male _f_female				0.3
F	10 (71%)	5 (42%)	13 (59%)	
M	4 (29%)	7 (58%)	9 (41%)	
race				0.029
Asian	5 (36%)	2 (18%)	1 (5.9%)	
NHPI	4 (29%)	2 (18%)	12 (71%)	
Other	2 (14%)	1 (9.1%)	2 (12%)	
White	3 (21%)	6 (55%)	2 (12%)	
Unknown	0	1	5	
NHPI				0.021
NH	1 (7.1%)	0 (0%)	1 (5.9%)	
Non-NHPI	10 (71%)	9 (82%)	5 (29%)	
PI	3 (21%)	2 (18%)	11 (65%)	
Unknown	0	1	5	
[1] n (%)				

Table 1. Individuals enrolled in our study were stratified by BMI into normal weight, overweight, or obese groups. ANOVA analysis of differenes between groups in age, sex, race, and Native Hawaiian and Pacific Islander (NHPI) or non-NHPI are shown *P-value shown*.

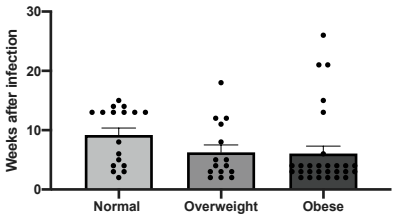

Figure 1. Individuals enrolled in our study were stratified by BMI into normal weight, overweight, or obese groups. Self-reported data collected from participants included the number of weeks after infection (as determined by a PCR test to confirm diagnosis) from when they enrolled in our study. Dot-plot shows no signficant differences between groups for duration of post-infection recovery from when participants were enrolled in the study, at which time biospecimens were collected over a 6-week period.

3. **Associative Analyses:** In stratifying individuals in our cohort by BMI group, we evaluated the following key features: (1) the number of common long-COVID symptoms experienced (e.g. fatigue, headache, sleep problems, brain fog, change in smell or taste, *etc*); (2) relationship bewteen long-COVID symptoms and anti-SARS-CoV-2 antibody (IgG) production; (3) viral neutralization capacity in vitro; and (4) immune cell composition. We observed that both obese and overweight individuals experienced signficant delays in recovering from common long-COVID symptoms (**Fig. 2a**), that was unrelated to the levels of anti-SARS-CoV-2 antibody production (**Fig. 2b**). Interestingly, however, we observed that obese and overweight individuals experienced a significant attenuation of anti-SARS-CoV-2 antibody production over the course of their post-COVID recovery, relative to normal weight individuals who maintained high levels of antibody production over the 6-week follow up period (**Fig. 2c**). Unlike normal or overweight individuals, we also observed that obese individuals failed to maintain a high degree of viral neutralization capacity in vitro that they first experienced early into recovery (**Fig. 2d**). Finally, using immunophenotyping approaches from cryopreserved peripheral blood mononuclear cells (PBMCs), we observed that obese individuals tended to harbor and maintain elevated levels of monocytes, yet exhibited significantly lower numbers of B-cells than that of their normal weight counterparts (**Fig. 2e,f**). Altogether, these results indicated that obese individuals experienced significant alterations to their immune regulation that associated with a higher frequency of long-COVID symptoms.

