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**Loss of Neu5Gc during Human Evolution: Impact on Macrophage
Inflammation and Muscle Metabolism**

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biomedical Sciences with a Specialization in Multi-Scale Biology

by

Jonathan Okerblom

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Professor Ajit Varki, Chair
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2017

The dissertation of Jonathan Okerblom is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2017

Dedication

To Jadyn.

Epigraph

When we try to pick out anything by itself,

we find it hitched to everything else in the universe.

—John Muir

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Thanks to Professor Michael "Mick" Bondello for being the most exceptional teacher I have ever encountered. I aspire to one day continue your teaching legacy.

The text of Chapter 1 chapter is, in full, a reprint of material originally published ChemBiochem by authors Okerblom J and Varki A, 2017. The dissertation author was the primary author of this review article.

The text of Chapter 2, in full, is a reprint of the material as it appears in the article of the same title by Okerblom J, Schwarz F, Olson J, Fletes W, Ali SR, Martin PT, Glass CK, Nizet V, and Varki A in the Journal of Immunology, 2017. The dissertation author was the primary author of this article.

The text of Chapter 3, in part, is currently being prepared for submission for publication by Okerblom J, Fletes W, Lee J, Patel H, Schenk S, Varki A, and Breen E. The dissertation author was the primary author of this material.

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2017   **Okerblom J** and Varki A. Cellular, Physiological and Pathological Consequences of Human loss of N-glycolylneuraminic Acid. ChemBioChem 2017 Apr 19. doi: 10.1002/cbic.201700077. [Epub ahead of print] PubMed PMID: 28423240.

2017   **Okerblom J**, Schwarz F, Olson J, Fletes W, Ali SR, Martin PT, Glass

CK, Nizet V, Varki A. Loss of CMAH During Human Evolution Primed the Monocyte-Macrophage Lineage Towards a More Inflammatory and Phagocytic State. Journal of Immunology. 2017 Mar 15;198(6):2366-2373. doi:10.4049/jimmunol.1601471. Epub 2017 Feb 1. PubMed PMID: 28148732; PubMed Central PMCID: PMC5340615.

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2014   Martin PT, Golden B, **Okerblom J**, Camboni M, Chandrasekharan K, Xu R, Varki A, Flanigan KM, Kornegay JN. A comparative study of N-glycolylneuraminic acid (Neu5Gc) and cytotoxic T cell (CT) carbohydrate expression in normal and dystrophin-deficient dog and human skeletal muscle. PLoS One. 2014 Feb 5;9(2):e88226. doi: 10.1371/journal.pone.0088226. PubMed PMID: 24505439; PubMed Central PMCID: PMC3914967.

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Oral   **Okerblom J, Schwarz F, Olson J, Fletes W, Ali SR, Nizet V, and Varki A**, Human-specific Loss of CMAH Primes the Monocyte-Macrophage Lineage Towards a More Inflammatory and Phagocytic State, San Diego Glycobiology Symposium - NexGen. San Diego, CA 2015.

Poster   **Okerblom J, Fletes W, Lee J, Varki A, and Breen E.**, Loss of CMAH Function Increases Muscle Endurance, San Diego Glycobiology Symposium. San Diego, CA 2017.

Okerblom J, Schwarz F, Olson J, Fletes W, Ali SR, Martin PT, Glass C, Nizet V, and Varki A, Loss of CMAH During Human Evolution Primed the Monocyte-Macrophage Lineage Towards a More Inflammatory and Phagocytic State, Sialoglyco Symposium. Santa Barbara, CA 2016.

Okerblom J, Schwarz F, Lizcano A, Lizardo E, Naito-Matsui Y, Martin PT, and Varki A, Loss of N-Glycolylneuraminic acid expression and function increases the innate inflammatory response, Society for Glycobiology Annual Meeting. Honolulu, HI 2014.

Okerblom J, Martin PT and Varki A, A model for Nglycolylneuraminic acid expression and function in mammalian muscle, Society for Glycobiology Annual Meeting. St. Petersburg, FL 2013.

Okerblom J and Antonio De Maio, Interaction of heat shock protein 70 with membranes depends on the lipid environment, SACNAS Annual

Meeting. Irvine, CA 2010.

ABSTRACT OF THE DISSERTATION

Loss of Neu5Gc during Human Evolution: Impact on Macrophage

Inflammation and Muscle Metabolism

by

Jonathan Okerblom

Doctor of Philosophy in Biomedical Sciences

with a Specialization in Multi-Scale Biology

University of California, San Diego, 2017

Professor Ajit Varki, Chair

Around 2-3 million years ago, *Alu*-mediated deletion of a critical exon in the *CMAH* gene became fixed in the hominin lineage ancestral to humans and may have contributed to the speciation of *Homo*. Although *CMAH* loss has occurred independently

in some other animal lineages, it is functionally intact in Old World primates, including our closest relatives, the chimpanzees. Chapter 1 is a thorough but concise review of the known consequences of Neu5Gc loss. We have speculated that hominin *CMAH* loss contributes to human evolution, at a time where our ancestors were using stone tools, increasing their consumption of meat, and possibly hunting. In Chapter 2, we show that when modeling Cmah loss in mice, they manifest a decreased survival in endotoxemia following bacterial lipopolysaccharide (LPS) injection. Macrophages and whole blood from *Cmah*$^{-/-}$ mice also killed *E. coli* K12 bacteria more effectively and this appears to be a conserved difference between humans and chimpanzees. While multiple mechanisms are likely involved, one cause is altered expression of C/EBPβ, a transcription factor affecting macrophage function that can be differentially expressed by simply feeding Neu5Gc to *Cmah*$^{-/-}$ macrophages. In Chapter 3, we show that *Cmah*$^{-/-}$ mice have a greater exercise capacity. Remarkably, the gastrocnemius complex time to fatigue measured *in situ* was more than 2-fold higher in non-exercise trained *Cmah*$^{-/-}$ mice when compared to WT controls. Mechanistically, the capillary to muscle fiber ratio is higher in *Cmah*$^{-/-}$ soleus, which could be contributing to a greater oxygen delivery during fatigue testing. After exercise training, metabolites in the pentose phosphate pathway and amino acid metabolism are also enriched in exercise-trained *Cmah*$^{-/-}$ soleus compared to WT, indicating a greater anabolic response. C/EBPδ, a transcription factor involved in regulation of metabolic and inflammatory pathways is also differentially expressed in *Cmah*$^{-/-}$ muscle. Therefore, we propose that the loss of *CMAH* in *Homo* likely allowed for greater bacterial killing and a greater maximum aerobic capacity. We speculate that this could have been a selective advantage when *Homo* transitioned towards persistence

hunting and greater consumption of meat, but is likely coupled with a greater susceptibility to inflammatory pathologies and endotoxic shock.

Chapter 1

Biochemical, Cellular, Physiological and Pathological Consequences of Human Loss of *N*-Glycolylneuraminic Acid

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Abstract (250 words)

About 2-3 million years ago, *Alu*-mediated deletion of a critical exon in the *CMAH* gene became fixed in the hominin lineage ancestral to humans, possibly through a stepwise process of selection by pathogen targeting of the CMAH product (the sialic acid Neu5Gc), followed by reproductive isolation via female anti-Neu5Gc antibodies. Loss of *CMAH* has occurred independently in some other lineages, but is functionally intact in Old World primates, including our closest relatives, the chimpanzees. While the biophysical and biochemical ramifications of losing tens of millions of Neu5Gc hydroxyl groups at most cell surfaces remains poorly understood, there are multi-scale effects functionally relevant to both sides of the host-pathogen interface. Hominin *CMAH* loss may also contribute to understanding human evolution, at a time where our ancestors were starting stone tool use, increasing their consumption of meat, and possibly hunting. Comparisons with chimpanzees within ethical and practical limitations have revealed some consequences of human *CMAH* loss, but more has been learned using a mouse model with a human-like *Cmah* inactivation. For example, such mice can develop antibodies against Neu5Gc that could affect inflammatory processes like cancer progression in the face of Neu5Gc metabolic incorporation from red meats, display a hyper-reactive immune system, a human-like tendency for delayed wound healing, late onset hearing loss, insulin resistance, susceptibility to muscular dystrophy pathologies, and increased sensitivity to multiple human-adapted pathogens involving sialic acids. Further studies in such mice may provide a model for other human-specific processes and pathologies involving sialic acid biology that have yet to be explored.

1. Introduction

1.1. Background

The most complex and rapidly evolving class of biological macromolecules appear to be glycan chains, which coat virtually all cell surfaces in nature,[1] display remarkable diversity in length, order, linkage type, modifications and branching structure and have numerous biological roles.[2] This review focuses on a human change in sialic acids, which are a family of 9-carbon backbone acidic monosaccharides that commonly terminate the glycan chains of the animals of the deuterostome lineage, as well as some successful bacterial pathogens of deuterostomes.[3] In mammals, there are $\sim10^6$-10^8 sialic acids present on each cell of all major tissues, prominently composed of *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) (Figure 1-1).[4–15, 15–20] This review focuses on the human loss of Neu5Gc, which differs from the precursor sialic acid Neu5Ac through enzymatic addition of a hydroxyl group to the *N*-acetyl moiety at C-5.[21, 22]

1.2. Discovery of Sialic Acids

In 1936, Gunnar Blix reported that acid hydrolysis combined with fractionation of bovine submaxillary mucin resulted in the crystal formations of an unknown sugar,[23, 24] which he did not name "sialic acid" until 1952.[25] Meanwhile in 1935, Ernst Klenk discovered gangliosides in the brain tissue of a patient with Niemann–Pick's disease[24, 26] and in 1941 independently described an acid hydrolyzed carbohydrate component that he named "neuraminic acid".[27] Ultimately it was confirmed that Blix and Klenk were describing the same family of sugars[28] and in 1957 Blix, Klenk, and Gottschalk (who

was studying influenza virus receptors) all agreed to "avoid further confusion" by officially calling them sialic acids.[29] But the two names have persisted to this day.[30] More than 50 types of sialic acids originating from the two "primary sialic acid backbones" (Neu5Ac and Kdn) have now been described in nature,[31–34] but the two most prevalent in mammals are Neu5Ac and Neu5Gc.[21, 35]

1.3. Werner Reutter's Major Contributions to Sialic Acid Biology

This journal issue is dedicated to the memory of Professor Werner Reutter, a pioneer in the study of sialic acids. A little over a decade after reporting the famous D-galactosamine study,[36] Reutter and colleagues began making key contributions to the field of sialic acid biology by studying the biosynthesis[37] and half-life of Neu5Ac in normal, cancerous (hepatomas), regenerating, and neonatal livers.[38, 39] After discovering that UDP-GlcNAc 2-epimerase (GNE), a key enzyme in sialic acid biosynthesis, was downregulated in hepatomas compared to normal livers,[37] they cloned and characterized the activity and reported it to be a bi-functional enzyme having both UDP-GlcNAc 2'-epimerase and ManNAc kinase activity.[40–42] *GNE* was subsequently discovered to be the gene mutated in Inclusion Body Myopathy 2,[43] the most common hereditary inclusion body myopathy affecting humans.[44]

Simultaneously, Reutter's group revolutionized the field of metabolic sialic acid glycoengineering when they discovered that adding 2-deoxy-2-propionamido-D-mannose (ManNProp) or to a lesser degree 2-deoxy-2-propionamido-D-glucose (GlcNProp) to liver homogenates resulted in the biosynthesis of *N*-propylneuraminic acid (NeuProp), a completely unnatural sialic acid that retained the propyl group from its modified

metabolic precursors.[45] A series of subsequent studies by Reutter, Bertozzi and others revealed that modified metabolic precursors (particularly modified mannosamines) could be incorporated as modified sialic acids onto cell surfaces as a tool for understanding biological relevance.[46, 46–63]