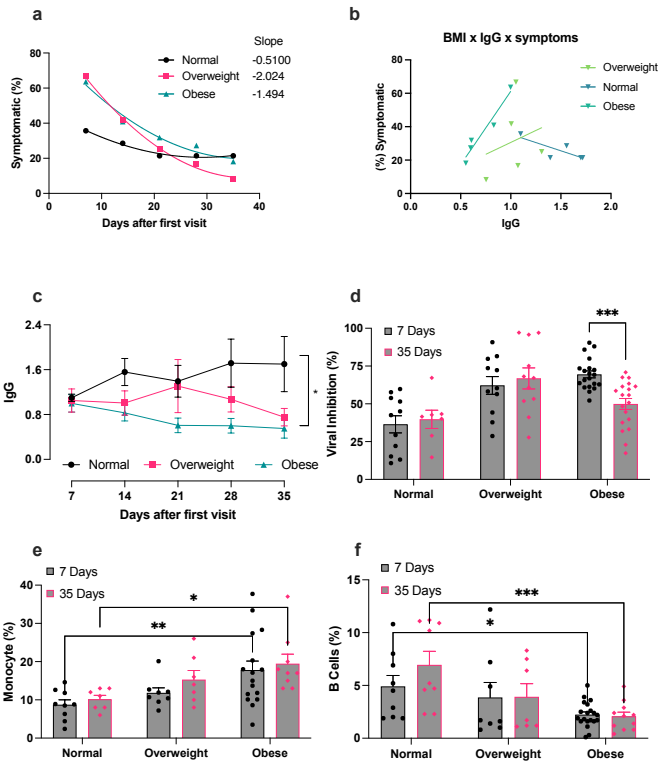

Figure 2. Initial assessment of differences in key metrics representative of adaptive immunity capacity across BMI subgroups for normal, overweight, and obese classifications indicated constraints on higher BMI groups. **a**, Overweight and obese participants experienced twice the rate of acute symptoms at baseline compared with normal BMI individuals, but at time of final follow-up all weight classes had similar symptoms reported. **b**, PCA plot of BMI, IgG, and symptoms. **c**, Longitudinal assessment of Immunoglobin G (IgG), a common antibody and general indicator of infection level and immunity, was diminished by roughly two-fold in overweight and obese individuals over a 6-week period. This suggests symptom duration corresponds with attenuated adaptive immunity. **d**, Although depletion of IgG in occurred in non-normal weight individuals, only the sustained loss IgG in obese groups tended to correspond with a significant reduction in viral inhibition capacity over the course of the study. Immune profiling was conducted to determine if BMI influences monocytes (**e**) and B-cell populations (**f**) implicated in attenuated antibody response. No significant changes to cell populations occurred in either of the groups, however obese individuals had elevated monocytes and depleted B cells relative to normal weight individuals. *, $P < 0.05$.

4. **Mechanistic Insights:** Crosstalk between gut environmental factors and intestinal epithelial cells regulate immune cell responses and subsequent adaptive immunity; thus, we explored the potential for the gut microbiome as a mediator of the attenuated antibody response in obese individuals recovering from COVID-19. Indeed, obesity related gut dysbiosis can induce divergent functionalities of the gut microbiome often resulting in proinflammatory cascades. Two microbial features associated with superior functionality include increased Simpson alpha diversity index and a lower Firmicutes to Bacteroidetes (F:B) ratio, which we evaluated from gut microbiome data to glean insight into microbial capacities to effectively modulate immunity during recovery. BMI distributions were clustered and correlated to Simpson diversity. As expected, overweight and obese groups represented a lower degree of diversity (**Fig. 3a**). To understand the relationship betewen alpha diversity and outcome (symptom prevalence) we binned individuals into low and high diversity groups – lower 50% and upper 50%. When these groups were compared, the high diversity group experienced a much lower percentage of residual long-COVID symptoms. The proportion of BMI groups representative of the low and high diversity cut-off indicate being of normal weight does not guarantee enhanced diversity, but drastically increases those chances. Similarly, not all obese individuals were found to have low diversity although it was still the majority (**Fig. 3b**). Continuing with low and high diversity groups, we assessed IgG fold-change over the duration of the study and observed a significant loss of IgG production in low diversity individuals (**Fig. 3c**), which corresponded to individuals with low F/B ratio that significantly inversely correlated with viral neutralization capacity (**Fig. 3d**). Altogether, these data suggested that obese individuals experienced gut microbial dysbiosis that increased their risk for attenuated antibody production upon infection with SARS-CoV-2 and subjected them to increased vulnerability to long-COVID compared to their normal weight counterparts. In the previous report, we noted that this increased vulnerability and gut microbial dysbiosis was associated with increased systemic inflammation as measured by highested levels of CRP, MCP-1, IL-6, and HMGB1 among others. Interestingly, obese individuals maintained elevated levels of these pro-inflammatory biomarkers in circulation (measured in plasma) compared to their normal weight counterparts who experienced significant reductions in these markers over recovery (as indicated in the prior report). To gain further insight into the potential mechanisms that may lead to this