2. *N*-Glycolylneuraminic Acid (Neu5Gc), a Common Sialic Acid

2.1. History of Medical Primatology and Genomics.

Over a century ago at the Pasteur Institute in France, Dr. Élie Metchnikoff and Dr. Pierre Paul Émile Roux combined their Madrid Medical Congress and Ifla-Oziris awards to purchase a cohort of chimpanzees and successfully develop the first animal model for an infection affecting over 10 percent of the human population in Paris at the time: syphilis.[64] Metchnikoff and colleague Besredka subsequently developed the first non-human primate model for studying typhoid/enteric fever[65, 66] and together, this pioneering work strongly contributed to the modern era of medical primatology.[64] Many years later, the introduction of protein and nucleic acid sequencing revealed the remarkable genetic similarity of humans and chimpanzees, eventually leading to the famous hypothesis of King and Wilson, that "their macromolecules are so alike that regulatory mutations may account for their biological differences".[67] It took more than 20 years for the discovery of the first clear-cut exception to this hypothesis, the selective absence of Neu5Gc in humans,[68] which was shown to be due to an inactivating exon deletion in CMP-Neu5Ac Hydroxylase (CMAH) that became fixed in the *Homo* lineage sometime after the divergence from chimpanzees.[69, 70]

This and subsequently discovered genetic and biomedical differences[71–74] motivated the initial sequencing of the chimpanzee genome[75] and ongoing sequencing of other non-human primate genomes with the aim of determining genetic components specifically accounting for these differences in phenotypes.[76, 77, 77] Today we also know that despite our genetic similarity with other primates, there are specific infections and diseases that primarily affect humans and some cannot be adequately modeled in non-human primates.[78] Some of these that involve sialic acid biology are discussed further below.

2.2. Sialic Acid Biosynthesis

In mammals, sialic acids are produced through the hexosamine biosynthesis pathway (HBP), which is rate limited by the conversion of fructose-6-phosphate to glucosamine-6-phosphate by Glutamine fructose-6-phosphate amidotransferase (GFAT). Although GFAT only utilizes 1-5% of total glucose, dysregulation of this pathway has been implicated in multiple metabolic diseases, such as diabetes, Alzheimer's, cardiovascular disease, and some cancers.[79, 80] In the final steps of sialic acid biosythesis, the primary sialic acids of vertebrates (Neu5Ac and Kdn) are formed by the condensation of ManNAc-6-P (Neu5Ac) or Man-6-P (Kdn) with phosphoenolpyruvate (PEP). Neu5Ac can then be further modified at C-5 or be further modified at C-4, C-7, C-8, and/or C-9 to generate over 50 different forms of sialic acids.[20, 32, 81, 82] In relation to glycan biosynthesis in deuterostomes, sialic acids are unique in that they require a monophosphate nucleotide donor (CMP) for activation[83] and must travel to the nucleus, in order to be activated into their CMP-conjugated form.[84, 85] Cytosolic CMP-conjugated

Neu5Gc is then produced by the hydroxylation of CMP-Neu5Ac by CMP-Neu5Ac Hydroxylase (CMAH), the only known enzyme capable of Neu5Gc biosynthesis from Neu5Ac in any living species.[6, 8, 15, 18, 86–88]

3. Human specific loss of Neu5Gc expression

Given their high abundance in animal tissues, Neu5Gc and other structural variants of Neu5Ac were already identified and characterized by the time the term "sialic acid" was officially agreed upon in the 1950s.[29] Subsequently, it was reported that CMAH was the hydroxylase/mono-oxygenase that converted the sugar nucleotide CMP-Neu5Ac to CMP-Neu5Gc in a complex mechanism requiring a variety of co-factors, including cytochrome b5/b5 reductase, iron, oxygen, and NADH.[6] Although humans were long known to lack easily detectable levels of Neu5Gc compared to other mammals, the inability of humans to synthesize Neu5Gc was not immediately apparent, because small amounts of this sialic acid were reported using antibodies, particularly on tumors and fetal tissues.[89–94] It was later shown that Neu5Gc in humans is incorporated from dietary sources and presented on some epithelial and endothelial cell surfaces.[95–98] In 1982, Roland Schauer noted that despite reports of Neu5Gc presence in tissues, Neu5Gc production was missing in humans, possibly antigenic, and potentially contributing to multiple pathological states[35], including "serum sickness" in human patients receiving infusions of animal sera.[99, 100] Sixteen years later, it was independently discovered by two groups that humans lack a functional CMAH enzyme and are therefore incapable of endogenous Neu5Gc production.[68–70] Both groups reported a genomic mutation eliminating a 92 bp exon in CMAH. While one report predicted the existence of a large

frame-shifted inactive protein,[69] the other correctly showed the frame-shift resulting in a small truncated inactive protein[70] (also see below). The ramifications of Neu5Gc loss continue to be explored.[97, 98, 101–106]

3.1. Genetic basis of Human-specific Loss of Neu5Gc Expression

The human 478 bp region of genomic DNA deletion in CMAH including the 92 bp exon, was later shown to be due to an Alu-Alu fusion that eliminated the sequences encoding the Rieske iron-sulfur-binding region, which is essential for its enzymatic activity.[107–109] Comparative genomic analysis revealed that chimpanzees, bonobos, gorillas, orangutans, gibbons, baboons, and rhesus monkeys all contain an ancient *AluSq* retroposon[110, 111] (subsequently designated sah*Alu*Sq) ~350 bp downstream from the human deletion site.[107] Although several other *Alu* elements were found in common between humans and primates, humans uniquely contain an *AluY* element arising from the fusion (subsequently designated sah*Alu*Y) that replaced both the sah*Alu*Sq and the missing 92 bp exon. Thus, sah*Alu*Y-mediated deletion of the genomic DNA (478 bp), including the 92 bp exon and intron fusion was proposed as the model for human CMAH inactivation.[107]

3.2. Timing of CMAH loss in the Hominin Lineage

Although technical limitations have prevented precise biochemical dating of CMAH pseudogenization in the hominin fossil record,[108, 112, 113] multiple genomic methods (see below) deduced that hominin CMAH loss likely occurred about 2-3 mya, during the biomechanical and immunological period of transition of early hominins from

forests to open savannahs.[114–118] Although technically challenging, biochemical methods were developed to successfully extract sialic acids from some *H. Neanderthal* fossils[108] and the lack of detectable Neu5Gc in these bones confirmed that CMAH inactivation occurred before the last common ancestor between *H. sapiens* and *H. Neanderthals* ~500,000 years ago.[108] *CMAH* inactivation has since been independently confirmed genetically by analyzing the limited Neanderthal and Denisovan genomic information that has become available.[119–121] Unfortunately, the same biochemical methods originally used by our group on *H. Neanderthals* fossils failed to obtain a detectable amount of sialic acid from *H. erectus* fossils,[122, 123] which likely decayed more rapidly in subtropical or tropical climates.[124] Therefore, three independent genomic methods were employed to approximate hominin CMAH loss.[108, 109] First, the timing of human *Alu* mediated exon deletion based upon *Alu* sequence analysis approximated that CMAH inactivation occurred 2.7 ± 1.1 mya. Second, molecular clock analysis of the CMAH pseudogene based on the substitution rate at nonsynonymous sites vs. synonymous sites estimated that CMAH inactivation took place 2.8 mya.[108] However, these estimations were based upon the divergence of humans and chimpanzees taking place ~5.3 mya, which was subsequently estimated to have occurred ~6 mya[125, 126] and the estimation of CMAH inactivation was changed to 3.2 mya.[109] Finally, genealogical analysis of haplotypes under significant linkage disequilibrium on a 7.3-kb *CMAH* intronic region was carried out on 132 chromosomes from 18 human populations worldwide and the most common recent ancestor was approximated at 2.9 ± 0.5 mya.[109] In summary, all three genomic approximations performed on *CMAH* placed the initial hominin *CMAH*

loss in the era of the *Australopithecines*[114, 127] and just prior to the emergence of genus *Homo*.

3.3. General Evolutionary Implications

The placement of hominin *CMAH* inactivation ~3 million years ago coincides with major evolutionary changes in hominins transitioning from forests to open savannahs, including biomechanical adaptation towards fully striding bipedalism,[118, 128, 129] increased consumption of other animals (expansion of prey base),[130–132] increased body and brain size,[133–139] and the earliest developments of Oldowan stone tool use.[116, 132, 140–143]

4. Proposed Mechanisms for Selection and Fixation of Human CMAH Pseudogene

4.1. Pathogen-mediated Selective Pressures

Many pathogens bind, synthesize, and/or utilize host sialic acids as a mechanism of survival or virulence[144–148] and many deadly human pathogens such as human influenza,[149–153] *Salmonella typhi,*[154] *and Plasmodium falciparum*[155–158] prefer Neu5Ac over Neu5Gc. Also, most pathogenic and viral sialic acid cleaving enzymes (sialidases/neuraminidases) studied prefer Neu5Ac over Neu5Gc substrates,[159–162] which could increase susceptibility for many infections.

Conversely, several pathogens have a binding preference for Neu5Gc,[163–167] including *Plasmodium reichenowi,* a close relative of *P. falciparum* that primarily infects chimpanzees.[157] *P. reichenowi* and *P. falciparum* were originally proposed to have diverged from a common ancestor around the same time as their preferred hosts (the

divergence of human and chimpanzee lineages): 5-7 million years ago (mya).[168–170] Subsequent molecular clock analyses indicated that *P. falciparum* is more likely the outcome of a much more recent transfer,[158] from a gorilla to humans.[171] Thus, a current hypothesis is that hominins initially selected for the loss of *CMAH* were able to escape an ancestral Neu5Gc binding pathogen related to *P. reichenowi (discussed further below)*.

4.2. Sexual Selection via Cryptic Female Choice (female immunity to paternal antigens)

Pathogen-mediated selection alone is unlikely to have led to a complete fixation of *CMAH* loss and was more likely a selection force for a balanced polymorphism.[172] or even expression polymorphisms, which occurs in cats and some dogs.[173–177] Therefore, a second mechanism was proposed for fixation: sexual selection through detection of Neu5Gc antigen on sperm by antibodies in the *CMAH* null female reproductive tract. This hypothesis was tested in female $Cmah^{-/-}$ mice that were systemically immunized against Neu5Gc and had circulating anti-Neu5Gc antibodies. When breeding with male WT mice (whose sperm are decorated with Neu5Gc), a major reduction (~30%) in fertility was recorded.[178, 179] It was also shown that human serum with high levels of anti-Neu5Gc IgG kills chimpanzee sperm in vitro.[178] Models of selection based on the frequency and strength of female incompatibility indicated that past an initial frequency threshold, which could have been reached by drift or by pathogen mediated selection, strong female sexual selection could have lead to rapid fixation of the loss of CMAH function mutation.[178]

5. A Mouse model for Human CMAH loss

Understanding the immediate ramifications of human *CMAH* is difficult, since our closest genetic ancestors diverged from us ~6 million years ago.[168, 170] and we have since evolved independently. Also ethical, legal and practical issues limit research on chimpanzees.[180] A Cmah null mouse (*Cmah$^{-/-}$*) with the human-like exon deletion was therefore generated to provide a practical model for studying the immediate loss of *CMAH* as it would have happened in hominins ~3 million years ago. *Cmah$^{-/-}$* mice have several human-like phenotypes (Table 1-1), including the induction of anti-Neu5Gc antibodies.[181], enhancement of cancer inflammation and progression of Neu5Gc containing tumors.[97, 182–185], enhanced immune clearance of recombinant Neu5Gc containing therapeutics.[186], delayed skin wound healing.[187], enhanced age-related hearing loss.[187, 188], altered immune responses.[189–191], sexual selection through Neu5Gc antigenicity.[178, 179], altered susceptibility to metabolic disorders.[192–194], altered susceptibility to muscular dystrophy.[195–197], and a xeno-antibody response against the vascular endothelium after nutritional incorporation of Neu5Gc.[198]

6. Biochemical Consequences of CMAH Loss in Humans

6.1. Redox Metabolism

CMAH oxidoreductase activity requires Fe^{2+} and a reducing cofactor (NADH or NADPH) for its enzymatic activity.[6] Therefore, disruption of CMAH activity could potentially change redox metabolism.[199], possibly by altering of the NAD^+:NADH steady state. Genetic evidence has led to speculation that CMAH loss could indirectly lead to increased oxidative damage.[188] and these mechanisms have been proposed to

explain gene expression differences observed during changes in metabolism or age-related hearing loss observed in *Cmah*^{-/-} mice.[187, 188, 192, 193] A detailed biochemical quantification of NAD^+:NADH and ROS levels in relevant Cmah^{-/-} tissues is of major interest and has not yet been performed.