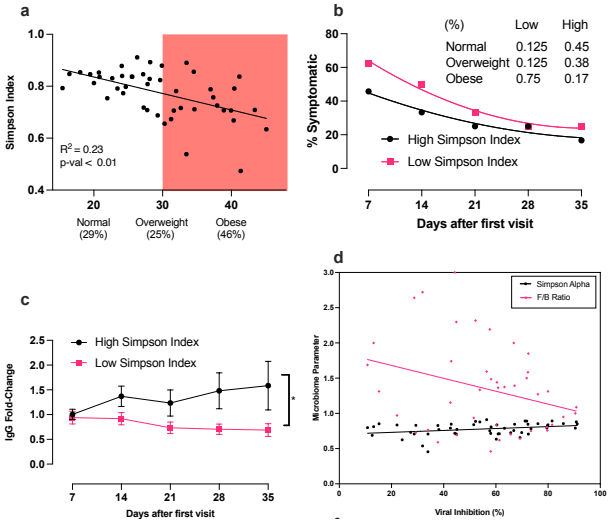

Figure 3. Initial assessment of differences in key metrics representative of adaptive immunity capacity across BMI subgroups indicated constraints on higher BMI groups. **a**, Overweight and obese participants experienced twice the rate of acute symptoms at baseline compared with normal BMI individuals, but at time of final follow-up all weight classes had similar symptoms reported. **b**, PCA plot of BMI, IgG, and symptoms. **c**, Longitudinal assessment of Immunoglobin G (IgG), a common antibody and general indicator of infection level and immunity, was diminished by roughly two-fold in overweight and obese individuals over a 6-week period. This suggests symptom duration corresponds with attenuated adaptive immunity. **d**, Although depletion of IgG in occurred in non-normal weight individuals, only the sustained loss IgG in obese groups tended to correspond with a significant reduction in viral inhibition capacity over the course of the study. Immune profiling was conducted to determine if BMI influences monocytes (**e**) and B-cell populations (**f**) implicated in attenuated antibody response. No significant changes to cell populations occurred in either of the groups, however obese individuals had elevated monocytes and depleted B cells relative to normal weight individuals. *, $P < 0.05$.

vulnerability in obese individuals, especially as it might relate to diet/nutrition, we applied untargeted metabolomic analyses of proteins isolated from stool samples of these participants.

5. **Metabolomic Data**: From funds of this award, we subcontracted Creative Proteomics who performed untargeted metabolomic analyses from proteins isolated from stool samples we collected from each participant at study entry (at the same timepoint as that collected for gut microbiome analyses). Given the enrichment of butyrate-producing (a SCFA known to modulate inflammation) bacterial species we observed, coupled with the higher degree of systemic inflammation in obese individuals, we directly examined the abundance and expression of butyrate-producers in stool samples (% DNA and % RNA of butyrate kinase relative to the butyryl-CoA:acetate CoA-transferase control gene, respectively) using quantitative PCR, which we reported previously. Higher levels of butyrate-producers appeared to be protective of SAR-CoV-2, as indicated by the significant positive correlations with neutralizing capacity. Given this finding, we expected to observe significant differences in butyrate metabolism pathways from the metabolomic data (as a positive control). Indeed, relative to normal weight individuals, we observed significant decreases in metabolite levels cooresponding to dysregulation of butryate metabolism in obese individuals, concomitant with decreases in vitamin B6 metabolism (**Fig. 4**), and implicates these pathways as key mediators of the heightened and sustained inflammation these individuals exhibited over the course of the follow-up period. These results confirm our prior findings and implicate that key dietary components may modify risk to severe and/or long-COVID, particularly of obese individuals.

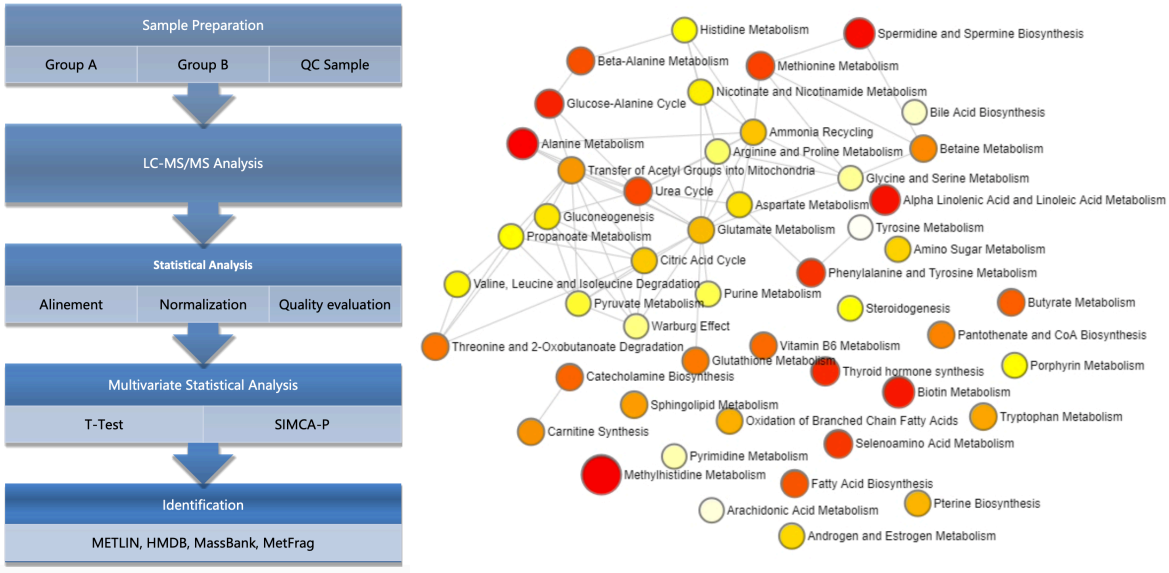

Figure 4. Pathway enrichment analysis of pertured metabolites. (left) Sample processing and analysis workflow by Creative Proteomics. (**right**) Under ESI+ scan, network analysis of stool proteomic data revealed significant differences between obese and normal weight individuals in post-acute stages of recovery from COVID-19. We note changes specific to dietary modifications that influence inflammation, including vitamin B6 and butryate metabolism pathway.