6.2. Metabolic Incorporation, Recycling, and Degradation

Although human pseudogenization of *CMAH* results in complete loss of enzymatic activity, the *CMAH* pseudogene (*CMAHP*) is still transcribed, particularly in human stem cells where Neu5Gc uptake was reported to modulate Wnt/β-catenin signaling.[94] Thus far, this finding has not been mechanistically explained. This work, along with evidence that feeding Neu5Gc to primary human T cells suppresses their cell proliferation after activation.[190], suggests that independent of *CMAH* oxidoreductase activity, metabolic incorporation of Neu5Gc from exogenous sources can affect some cell signaling processes. More recently, Neu5Gc feeding has been found to suppress bacterial killing by macrophages from Cmah^{-/-} mice and humans, where the expression of the transcription factor C/EBPβ was also suppressed.[191] Further studies are needed to systematically elucidate the possible mechanisms contributing to the observed phenotypes (Table 1-2).

Free exogenous sialic acids can be taken up by cells through macropinocytosis and transported into the cytosol by the lysosomal transporter sialin.[200], which can be upregulated in hypoxic conditions including in cancer tissue.[201] Thus, the feeding of sialic acid is regulated by endolysosomal transport, which is very different from the feeding of the artificial peracetylated mannosamines that diffuse across cell membranes

and can become unnaturally hyper-enriched within the cytosol.[202] Cytosolic sialic acids can then be activated into CMP-Sias and utilized, as if they were endogenously produced. Similarly, endogenous cell surface sialic acids cleaved by the most abundantly expressed endolysosomal sialidase NEU1 experience a similar recycling via the transporter sialin.[200]

NEU1-mediated sialic acid catabolism and transportation occurs frequently, in order to maintain cell "steady state". Furthermore, NEU1 has long been considered the only "clinically relevant" neuraminidase, since loss of its expression and/or function results in the lysosomal storage and neurodegenerative disorder sialidosis.[203] However, there are three other vertebrate sialidases primarily involved in sialic acid catabolism that are less abundantly expressed (NEU2-NEU4) but play significant roles in many biological functions. Neu2 is cytosolic and highly expressed in muscle, where it is also found in the nucleoplasm.[204] NEU3 is plasma membrane associated and primarily targets cell surface gangliosides. NEU4.[205] is associated with intracellular membranes such as the endoplasmic reticulum and mitochondria.[206–208] Sialic acids are constantly being cleaved by these host sialidases and reutilized in new glycoconjugates before degradation, which may explain why the rate of sialic acid turnover is particularly slow in some tissues, such as brain.[209] The half-life in normal liver, where glycans are primarily protein bound.[30] is ~33 hours.[35, 38, 210] and the half-life in brain, where glycans are primarily lipid bound.[30] varies greatly between 4 and 45 days.[211, 212] Pulsing with Neu5Gc in a human B-cell lymphoma cell line yielded a similar half-life to what has been observed in brain tissue (~4 days).[213]

Although Neu5Ac and Neu5Gc are handled similarly by enzymes of the hexosamine biosynthetic pathway, terminal degradation of Neu5Gc results in the production of glycolate while degradation of Neu5Ac results in the production of acetate.[213, 214] (Figure 1-2). Thus, once CMAH converts Neu5Ac to Neu5Gc, the acetyl- to glycolyl- conversion is irreversible and potentially affects the metabolic homeostasis of acetate:glycolate ratios in cell metabolism. Since millions of sialic acids are constantly being recycled and degraded within a cell on a regular basis to maintain a steady state, it's not clear if intracellular acetate, which is quickly converted to Acetyl-CoA.[215] drives metabolism in a different direction than glycolate, which is converted to oxalate.[216] or glyoxylate.[214] Beyond limited gene expression studies.[188, 193], the true ramifications of these altered metabolic fates during constant degradation to maintain steady state have not been fully explored.

7. Cell Biological Consequences of CMAH Loss in Humans

Sialic acids have a multitude of functions on cell surfaces, such as repulsion of other cells.[217–221], protection from proteases.[222, 223], and modulation of certain cell signaling pathways.[98, 224–230] Some areas of interest regarding the ramifications of Neu5Gc loss on specific cellular processes are discussed below.

7.1 Potential Biophysical Effects

Although the effects of sialic acids on cell repulsion and adhesion has been extensively characterized.[30], very little is known about whether the structural differences between Neu5Ac and Neu5Gc could alter these processes.[231, 232] Theoretically, the loss

of Neu5Gc and subsequent loss of millions to tens of millions of hydroxyl groups at the terminal cell surface could systemically culminate in global and compartmental changes in membrane hydrophobicity between humans and other species (e.g. mice and chimpanzees). For example, in "gangliosides patches".[228] or lipid raft compartments, small changes in interactions involving Neu5Ac vs. Neu5Gc are potentially magnified in concentrated compartments or through multivalent interactions.[233] Changes in the partition coefficient of a drug alters its diffusion rate across membranes.[234], therefore changes in membrane hydrophobicity (through loss of Neu5Gc) could affect the diffusion of hydrophobic molecules across membranes. Although drug permeability has been studied extensively between species.[235, 236], the effect of human Neu5Gc loss on drug permeability or the permeability of gasses and molecules that diffuse across cell membranes (such as oxygen and carbon dioxide).[237, 238] has yet to be tested.

7.2. Changes in Siglec Binding

Changes in the ratio of Neu5Gc:Neu5Ac could potentially alter cell reactivity through changes in the binding of sialic acid ligands to complementary receptors, the Siglecs. Siglecs are Ig superfamily sialic acid binding lectins that commonly interact with host sialic acids on immune cells as self-associated molecular patterns (SAMPs) that suppress MAP kinase signaling and subsequent inflammatory responses.[239–243] This phenomenon is also exploited through Neu5Ac sialic acid mimicry by multiple invading pathogens.[147, 148, 244–246] Siglecs are rapidly evolving and highly variable across species.[247, 248], making it difficult to model human siglec biology in mice that have no functional equivalents to human Siglec-5, Siglec-6, Siglec-7, Siglec-11, Siglec-XII,

Siglec-13, or Siglec-14.[249, 250] Some human Siglecs have also evolved particularly rapidly such as human Siglec-9, which binds both Neu5Ac and Neu5Gc relatively equally whilst chimpanzee and gorilla Siglec-9 strongly prefers to bind Neu5Gc.[251] Furthermore, some Siglecs, such as sialoadhesin (Siglec-1),[252, 253] and MAG (Siglec-4).[254–256] have a conserved preference from mice to humans for Neu5Ac over Neu5Gc and it is therefore likely that human Neu5Gc loss increased their binding and signaling activity.[251] In mice, CD22 (Siglec 2) has a strong preference for Neu5Gc. Since CD22 is highly expressed on B cells (and to a lesser degree in T cells.[257–259]), loss of inhibitory signaling through loss of Neu5Gc ligands has been the proposed mechanism for the B-cell hyperreactivity observed in *Cmah*-/- mice.[189, 190, 260]

7.3. Changes in Neuraminidase Susceptibility

Although glycosphingolipids (particularly gangliosides) account for ~80% of the total glycan mass in the brain.[30], neuronal plasticity and development is heavily regulated by very long polysialic acids (PSA) that are primarily (~95%) conjugated to NCAM (NCAM-PSA).[261–265] and catabolized by host neuraminidases.[266–268] Despite its presence in most mammalian tissues, Neu5Gc is present in brain endothelium but absent from the neuronal brain tissue of all animals tested.[269] A proposed mechanism for this phenomenon has been attributed to a NEU1 preference for the α2-8Neu5Ac linkages common in brain polysialic acids.[269–271] over the α2-8Neu5Gc linkages commonly found in fish eggs.[272–274] A recent study has shown that Neu5Gc overexpression in the nervous system has multiple detrimental effects, including the loss of MAG ligand, impaired CNS

mylination, increased PNS degeneration, impaired locomotor activity, and impaired recognition memory.[271]

A series of studies have implicated endolysosomal NEU1 as a modulator of cell signaling at the cell surface, where it is thought to relocalize and cleave relevant sialic acids in a multitude of different signaling conditions, including the activation of receptor tyrosine kinases and TLRs.[275–279] Since most microbial sialidases have a Neu5Ac or Neu5Gc preference and host NEU1 prefers α2-8 linked Neu5Ac over Neu5Gc, it may be that NEU1 has a preference for the α2-3 or α2-6 Neu5Ac vs. Neu5Gc sialic acid linkages commonly found on all host cell surfaces. If there is indeed a difference, this could potentially contribute towards the differences in signaling observed when feeding Neu5Gc *in vitro*.[94, 190, 191]

7.4. Altered Cell Surface Sialic Acid 9-*O*-Acetylation

Another common modification of Neu5Ac is its 9-*O*-acetylation, which can inhibit surface recognition of sialic acids by some Siglecs (e.g. CD22) and certain pathogens, while also preferentially binding other pathogens, such as influenza C.[280, 281] Compared to chimpanzees, humans were found to contain higher levels cell surface 9-*O*-acetylation.[282] and this phenomenon is similarly observed in *Cmah*^{-/-} vs. WT mice.[187] Although 9-*O*-acetylation is found to disrupt CD22 (Siglec-2) binding *in vitro*.[283], genetic deletion in mice leads to the development of autoantibodies.[260] and more work is needed to determine how secondary changes in surface *O*-acetylation through CMAH loss could be contributing to human inflammation and autoimmunity.[284, 285]

7.5 Alterations in Cell Signaling

7.5.1. Hyperreactive Immunity

Post-translational glycosylation on B cell, T cell, and Toll-like receptors has been shown to modulate recycling, activation, and apoptosis susceptibility through clustering or other multivalent interactions.[286, 287] Recent studies have rendered the removal or reintroduction of Neu5Gc capable of modulating adaptive and innate immune cell responses in both humans and mice.[189, 190, 206, 207, 225, 276, 277, 288–300] Specific examples of the role of sialic acid in hyperreactivity are discussed below.

7.5.1.1 T-cell Receptor (TCR) Activation

Compared to chimpanzees tested in captivity, humans mount a greater proliferative response to a multitude of canonical T-cell receptor agonists, including α-TCR antibodies of multiple isotypes, l-phytohemagglutin, *S. aureus* super antigen, a superagonist α-CD28 Ab, and in mixed leukocyte reactions (MLRs).[296, 301] The same phenomenon was observed in *Cmah*^{-/-} mice compared to WT controls..[190] Although these differences were initially attributed to differences in Siglec expression, suppression of human T-cell proliferation can be achieved simply by feeding Neu5Gc during TCR activation, in conditions where there is no known difference in siglec expression or Neu5Gc preference.[190] There remain several unanswered questions about the influence of Neu5Gc on T-cell function, some of which are discussed in regards to HIV further below.

7.5.1.2. B Cell Receptor (BCR) Activation

The BCR forms complexes with multiple glycoproteins including Siglec-2 (CD22) and Siglec-G (Siglec-10 in humans) to modulate its threshold of activation. Chronic desensitization through exposure to self associated molecular patterns (SAMPs) are of particular importance to anergic B cells to help prevent autoimmunity and this has been reviewed extensively elsewhere.[287, 302–304] It has been reported that *Cmah*^{-/-} mice display BCR hyperreactivity *in vivo*.[189], which is partially attributed to loss of CD22 (Siglec-2) ligand. Furthermore, BCR hyper-reactivity may also occur in human B-cells.[296], which express a Siglec-2 that does not have a preference for Neu5Ac vs. Neu5Gc ligands *in vitro*.[251] Thus, CD22 preference alone may not explain the hyper-reactivity observed in B cells, particularly since hyperreactivity is also observed in human and mouse T cells.[190, 289]

7.5.1.3. Toll-Like Receptor 4 (TLR4) Activation and Bacterial Killing

Because *Cmah*^{-/-} mice experience delayed wound healing.[187], greater inflammation in some models of muscular dystrophy.[195], and increased growth of transplanted human tumor cells.[182], we investigated the innate immunity of *Cmah*^{-/-} mouse macrophages and re-examined the dogma that humans and chimpanzees mount similar innate inflammatory responses to endotoxin. Results were consistent with previous studies that established humans and chimpanzees to respond at the same order of magnitude to endotoxin.[305–309] A small increase in sensitivity of *Cmah*^{-/-} mice to endotoxin *ex vivo was also observed, with* a more profound effect *in vivo*. We further investigated a functional ramification of hyperinflammation (bacterial killing) and found that both *Cmah*^{-/-} mice and humans exhibited a greater capability to kill non-pathogenic

bacteria vs. their WT and Chimpanzee counterparts.[191] Thus, it can be speculated that human *CMAH* loss might have been beneficial for clearing minor infections but could be potentially deleterious in conditions of severe infection and endotoxic shock.

8. Physiological Consequences of CMAH Loss in Humans

8.1. Metabolic Disorders

Although the spontaneous development of Type 2 diabetes mellitus (T2DM) has been reported in apes.[310, 311], it is now an epidemic (along with obesity) in unhealthy humans and a major pathological consequence of diabetes is pancreatic islet β-cell loss due to apoptosis.[312] It has been reported that Cmah^{-/-} mice may experience an altered glucose metabolism at baseline.[193] and after consumption of a high fat diet.[192, 194] Impairment of glucose metabolism after high fat diet in Cmah^{-/-} mice was attributed to pancreatic β-cell failure rather than insulin resistance as determined by reduced pancreatic islet area.[192] Subsequently, WT and Cmah^{-/-} true littermate controls were independently examined under different conditions.[194] Although a detailed quantification was not reported, a reduction in both pancreatic islet size and β-cell number was independently observed and reported in Cmah^{-/-} mice compared to WT controls.[194] Thus, Cmah^{-/-} may have smaller pancreatic islets and reduced β-cells, which is interesting, since human pancreatic islets are smaller than monkeys.[313] and contain fewer β-cells (and more α-cells) compared to rodents and most non-human primates.[314, 315] A deeper investigation (including a detailed quantification of WT vs. Cmah^{-/-} pancreatic islets) into littermate controls under normal and high fat diet conditions is necessary to further determine the effects of Cmah inactivity on islet cell distribution and

glucose homeostasis. Thus, the role of Neu5Gc loss in diabetes mellitus susceptibility remains to be clearly determined.

8.2. Delayed Wound Healing

It has been reported that *Cmah*[^-/-] experience delayed skin wound healing.[187], with no obvious differences in immune cell recruitment, angiogenesis, or keratinocyte morphology reported. There has never been a follow-up study and the mechanisms involved in this phenotype have never been characterized or described. It could be speculated that the redox and/or macrophage changes mentioned before might be involved in delayed wound healing in *Cmah*[^-/-] mice.

8.3 Age-related hearing loss

By 9 months of age, *Cmah*[^-/-] mice display reduced hearing sensitivities across all frequencies, increased outer hair cell degeneration throughout the cochlea, and collapse of the outer organ of Corti compared to WT controls.[187] This phenotype has been independently confirmed.[188], but the biochemical mechanisms involved in this phenotype have not been characterized or described.

8.4. Gut Microbiome

The human body harbors trillions of microbes in the gastrointestinal (GI) tract.[316, 317] that are proposed to influence virtually every aspect of human health, including immune cell function.[318], metabolic disorders.[319], neurodegeneration.[320, 321], and cancer.[322, 323] Many gut microbes, including pathogenic types, have developed the

capability to utilize host sialic acids as an energy source in multiple ways, either through cleavage and/or scavenging with subsequent differential transport capabilities.[146, 324–328] Although sialic acid synthesis and neuraminidase preferences of many bacteria have been studied in relation to the host-pathogen interface, whether or not these individual microbes prefer Neu5Gc vs. Neu5Ac as an energy source in culture or within the intestine remains an open question to be explored in WT vs. *Cmah*^{-/-} mice.

9. Pathological Consequences of CMAH Loss in Humans

9.1 Antibody Production and Antigenicity

All humans who have consumed Neu5Gc express variable levels of circulating anti-Neu5Gc antibodies. The antigenicity of humans against Neu5Gc is not inherited from the mother and has been attributed to its cell surface presentation by common commensal bacteria (e.g. *H. influenza)* after consumption of Neu5Gc after birth.[95, 181], potentially coinciding with the introduction of cow based infant formula and baby food. Thus, all humans who continue to consume Neu5Gc could experience "Xenosialitis", the host response to a foreign but metabolically tolerated antigen (reviewed extensively elsewhere).[97, 98, 106, 329] Briefly, red meat is particularly high in Neu5Gc compared to poultry and fish, which contain low or undetectable Neu5Gc content (with the exception of caviar),.[97, 330] When Neu5Gc-rich food is consumed, it is absorbed and either eliminated in urine or metabolically incorporated into some tissues.[95, 331] The display of this foreign antigen induces an immune response via antibody and complement-mediated xeno-auto-antigen immunity. Chronic inflammation induced in this way was recently shown to increase the propensity for carcinoma formation in the *Cmah*^{-/-} mouse.[97] In this

regard, Neu5Gc has been reported for decades as a potential antigen in multiple cancer pathologies including lung.[92], liver.[92, 332], colon.[89, 92, 333], kidney.[334], breast.[93, 95, 335], skin.[335–337], ovary.[337], throat.[338] cancers and malignant lymphoma.[92] Although nutritional incorporation alone does not lead to anti-Neu5Gc immunization in *Cmah*^{−/−} mice, anti-Neu5Gc immunization is achieved by co-stimulation through injection with chimpanzee red blood cells (RBCs) or with Neu5Gc-containing tumor cell lines.[181] *Cmah*^{−/−} mouse anti-Neu5Gc antibody production has become an important model for the study of Neu5Gc antigenicity in humans..[97, 98, 106, 329] Anti-Neu5Gc antibodies have been directly shown to enhance tumor growth in *Cmah*^{−/−} mice by promoting cancer-associated inflammation.[97, 182] and anti-Neu5Gc antibodies have been identified as potential serum biomarkers for tumors in humans.[185] The possibility that higher levels of antibodies might be tumoricidal needs further study, since anti-Neu5Gc passively transferred into mice bearing a syngeneic MC-38 colon adenocarcinoma display a hormetic relationship between tumor growth and antibody dose.[183, 184] Potential effects of anti-Neu5Gc antibodies on the vascular endothelium have been modeled *in vitro* but have yet to be described *in vivo*.[198] Neu5Gc aggregates have also been found in dystrophic human and Cmah null mouse muscle tissue.[197] but the implications of this have not yet been fully understood.

9.2 Infectious Disease

Infectious diseases remain a major cause of death, disability, and suffering for hundreds of millions of people throughout the world.[339, 340] Many pathogens and toxins bind specific linkages of sialic acids.[341] and some major human-specific pathogens have

been found to prefer Neu5Ac over Neu5Gc linkages. Multiple infectious disease pathologies are further complicated by an apparent hyperactive immune system associated with Neu5Gc loss. Some examples are described below.

9.2.1. Malaria

The most common and most severe form of malaria parasite in humans is *Plasmodium falciparum,* and the meorozoite stage contains a 175 kDa erythrocyte-binding protein (EBA-175) that binds sialic acid residues on glycophorin A during invasion of the erythrocyte. It was demonstrated that EBA-175 binds human RBCs better than chimp RBCs and that Neu5Gc feeding could suppress the EBA-175 binding to a human erythroleukemia line.[157] In contrast, EBA-175 from the chimpanzee parasite *Plasmodium reichenowi* strongly prefers *Neu5Gc* and this *difference in binding preference* may account for the difference in species infectivity observed between *P. falciparum* and P. *reichenowi* for humans and chimps. Furthermore, primary RBCs from new world monkeys, which also lack cell surface Neu5Gc, also showed a similar susceptibility to EBA-175 binding compared to human RBCs.[157] Taken together, this data illustrates a human Neu5Ac binding specificity for *Plasmodium falciparum* EBA-175 protein. Whether or not *Plasmodium falciparum* has binding preferences for WT vs. *Cmah*$^{-/-}$ mouse RBCs have not been quantified.