6. **Putative Model.** We present the following immunoepigenetic-microbiome axis as a pathway to link the intestinal microbiome environment and subsequential influence on immune responses to SARS-CoV-2 infection. Maintenance of homeostatic regulation of this axis is implicated in proper adaptive immune system response. However, microbial dysbiosis associated with obesity induce inflammation and impairs proper adaptive immune responses upon SARS-CoV-2 viral infection and recovery. This initiates pro-inflammatory cytokine signaling that becomes exacerbated upon infection and subsequently leads to monocyte overactivation via increased

TNF-a and IFN-g signaling. Cascading responses in the ERK/MAPK and NF-kB signaling pathways result in enrichment of HMGB1 expression and elicits successive inflammatory activation in endothelial cells, vascular smooth muscle cells, neurons, macrophages, and additional monocytes upon binding with RAGE receptors. This consequently expands differentiation of immune cells to proinflammatory monocytes eliciting the release of nitric oxide and inhibiting BAFF production. This culminates as decreased B cell maturation, proliferation, and antibody production impairing SARS-CoV-2 inhibition capacity. Our data from this cohort supports this model (**Fig. 5**).

7. **Implications:** SARS-CoV-2 infection and long COVID-19 is confirmed to be exacerbated in individuals with pre-existing conditions associated with immune dysregulation. Our results further demonstrate resolution is reduced under immunosuppressive conditions stemming from obesity-related chronic inflammation. Over the duration of the study, previously infected obese individuals maintained immune-profile characteristics indicative of inflammaging and pulmonary inflammation as established in other reports. We found obesity associated with persistent SARS-CoV-2 as characterized by prolonged COVID-19 symptom duration and delayed recovery resulting from impeded adaptive immunity. Investigation of long COVID-19 indicates obesity-imposed constraints on humoral immunity through inflammatory attenuated SARS-CoV-2 antibody production and decreased viral neutralization capabilities. Recent studies associate the loss of neutralizing antibodies in obese COVID-19 patients with circulating autoimmune antibodies that positively correlate with CRP levels and COVID-19 hospitalizations. While we did not expand upon the accumulation of autoantibodies in our cohort, these studies considered along with our data further imply deficiencies in adaptive immunity is inflammatory-mediated and likely dependent on BMI. Finally, our metabolomic profiling analyses implicate that these inflammatory-associated factors elevated in obesity may be modified by dietary components, including vitamin B6 and butyrate regulation. Overall, these findings provide key mechanistic insight into the attenuated antibody production experienced largely, but not exclusively by, obese individuals, which associates with gut microbial dysbiosis and metabolic dysregulation associated with prolonged and sustained inflammation, culminating in slower recovery and higher prevalence of long-COVID symptoms. Our study supports dietary supplementation as potential interventions against severe and long-COVID conditions, in particular among individuals predisposed to such conditions. As such, we are preparing a Phase 2 application with a clinical partner in Honolulu to test our model and identify dietary interventions that may reduce the risk to severe COVID-19 and improve recovery. Given the new viral variants, waning vaccine efficacy, and breakthrough infections, such interventions remain in demand.

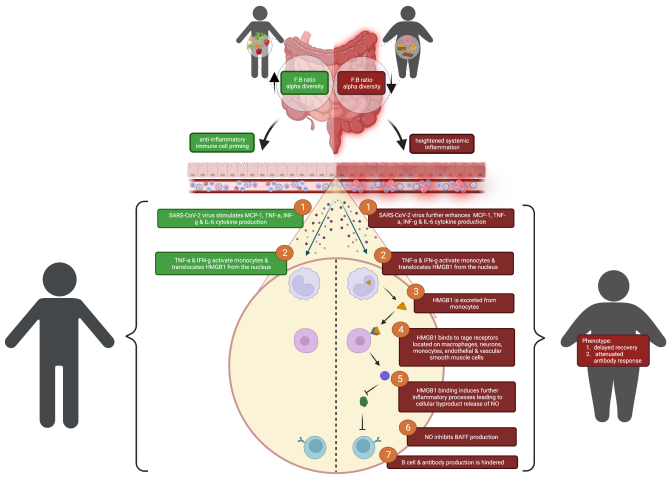

Figure 5. Immunoepigenetic-Gut Microbiome Axis in COVID-19.

Problem areas: None identified.

No classified information included in this report