9.2.2. Viral Infections

9.2.2.1. Influenza

Human influenza is a common upper respiratory pathogen still considered one of the greatest global pandemic threats.[342, 343] Historically, the 1918 swine flu pandemic killed more people than the entire First World War.[344] Influenza virus strains are named after their surface glycoproteins hemagglutinin (H) and neuraminidase (N) (e.g. H1N1), which bind and cleave sialic acids, respectively. Influenza type A hemagglutinin was the first microbial hemagglutinin ever described.[345] and its host specificity is dependent upon its sialic acid linkage preference.[149] For example, human influenza type A hemagglutinin preferentially binds α2-6-linked sialic acids (Neu5Ac), while avian influenza hemagglutinin preferentially binds α2-3-linked sialic acid.[346–348] Alterations in hemagglutinin binding specificity from α2-3 to α2-6 or from Neu5Ac to Neu5Gc can be achieved through minor amino acid substitutions.[349, 350] Influenza Neu5Ac or Neu5Gc binding preferences are also species dependent. For example, horse trachea expresses ~90% Neu5Gc and some equine influenza types prefer Neu5Gc for invasion and replication.[351] Swine trachea is ~50% Neu5Gc and swine influenza types can vary between Neu5Ac or Neu5Gc preference.[352] Interestingly, some human influenza strains are still capable of binding Neu5Gc,.[349] yet Neu5Gc feeding has also been found to suppress human epithelial cell infectivity in influenza type A strains with Neu5Gc binding capability.[353] Thus, both sialic acid linkage and sialic acid type affect the infectivity of influenza viruses in a species-specific manner and animals with similar airway sialic acid architecture to humans such as ferrets, who also lack a functional CMAH (see below), are also susceptible to airborne transmission of human influenza.[152]

9.2.2.2. Human immunodeficiency virus.

Human immunodeficiency virus (HIV) is a retrovirus that continues to infect and kill millions of people every year, particularly in regions of socio-economic disparity.[354] Although chimpanzees suffer from an AIDS-like SIVcpz immunopathology.[355], HIV progression to AIDS occurs more frequently and is more severe in humans compared to chimpanzees.[78, 356, 357] The causative mechanisms for this have never been unequivocally determined. HIV and HIV envelope proteins gp120 and gp41, and HIV gag protein p24 elicit a strong proliferative response in chimpanzee lymphocytes, even after years of HIV infection.[356] Conversely, human lymphocyte proliferative responses to HIV are relatively impaired compared to chimpanzee lymphocytes.[358, 359] and this has been proposed as a mechanism of human AIDS susceptibility.[356] Others have proposed that human AIDS is attributed to a greater susceptibility of lymphocytes to apoptosis.[360, 361], potentially through differential expression of Siglecs.[301, 361] Although Neu5Gc feeding alone is capable of altering T-cell proliferation after TCR activation.[190], whether or not Neu5Gc feeding can alter susceptibility to apoptosis in these systems is of interest and has not yet been systematically explored. It is also notable that Siglec-1 (sialoadhesin) which initiates formation of the virus-containing compartment and enhances macrophage-to-T cell transmission of HIV-1.[362] has a very strong preference for recognizing Neu5Ac over Neu5Gc, and shows increased positivity and altered distribution in humans compared with chimpanzees.[251]

9.2.3 Susceptibility to Bacterial Infections

9.2.3.1. *Streptococcus pneumoniae*

Streptococcus pneumoniae infections cause ~11% of all deaths among children younger than 5 years old[363] and are the major cause of community-acquired pneumonia in the elderly.[364] Like many other sialic acid utilizing pathogens, *Streptococcus pneumoniae* expresses a neuraminidase (nanA) and a sialic acid transporter (SatABC) that are together capable of harvesting and uptaking sialic acids from host mucins and other glycoconjugates in the nasopharynx. Interestingly, *Streptococcus pneumoniae* TIGR4 (serotype 4) has evolved to preferentially respond to Neu5Ac over Neu5Gc in conditions of low glucose (e.g. nasopharynx) with a positive feedback loop, upregulating both nanA and htrA, which protects from oxidative stress. This phenomenon may ultimately explain why *Cmah$^{-/-}$* mice experience a faster pneumococcal disease progression after intranasal but not intravenous challenge.[365]

9.2.3.2. Typhoid Fever

Salmonella enterica serovar Typhi (*S. Typhi*) is the human specific pathogen that continues to infect tens of millions and kill hundreds of thousands every year, particularly children who live in regions of poor sanitation and lack access to clean food or water.[366] Although *S.* Typhi is not capable of infecting mice, a mouse model was developed by injection with typhoid toxin, which preferentially binds Neu5Ac.[367] Thus, typhoid toxin is capable of producing typhoid fever symptoms in WT mice, but not in transgenic mice that overexpress *Cmah* (~98% Neu5Gc on all tissues).[154] Although this does not explain why humans and chimpanzees develop Typhoid infection after consumption of *S. Typhi,* it may help explain why humans experience a more severe form of Typhoid fever compared to Chimpanzees.[65, 66]

9.2.3.3. SubAB Toxins

Shiga toxigenic Escherichia coli (STEC) are a common foodborne pathogen.[368] that can cause serious disease, including bloody diarrhea, and sometimes haemolytic-uremic syndrome (HUS). STEC secretes a SubAB toxin that was found to preferentially bind Neu5Gc *in vitro* and *ex vivo*. Although metabolic introduction of Neu5Gc into human cell lines increased susceptibility to SubAB toxicity, infection of *Cmah*$^{-/-}$ mice resulted in a faster disease progression than in WT controls. This was found to be due in part by a lack of competitive inhibition of serum proteins in *Cmah*$^{-/-}$ mice.[166] Regardless, due to its Neu5Gc binding preference, humans who consume red meats rich in Neu5Gc may incorporate it into their gut epithelium and this can allow for toxicity of a subsequent SubAB positive infection.[369]

9.3. Muscular Dystrophy

In humans, Duchenne Muscular Dystrophy (DMD) is the most common and most severe muscular dystrophy affecting children.[370, 371] Concerted efforts towards the development of new practical therapeutics (gene and cell-based therapies) have resulted in many new promising paradigms, some on the forefront of clinical utilization.[371, 372] But until recently, a critical barrier to the progress in the field has been the stark difference in muscular dystrophy severity observed between mice and humans.[373–376], with mice showing minimal phenotypes. In this regard, *Cmah*$^{-/-}$ mice experience a profound increase in DMD and Limb Girdle Muscular Dystrophy 2D (LGMD2D) severity, most notably in DMD life expectancy.[195–197] *Cmah*$^{-/-}$/mdx mice experience greater muscle weakness, greater skeletal muscle fibrosis, greater immune cell recruitment to both cardiac and skeletal muscle tissue, more inflammatory cytokine

production in skeletal muscle, and decreased survival compared to WT/mdx controls.[195–197] Further work is needed to determine whether this difference is attributed to an intrinsic difference in muscle physiology, the adaptive immune system, and/or the innate immune system. This is testable by HSA-Cre (muscle), CD4-Cre (T-cells of adaptive immune system), or CD14-Cre (innate immune system) expression of *Cmah* in *Cmah$^{-/-}$/mdx mice*, but these experiments have yet to be reported. Besides an apparent increase of Neu5Gc vs. Neu5Ac affinity for α-laminin *in vitro*.[195], the underlying mechanisms responsible for this phenomenon are completely unknown and it is possible that "xenosialitis" could be an aggravating factor.[197] Understanding these mechanisms could reveal new therapeutic targets, possibly beneficial to the human lineage. They could also help us to understand what has set human muscle apart from mouse and chimpanzee muscle tissues, which are both relatively high in Neu5Gc.[197]

10. Evolutionary Implications of Human CMAH loss

Although sexual and microbial selection may have led to the fixation of *CMAHP*, subsequent changes in systemic inflammation and metabolism could have benefited ancient hominins transitioning towards an exposure to novel pathogen regimes during the transition from forests to open savannahs, an increased consumption of other animals, and the earliest developments of stone tool use.[114–116, 142, 377, 378] This activity likely resulted in many superficial injuries, potentially leading to a greater prevalence of infection. Under these conditions, the ability to kill and clear sublethal doses of bacteria at a greater capacity would be beneficial. However, CMAH loss may be a double-edged sword, since experimental evidence in mice suggests that *C*mah loss could be a

deleterious contributor in conditions of endotoxic shock.[191] A definite evolutionary cost of CMAH loss is at least two fold: first, the inability to modulate the ratio of Neu5Gc and Neu5Ac in the the glycocalyx of various tissues and their secretions, and second, the inability to use the presence of abundant Neu5Gc as an honest and costly signal of self.[379]

11. Other Medical Implications of CMAH Loss in Humans

11.1. Xenotransplantation

In addition to anti-Neu5Gc antibodies, humans, apes, and old world monkeys also lack a terminal α-galactose (α-gal) residue, which is a major antigen (along with Neu5Gc) causing hyper acute rejection after xenotransplantation to humans or decreased half-life of animal-based transplantations.[380, 381] To address this, pigs lacking α-gal.[382] or both α-gal and Neu5Gc.[383, 384] were created to decrease the immunogenicity of pig xenografts with some success.[385–388] Indeed, this work confirmed that in their bound form (e.g. in tissue), both α-gal and Neu5Gc are major foreign antigens that trigger inflammation and contribute to tissue rejection.[389–391] Ongoing studies are seeking to systematically improve potential sources for tissue xenotransplantation.[392]

11.2. Stem Cells and Recombinant Proteins

Nutritionally, there is a major difference in immunogenicity between Neu5Gc and α-gal that occurs during catabolism, where α-gal becomes free galactose (and further utilized normally), but free Neu5Gc is incorporated and presented on host cell surfaces as the same bound foreign antigen.[106, 393, 394] Therefore, all glycosylated human cells and

recombinant proteins grown in the presence of serum from other animals or on mouse feeder cells are potentially contaminated with the cell surface antigen Neu5Gc, potentially causing rapid clearance from circulation.[186] and/or triggering an antibody-mediated inflammatory response.[94, 186, 395–398]

12. Independent CMAH Loss in other Taxa

Since the original discovery of human-specific Old World primate CMAH loss, Monotremes (platypus), Sauropsids (birds and reptiles), Pinnipeds and Mustelids (walruses, sea lions and seals, ferrets), and platyrrhines (new world monkeys) have all been shown to have independently lost or inactivated CMAH and endogenous production of Neu5Gc.[152, 399–401] Similarly to humans.[347], ferrets also express high levels of α2-6 linked Sias in their airway epithelium, which along with Neu5Gc loss now explains their success as a model for human influenza infections.[152] Interestingly, new world monkeys are the only other primates known to lack Neu5Gc and are the standard model for *P. falciparum* infection in vivo.[157, 402, 403] Further field studies are necessary to determine if new world monkeys are potentially a reservoir for human *Plasmodium falciparum* infection in the wild.[404]

13. Summary and Outlook

Due to the high prevalence of sialic acids on all cell surfaces, the loss of *CMAH* in the hominin lineage likely had complex physiological ramifications, as evident in the multiple organ and cell types affected in *Cmah* mice. Many of these phenotypic differences observed between WT and *Cmah*-/- mice are possibly analogous to differences

between humans and chimpanzees, but much more work is needed to understand the many mechanisms likely at play. Importantly, these mechanisms may have implications for the treatment of diseases specifically affecting humans that are difficult to model in rodents, such as the case with muscular dystrophy. Exogenously, many deadly human pathogens have a Neu5Ac preference and possibly a linkage specific preference contributing to its species-specific infectivity, such as the case with influenza. Furthermore, many pathogens, commensal bacteria and/or symbiotic bacteria may have an optimal metabolic preference for Neu5Ac vs. Neu5Gc, such as the case with *S. pneumoniae*. Many of these and other open questions of human sialic acid biology remain unexplored or unreported. The hope is that these studies will highlight the significant importance of sialic acids and spur further investigations towards the development of therapeutic possibilities.
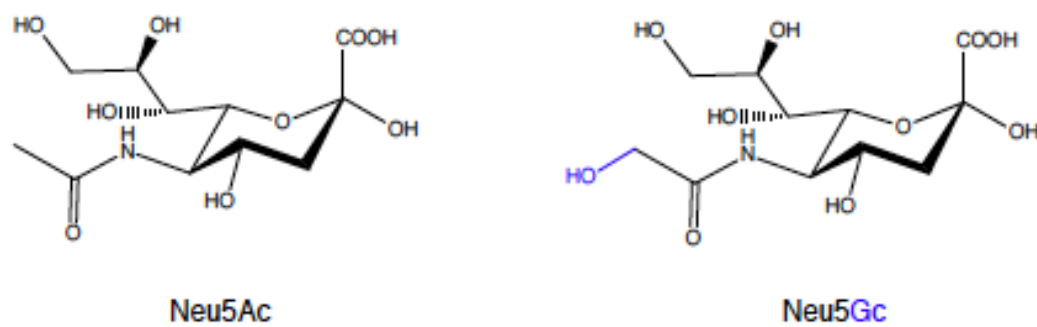
Figure 1-1: The two most common sialic acids in mammals, **Neu5Ac and Neu5Gc**, which differ by a single hydroxyl group, shown in alpha configuration. CMP-Neu5Ac is converted to CMP-Neu5Gc by the enzyme CMAH, which is pseudogenized in humans.
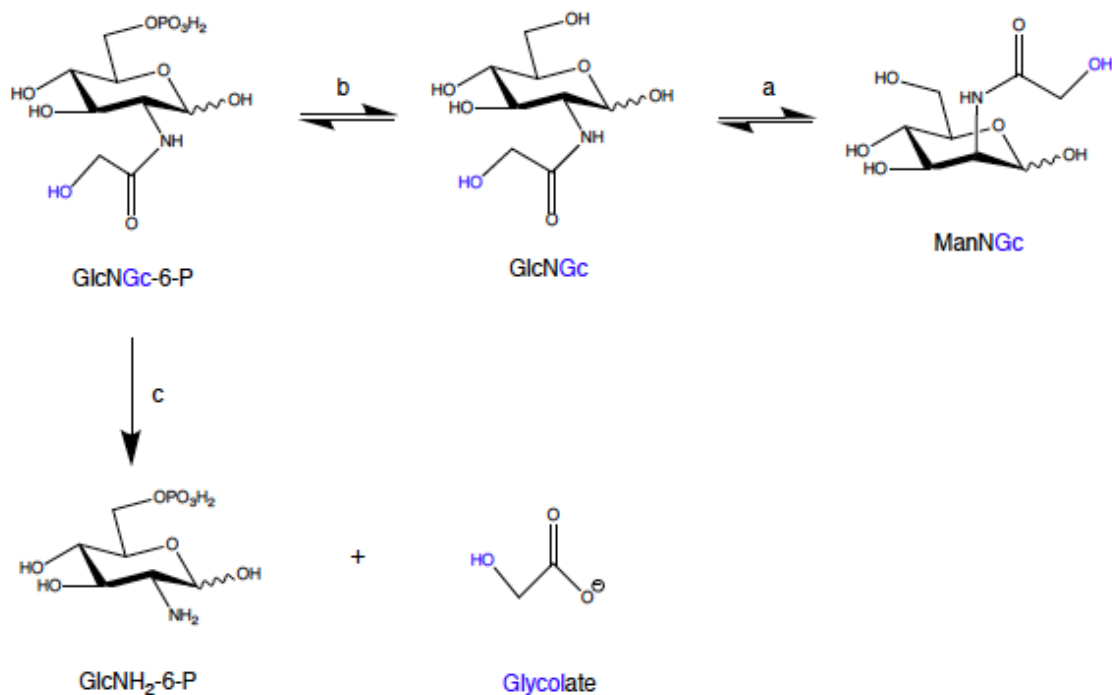
Figure 1-2: **Proposed pathway for the metabolic turnover of excess Neu5Gc (including blue moiety) or Neu5Ac (excluding blue moiety) in mammalian cells.** Neu5Gc and Neu5Ac are substrates for the pyruvate lyase, which results in the formation of ManNAc or ManNGc (*a*). GlcNAc-2-epimerase potentially modifies ManNAc or ManNGc into GlcNAc or GlcNGc, which could then potentially be phosphorylated at the 6 position by action of the GlcNAc kinase (*c*). Thereafter, the *N*-acetyl or *N*-glycolyl group might be irreversibly removed from GlcNGc-6-P by the GlcNAc-6-P deacetylase (*d*), which would result in GlcNH2-6-P and either acetate or glycolate, which may have different metabolic fates. Modified from.[213]

Table 1-1. Known host organs and cell types affected by Neu5Gc loss*

Organ	Cell Type	Phenotype	Proposed Mechanism
Skin	Multiple	Delayed Wound Healing	Unknown
Inner Ear	Multiple	Degeneration, Hearing Loss	Oxidative damage?
Blood/Multiple	B-cell	Anti-Neu5Gc Antibody Production	Nutritional incorporation of commensal bacteria
		BCR Hyperreactivity	Unknown (human)
			Reduced CD22/Siglec G ligand? (mouse)
	T-cell	Hyperproliferation	Unknown
	Monocyte/Macrophage	Hyperreactivity	Altered C/EBP expression?
	N/A (plasma)	Increased 9-*O*-Acetylation	Unknown
Liver/Multiple	Tumor	Increased Tumor Prevelance after Neu5Gc Immunization and Feeding	Xenosialitis
Muscle	Multiple	Increased Sensitivity to Dystrophin-Associated Muscular Dystrophies	Altered Scaffold Adhesion?
			Xenosialitis?
			Hyperinflammation
Pancreatic islets	α-cells, β-cells	Reduced pancreatic islet size, Insulin resistance	Altered redox metabolism?
Uterus	Multiple	Cryptic female sexual selection	Anti-Neu5Gc antibody mediated sperm killing
Blood vessels	Endothelial	Xeno-antibody response after Neu5Gc incorporation	Anti-Neu5Gc antibody mediated inflammation
Multiple	Multiple	Altered Interactions with Pathogens	Various

*See Text for discussion and literature citations

Table 1-2. General mechanisms by which loss of Neu5Gc can alter host biology

Mechanism	Ramifications
Loss of millions of cell surface hydroxyl groups	Increased membrane hydrophobicity?
	Altered cell recognition and receptor clustering?
Loss of CMAH oxidoreductase activity	Altered redox metabolism?
Increase in host neuraminidase activity	Altered cell signaling, endocytosis, adhesion?
Greater prevalence of acetate vs. glycolate metabolites	Altered cell metabolism?
	Altered bacterial flora homeostasis, particularly in conditions of low glucose (airway epithelium)?
Changes in siglec binding	Altered cell reactivity, particularly in immune cells
Production of anti-Neu5Gc antibodies	Xenosialitis (chronic inflammation after Neu5Gc consumption), cryptic female sexual selection against Neu5Gc containing sperm, rapid clearance of Neu5Gc containing biologics, transplantation rejection
Changes in microbial sialic acid binding at the cell surface	Altered susceptibility to many human Neu5Ac binding pathogens
Changes in microbial neuraminidase activity for host sialic acids	Increased susceptibility to many sialic acid scavenging bacteria, particularly in conditions of low glucose (airway epithelium)

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Chapter 2

Loss of CMAH During Human Evolution Primed

the Monocyte-Macrophage Lineage Towards a

More Inflammatory and Phagocytic State

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Abstract (250 words)

Humans and chimpanzees are more sensitive to endotoxin than mice or monkeys, but any underlying differences in inflammatory physiology have not been fully described or understood. We studied innate immune responses in $Cmah^{-/-}$ mice, emulating human loss of the gene encoding production of Neu5Gc, a major cell surface sialic acid. *CMAH* loss occurred ~2-3 million years ago, after the common ancestor of humans and chimpanzees, perhaps contributing to speciation of the genus *Homo*. $Cmah^{-/-}$ mice manifested a decreased survival in endotoxemia following bacterial lipopolysaccharide (LPS) injection. Macrophages from $Cmah^{-/-}$ mice secreted more inflammatory cytokines with LPS-stimulation and showed more phagocytic activity. Macrophages and whole blood from $Cmah^{-/-}$ mice also killed bacteria more effectively. Metabolic re-introduction of Neu5Gc into $Cmah^{-/-}$ macrophages suppressed these differences. $Cmah^{-/-}$ mice also showed enhanced bacterial clearance during sub-lethal lung infection. Although monocytes and monocyte-derived macrophages from humans and chimpanzees exhibited marginal differences in LPS responses, human monocyte-derived macrophages killed *E. coli* and ingested *E. coli* bioparticles better. Metabolic re-introduction of Neu5Gc into human macrophages suppressed these differences. While multiple mechanisms are likely involved, one cause is altered expression of C/EBPβ, a transcription factor affecting macrophage function. Loss of Neu5Gc in *Homo* likely had complex effects on immunity, providing greater capabilities to clear sub-lethal bacterial challenges, possibly at the cost of endotoxic shock risk. This trade-off may have provided a selective advantage when *Homo* transitioned to butchery using stone tools. The findings may also explain why the $Cmah^{-/-}$ state alters severity in mouse models of human disease.

Introduction

Approximately two to three million years ago (mya), our ancestors inactivated the gene encoding CMP-Neu5Ac hydroxylase (CMAH), an enzyme that adds a single oxygen atom to the *N*-acetyl group of the sialic acid *N*-acetylneuraminic acid (Neu5Ac), converting it to the hydroxylated form, *N*-glycolylneuraminic acid (Neu5Gc) [70]. Since *CMAH* is homozygously pseudogenized in all humans, this mutation resulted in the loss of tens of millions of hydroxyl groups from the plasma membrane surface of most cell types [226]. The overall consequences of human *CMAH* loss are being investigated by multiple approaches, including studies of mice harboring a human-like mutation in *Cmah* [187]. Studies in such mice indicate that partial reproductive incompatibility due to Neu5Gc loss ~2-3 mya likely contributed to speciation of the genus *Homo* [178], which also happens to be a time when the risk of injury and novel infections may have also increased due to scavenging and hunting, involving butchery of animal carcasses with stone tools [114–116, 142, 377, 378]. We also reported a difference in human and chimpanzee lymphocyte activation [296, 361] and showed that feeding Neu5Gc (which is metabolically incorporated and displayed) to activated human lymphocytes suppresses their proliferative response [190]. In addition loss of *Cmah* function accelerates the pathological progression of the mdx and LGMD2D mouse models of human muscular dystrophy [195, 196], concomitant with increased CD68^{+} innate immune cell recruitment to muscles and increased muscle production of inflammatory cytokines, including IL1β and MCP-1.

Tissue injury invokes a complex repair and regenerative response, which is substantially facilitated and potentiated by the resident and recruited host innate immune system [405–411]. Classically, the first responders to tissue injury are the resident

macrophage population, followed by neutrophils, which are rapidly recruited from circulating blood to the site of injury [412, 413]. Blood monocytes are also recruited and produce many inflammatory components [414–416]. The next wave of innate immune cell recruitment involves a more complex anti-inflammatory macrophage population [417–419], which facilitates wound healing [420–422]. Dysregulation of these inflammatory and anti-inflammatory pathways during chronic disorders can lead to unrestrained inflammation, ultimately leading to an increase in tissue fibrosis and in some cases, tissue loss [423–426].

Toll-like receptors (TLRs), which are innate pattern recognition receptors, are master regulators of the innate inflammatory status of cells [427, 428]. TLRs also recognize and potentiate the first line of host defense against pathogens. Recently, Toll-like receptor 4 (TLR4) has been implicated in the pathology of muscular dystrophy, wherein muscles are in a chronically inflamed state [407]. Classically, lipopolysaccharide (LPS), which can be released from the outer membrane of gram-negative bacteria, stimulates the production and secretion of pro-inflammatory cytokines such as TNFα, IL-6, and IL-1β [309, 429–432]. LPS is recognized by TLR4, which is part of the LPS receptor complex, along with CD14 and MD-2. TLR4 contains 9 N-linked glycans in its ectodomain, two of which are required for its membrane localization and others that are important for proper LPS binding [286].

Sialic acids such as Neu5Ac and Neu5Gc commonly terminate these N-linked glycan chains and their density and signaling influence can be affected by the action of endogenous neuraminidase 1 (a sialidase) [276–278, 298]. Sialic acid-bearing glycosphingolipids (gangliosides) that densely populate lipid rafts on cell membranes have also been shown to directly influence the translocation of TLR4 into lipid rafts after

LPS stimulation [433]. The specific impact of human CMAH/Neu5Gc loss has not been explored in these systems. Therefore, we focused our investigation on the impact of *Cmah* loss on innate immune responses, as a model for how Neu5Gc loss could have altered inflammatory physiology during the evolution of genus *Homo*.

Materials and Methods

Isolation of monocytes. Chimpanzee blood samples were collected into EDTA-containing tubes during routine non-invasive health screens of chimpanzee subjects at the Yerkes National Primate Center, Emory University, Atlanta, GA under local IRB approval by Emory University). All collections were made under the rules prevailing prior to the Sept 15, 2015 designation of captive chimpanzees as endangered species [180]. Chimpanzee blood samples were shipped overnight on ice to the University of California, San Diego. Human blood was collected at about the same time into identical tubes from healthy volunteer donors (following informed consent, under the approval from the University of California, San Diego Human Subjects IRB), and stored overnight on ice, to ensure similar treatment conditions prior to analysis. All health and safety issues related to handling of human and non-human primate samples are covered by an Institutional biosafety approval from the University of California San Diego Environmental Health and Safety Committee. All individuals who handle the samples received the required training regarding precautions for blood-borne pathogens. PBMCs were separated over Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden), followed by positive selection for monocytes using a CD14 MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's protocol.

Intracellular TNF labeling & cytokine release detection. One million monocytes were seeded per well of a 48-well plate or 100,000 macrophages per well in a 24 well plate were stimulated with varying doses of LPS, while unstimulated cells served as negative controls. Stimulation for all assays was conducted at 37°C and 5% CO2, in the presence of 0.5 mg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO) to inhibit cellular cytokine release. The intracellular cytokine content was determined after 4 hours of stimulation. Briefly, cells were fixed and permeabilized using Fix Buffer I and Perm/Wash Buffer I (BD Biosciences, San Jose, CA) and stained for intracellular TNFα. Samples were analyzed with a FACSCalibur (BD Biosciences). The frequencies of cytokine-positive cells were determined by subsequent analysis using FlowJo software (TreeStar).

Animals. All animal experiments were conducted under approved protocols and according to the regulations and guidelines of the Institutional Animal Care and Use Committee at the University of California, San Diego. Male and Female *Cmah*^{−/−} C57/B6 (Harlan) mice 8-10 weeks were injected with 30 mg/kg LPS (Sigma, L2880, Lot# 044M4004V) and monitored over the period of 72 hours for mortality.

Lung Infection Model. The murine sub-lethal lung infection model was performed with slight modifications, as previously described [434]. E. coli cultures were grown overnight in LB at 37 °C with shaking and then re-grown in the morning in fresh LB to a concentration of OD600 = 0.4. Bacteria were washed twice with PBS via centrifugation at 3220 ×g at room temperature and concentrated in PBS to yield 5×10^6 CFU in the 40 μL, the inoculation volume. Mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. Once sedated, the vocal chords were visualized using

an operating otoscope (Welch Allyn) and 40 μL of bacteria was instilled into the trachea during inspiration using a plastic gel loading pipette tip. Mice were placed on a warmed pad for recovery. Mice were sacrificed with CO_2 for bacterial counts 24 h after infection. To enumerate total surviving bacteria in the lungs, both lung lobes were removed and placed in a 2 ml sterile micro tube (Sarstedt) containing 1 ml of PBS and 1 mm silica beads (Biospec). Lungs were homogenized by shaking twice at 6000 rpm for 1 min using a MagNA Lyser (Roche), with the specimens placed on ice as soon as they were harvested. Aliquots from each tube were serially diluted for CFU enumeration on LB plates.

Mouse bone marrow derived macrophages. BMDMs were isolated from the femurs and tibia of 8-to-12-week-old WT and $Cmah^{-/-}$ C57BL/6 mice. Femurs and tibias were flushed with room-temperature Dulbecco's modified Eagle's medium (DMEM), and precursor cells were cultured in RPMI–20% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 50 ng/ml M-CSF at 37°C in 5% CO_2 for 7 days, with changes of media on day 4. One day prior to LPS stimulation, cells were lifted with 5 mM EDTA and 300,000 cells/well were seeded in a 24-well plate in RPMI–10% FBS.

Mouse peritoneal macrophages. Peritoneal macrophages from 8-to-12-week-old WT and $Cmah^{-/-}$ C57BL/6 mice were harvested 4 days after 2.5 ml of 3% thioglycolate intraperitoneal injection. A total volume of 10 ml ice cold PBS was used to extract 7-8 ml of peritoneal lavage. Cells were separated by adherence overnight, washed with PBS, lifted with 5 mM EDTA, then 300,000 cells/well in a 24-well plate or 100,000 cells/well in a 96-well plate were seeded in RPMI–10% FBS.

Human and Chimpanzee monocyte-derived macrophages. Human or chimpanzee PBMCs isolated from 20 ml of blood were plated at one 24 well. Monocytes were separated by overnight adhesion in RPMI containing 10% FBS and 1% Pen/Strep and cell culture media was carefully replaced with RPMI containing 10%FBS and 100 ng/ml Human M-CSF without washing. Cells were allowed to differentiate in the presence of M-CSF for 5 days. Additional RPMI + 10%FBS + 100 ng/ml Human M-CSF was added when needed in order to maintain normal pH. After 5 days, adherent cells were washed twice with PBS and resuspended in the appropriate experimental conditions.

Quantification of Sialic Acid Content by DMB-HPLC. Quantification of Sia content and type on acid-hydrolyzed samples of vertebrate tissues, polymers, and disaccharides was done using previously described methods of DMB derivatization at 50 °C for 2.5 h [435], followed by HPLC-fluorometry on a Phenomenex C18 column using an isocratic elution in 85% water, 7% methanol, and 8% acetonitrile.

Macrophage Phagocytosis Assay. pHrodo Red E. coli (ThermoFisher, P35361) and S. aureus (ThermoFisher, A10010) Bioparticles Conjugates for Phagocytosis were used exactly as indicated by manufacturer's protocol. 100,000 peritoneal macrophages/well were incubated with 100 uL pHrodo Bioparticle suspension for 1 or 2 hours. For confocal microscopy, cells were fixed in 2% PFA/PBS at 4˚C, then imaged by fluorescent microscopy at the exact same exposure.

Macrophage Bacterial Killing Assay. A single E. coli K12 colony was inoculated in LB and incubated overnight on a shaker at 37 ˚C. The next day, inoculum was diluted 1:100 and optical density (OD) was monitored up to 0.4 (corresponding to ~2x10^8 CFU/ml). Bacteria was washed once with PBS and resuspended in appropriate

cell culture media. Bacteria was then added to plates, centrifuged at 500g for 10 min, and then incubated with cells at 37°C. Macrophages were then lysed with 0.05% TX-100, scraped with a rubber stopper, and plated. Neutrophils and whole blood were diluted in the same lysis buffer before plating. Inoculum and all other samples were plated at four different dilutions (10^{-1} – 10^{-4}) in triplicates. Percent survival was calculated as the experimental sample CFU divided by the initial inoculum CFU.

LPS Binding Assay. Peritoneal lavage from WT and *Cmah*$^{-/-}$ mice was resuspended at 2 million cells/ml in cold PBS and handled on ice. Cell fluorescence was collected in real time by flow cytometry at a rate of approximately 1,000/second for 10 seconds. Tube was then removed, 10 ug fluorescent LPS (Sigma, F8666) was added followed by a quick vortex. Tube was then immediately returned to the flow cytometer and measured in real time for an additional 2 minutes.

RNA-Seq

Total RNA was assessed for quality using an Agilent Tapestation, with RNA Integrity Numbers (RIN) ranging from 8.8 to 10.0. RNA libraries were generated using Illumina's TruSeq Stranded mRNA Sample Prep Kit using 100 ng of RNA following manufacturer's instructions, modifying the shear time to 5 minutes. RNA libraries were multiplexed and sequenced with 50 basepair (bp) single end reads (SR50) to a depth of approximately 20 million reads per sample on an Illumina HiSeq4000.

Statistical Analysis.

Error data represents standard errors of the means (SEM) of the results. When comparing WT vs. *Cmah*$^{-/-}$ or human vs. chimpanzee macrophages, statistical analysis was performed using two-way analysis of variance (ANOVA) followed by the Tukey's

multiple comparisons test. For Neu5Ac and Neu5Gc feeding experiments on the same cells, Student's paired two-tailed *t* test was performed. For the survival assay, statistical significance was evaluated using the Logrank (Mantel-Cox) test with a 95% confidence interval. (Graph Pad Prism, version 7.0a). * (*P* < 0.05), ** (*P* < 0.01), and *** (*P* < 0.001) represent statistical significance. For RNA-seq, q values were determined using DESeq2 differential expression analysis.

Results

Mice and Macrophages with a Human-like loss of Neu5Gc Show Enhanced Sensitivity to LPS Activation. To simulate the immediate effects CMAH loss in the human lineage ~2-3 mya, we compared WT and congenic *Cmah*^{-/-} mice (Figure 2-1 A). First, we determined the impact of Neu5Gc on the LPS response *in vivo* by injecting WT and *Cmah*^{-/-} mice with potentially lethal doses of LPS and monitoring survival. At multiple doses, *Cmah*^{-/-} mice exhibited a significant reduction in survival rate compared to WT controls (Figure 2-1 B-D). Next we assessed the specific role of Neu5Gc in the macrophage inflammatory response by comparing LPS responses of macrophages derived from bone marrow or the peritoneal cavity. After 24 hours of stimulation, bone marrow-derived macrophages from *Cmah*^{-/-} mice produced more inflammatory IL-6 (Figure 2-1 E) and peritoneal macrophages secreted higher levels of MCP-1 (Figure 2-1 F). Notably, these phenotypes were not due to differential expression of the LPS receptor TLR4 (Figure 2-S1 A) or changes in LPS binding (Fig 2-S1 B). We concluded that macrophages from *Cmah*^{-/-} Mice exhibit higher sensitivity to LPS, possibly due to differences in signaling.

Macrophages from Mice with a Human-like loss of Neu5Gc Show Increased Phagocytosis and Killing of Bacteria. We next explored a more functionally relevant benefit of a stronger innate inflammatory response: the ability to clear and kill bacteria. We checked the capability of mouse peritoneal macrophages to kill *E. coli* and observed a ~50% reduction in recovery of bacteria incubated with *Cmah*^{-/-} macrophages (Figure 2-2 A). A similar increase in bacterial killing was observed in heparinized whole blood from *Cmah*^{-/-} mice compared to WT controls. (Figure 2-2 B). To prove that the observed differences were related to the absence of Neu5Gc, we studied peritoneal macrophages isolated from *Cmah*^{-/-} mice that had been fed equal concentrations of either Neu5Ac or Neu5Gc. This approach allows experimental manipulation of cells from the same individual to generate expression of either cell surface Neu5Ac or Neu5Gc, for direct comparison to each other. Free sialic acids in the media can only be taken up through macropinocytosis and utilized by the cell if they are transported into the cytosol by the endolysosomal transporter sialin [200]. Once there, free Neu5Ac or Neu5Gc must be transported to the nucleus to be CMP-activated [85] and then to the Golgi lumen to be conjugated onto glycoproteins or glycolipids. The half-life of free sialic acids fed to cells is ~4 days [213], so most of it should be utilized in this way after feeding. However, sialic acid degradation to their acetate (from Neu5Ac) and glycolate (from Neu5Gc) metabolites does occur during the constant recycling of endogenous Neu5Ac or Neu5Gc sialic acids during maintenance of steady state [209, 211–213]. Similar to results observed between WT and Cmah^{-/-} mice, the feeding of Neu5Gc to peritoneal macrophages isolated from *Cmah*^{-/-} mice indeed increased their Neu5Gc content (Figure 2-S3 A) and suppressed their capability to kill bacteria (Figure 2-2 C).

To determine the effect of Cmah loss on bacterial killing *in vivo*, we infected the lungs of WT and *Cmah*^{-/-} mice with *E. coli* K12 bacteria via intranasal inoculation. At 24h, *Cmah*^{-/-} mice exhibited a greater capability to clear *E. coli* K12 from the lungs *in vivo* compared to WT controls (Figure 2-2 D). WT and *Cmah*^{-/-} peritoneal macrophages were also incubated with *S. aureus* bioparticles that fluoresce when internalized into phagosomes and we observed a ten-fold increase in macrophage phagocytosis of the fluorescent bacterial particles in *Cmah*^{-/-} macrophages (Figure 2-2 E-F). Taken together, this data suggests that loss of Neu5Gc leads to increased phagocytic activity and bacterial clearance during infection.

Human Innate Immune Cells Show increased Phagocytosis and Killing of Bacteria Relative to Chimpanzees, which is Suppressed by Neu5Gc feeding. Chimpanzees have intact CMAH function and express high levels of Neu5Gc on their cell surfaces [157]. However, they have diverged independently for ~6-7 mya since the common ancestor with humans, and also have a significantly higher genetic diversity. The availability of samples was limited to chimpanzees in captivity undergoing routine blood testing during annual health checks (prior to the Sept 15, 2015 restriction on such ethically reasonable studies) [180]. We noted an unexplained technical pitfall associated with positively enriching for chimpanzee CD14^{+} PBMCs, since (unlike human samples) they are commonly contaminated with ~70% neutrophils. Therefore, we compared innate inflammatory response of limited samples of chimpanzee monocytes to those of humans and conducted the rest of our limited comparisons with monocyte-derived macrophages. Monocyte-derived macrophages from humans incubated with *E. coli* exhibited a greater killing capacity compared to chimpanzee controls (Figure 2-3 A). A

similar difference in bacterial killing was observed in whole blood from chimpanzees and humans inoculated with *E. coli* (K12) bacteria (Figure 2-3 B). Similar to *Cmah*$^{-/-}$ mouse macrophages, feeding human monocytes with Neu5Gc substantially reduced their capacity of killing bacteria (Figure 2-3 C). We tested the same human and chimpanzee monocyte-derived macrophages for both the intracellular TNFα expression after LPS stimulation and the phagocytosis of *E. coli* or *S. aureus* bioparticles. Macrophages were incubated with LPS in the presence of brefeldin A for 4h or with the bioparticles for 1h in the presence of autologous heat denatured serum. Although we did not observe a statistically significant difference in intracellular TNFα (Figure 2-3 D), the same human cells exhibited substantially greater phagocytosis of both *E. coli* and *S. aureus* bioparticles compared to chimpanzee cells (Figure 2-3 E-F).

Peripheral blood monocytes were stimulated with LPS for 4h and intracellular TNFα production was measured by flow cytometry. The percentage of TNFα positive cells were also measured by quantifying intracellular TNFα levels above baseline levels. Human CD14^{+} monocytes showed a marginal increase in TNFα production compared to chimpanzees *ex vivo,* but this difference was not statistically significant (Figure 2-S2 A-B). Considerable variation was also seen, as expected for the individuals from outbred species with varying genetic backgrounds, and environmental conditions.

We next differentiated human monocytes into macrophages while feeding the same cells either Neu5Ac or Neu5Gc for 4 days. We observed a significant effect on intracellular TNFα expression (Figure 2-4 A-B) and *E. coli* bioparticle phagocytosis (Figure 2-4 C-D) in Neu5Gc fed macrophages compared to Neu5Ac fed macrophages.

Neu5Gc Containing Mouse Macrophages Show Differential Expression of C/EBPβ. To determine if Neu5Ac or Neu5Gc feeding differentially altered gene expression, we performed RNAseq on macrophages from *Cmah*^{-/-} mice that had been fed either Neu5Ac or Neu5Gc for 3 days (Figure 2-5 A). Although several of the 36 genes that showed significant differential expression(Table 2-S1) could be affecting macrophage function, C/EBPβ (Figure 2-5 B) was of major interest because of its role in macrophage development and bacterial killing [436, 437]. Therefore, we examined the protein expression of LAP (a 34 kDa C/EBPβ isoform that is transcriptionally active in macrophages) and found that LAP expression is elevated in *Cmah*^{-/-} peritoneal macrophages compared to WT controls (Figure 2-5 C-D).

Discussion

We have suggested that initial hominin loss of CMAH may have been selected by an infectious agent, such as the malarial sporozoite recognition of erythrocyte sialic acids [157], and was then fixed relatively rapidly in a new population by virtue of cryptic female choice, mediated by intrauterine anti-Neu5Gc antibodies, selecting against ancestral Neu5Gc-positive sperm and/or embryos [178]. Here we present data suggesting that fixation of the *CMAH*-null state may have then been beneficial for the transition of these hominins towards increased tool usage, butchery of carcasses, and exposure to novel pathogens. Approximately 2-3 mya, when *CMAH* loss is likely to have been fixed in our lineage [70], the fossils of late *Australopithecus* and early *Homo* are found surrounded by Oldowan stone tools, presumably used to break bones and eat nutrient rich bone marrow

[114, 142, 378, 438]. This activity likely resulted in superficial injuries, combined with exposure to new bacterial pathogens, potentially leading to a greater prevalence of infection.

Under these novel circumstances, the ability to kill and clear sublethal doses of bacteria at a greater capacity could have been beneficial for the utilization of stone tools and the consumption of other animals. On the other hand, just as other ancestral mutations have become deleterious for specific modern pathologies [439, 440], CMAH loss may be a deleterious contributor to endotoxemia in humans today who experience overwhelming infections and sepsis [441].

Understanding the immediate effect of CMAH loss on ancient hominin physiology is impossible. Our best efforts are to compare humans vs. chimpanzees *ex vivo* and *Cmah*-/- vs. WT mice *ex vivo* and *in vivo*. When comparing humans and chimpanzee macrophages *ex vivo* we see a non-significant difference in inflammatory responses in the same conditions where we see significant differences in bacterial killing and phagocytosis. When studying the immediate effects of Cmah loss in mice, we see similar results: profound effects on bacterial killing, yet a relatively mild inflammatory phenotype *ex vivo*. Importantly*,* our experimental evidence also illustrates an increased sensitivity of *Cmah*-/- mice to endotoxic shock *in vivo*, which could be due to multiple mechanisms and other cell types. More work is needed to determine if an altered innate immune response in *Cmah*-/- mice contributes to other observed phenotypes, such as delayed wound healing [187], greater inflammation and fibrosis in the mdx model of muscular dystrophy [195], and increased growth of transplanted human tumor cells [182]. We speculate that these and other human chronic inflammatory states could be modeled in *Cmah*-/- mice..

Fully explaining all mechanisms underlying these phenotypic differences is complex and difficult. Given the global impact of eliminating tens of millions of N-glycolyl groups from cells via CMAH loss, there are multiple possible (and mutually non-exclusive) mechanisms contributing to our findings. For example, cytosolic degradation of excess Neu5Gc can result in generation of glycolate (instead of acetate generated from Neu5Ac breakdown) [213], with the possibility of a systematic alteration in cell metabolism. Previously published microarray data in WT and *Cmah*^{-/-} mouse muscle implicated CREB1, C/EBPα, and C/EBPβ as candidate transcription factors affected by Cmah loss [195]. Alterations in these transcription factors have been shown to affect both macrophage activation and systemic metabolism [436, 442–444] and appear to be differentially expressed and regulated in different tissue types of WT vs. *Cmah*^{-/-} mice. C/EBPβ expression is also suppressed in macrophages fed Neu5Gc and C/EBPβ (NF-IL6) knockout mice are known to experience a drastic reduction in the capability of their monocytes to kill bacteria [437, 444, 445].

It is also possible that Neu5Gc loss altered cell surface neuraminidase activity. Multiple reports have implicated neuraminidase 1 (Neu1) as an important regulator of the TLR4 response [276] and we previously reported that neuraminidase 1 (Neu1) prefers Neu5Ac to Neu5Gc in the α2-8 linkages common in polysialic acids [270]. The endogenous neuraminidase preference for α2-3 or α2-6 linked Neu5Ac and Neu5Gc, which are common on the surface of innate immune cells, needs further study. A further non-mutually exclusive possibility is that the loss of millions of hydrophilic cell surface hydroxyl groups on Neu5Gc (replaced by hydrophobic acetyl groups on Neu5Ac) led to global changes in cell surface biophysics, which could have global ramifications on cell

surface receptor localization, clustering, and signaling. It is reasonable to hypothesize that the cell membranes of humans and *Cmah^-/-* mice are overall more hydrophobic than chimpanzee or WT counterparts.. Further investigations are needed to determine if global changes in cell surface biophysics occur after Cmah loss.

Due to a limitation in the amount of samples combined with a high genetic variation, it is also difficult to study the differences in inflammatory physiology between humans and chimpanzees. Our results are consistent with previous studies that have established that humans and chimpanzees respond at the same order of magnitude to endotoxin [305–309]. However, direct comparisons of humans and chimpanzees with the same batch of LPS at multiple doses *in vivo* is no longer possible, nor is it possible to compare survival after endotoxic shock between humans and chimpanzees with the same batch and dose of endotoxin. Limited evidence using different batches of *E. Coli* endotoxin could suggest a difference between humans and chimpanzees, but are not directly comparable. For example, peak serum TNFα (68 to 1,374 pg/ml) and IL-6 (72 to 2,820 pg/ml) secretion of humans in response to 2ng/kg *E. coli* endotoxin bolus injection [305] is more sensitive than the serum TNFα (188 ± 54 pg/ml) and IL-6 (138 ± 37 pg/ml) concentrations of chimpanzees in response to 4 ng/kg *E. coli* endotoxin bolus injection [307]. Regardless, when we compared human and chimpanzee macrophages *ex vivo*, we did not observe a statistically significant difference in the inflammatory response to LPS in exactly the same conditions where we did observe a difference in both bacterial killing and bioparticle phagocytosis.

Furthermore, millions of years have passed since humans and chimpanzees diverged from each other [168, 170]. Therefore, studying differences in *Cmah^-/-* and WT

mice allows for greater sample sizes with much less genetic variation. Overall, we observed similar results in $Cmah^{-/-}$ vs. WT mice as we did in humans vs. chimpanzees and $Cmah^{-/-}$ mice are useful as a model for human evolution as well as human disease susceptibility.

While much further work is needed to define the multiple consequences of CMAH/Neu5Gc loss during human evolution, our current work suggests that there were likely complex effects on innate immunity, apparently providing a greater capability to clear sub-lethal bacterial challenges, possibly at the cost of increased risk of endotoxic shock. These findings may also help explain why the *Cmah* null state alters disease severity in mouse models of human disease associated with inflammation, like muscular dystrophy. They are also relevant to future modeling of human infectious disease states in mice.

Acknowledgements:
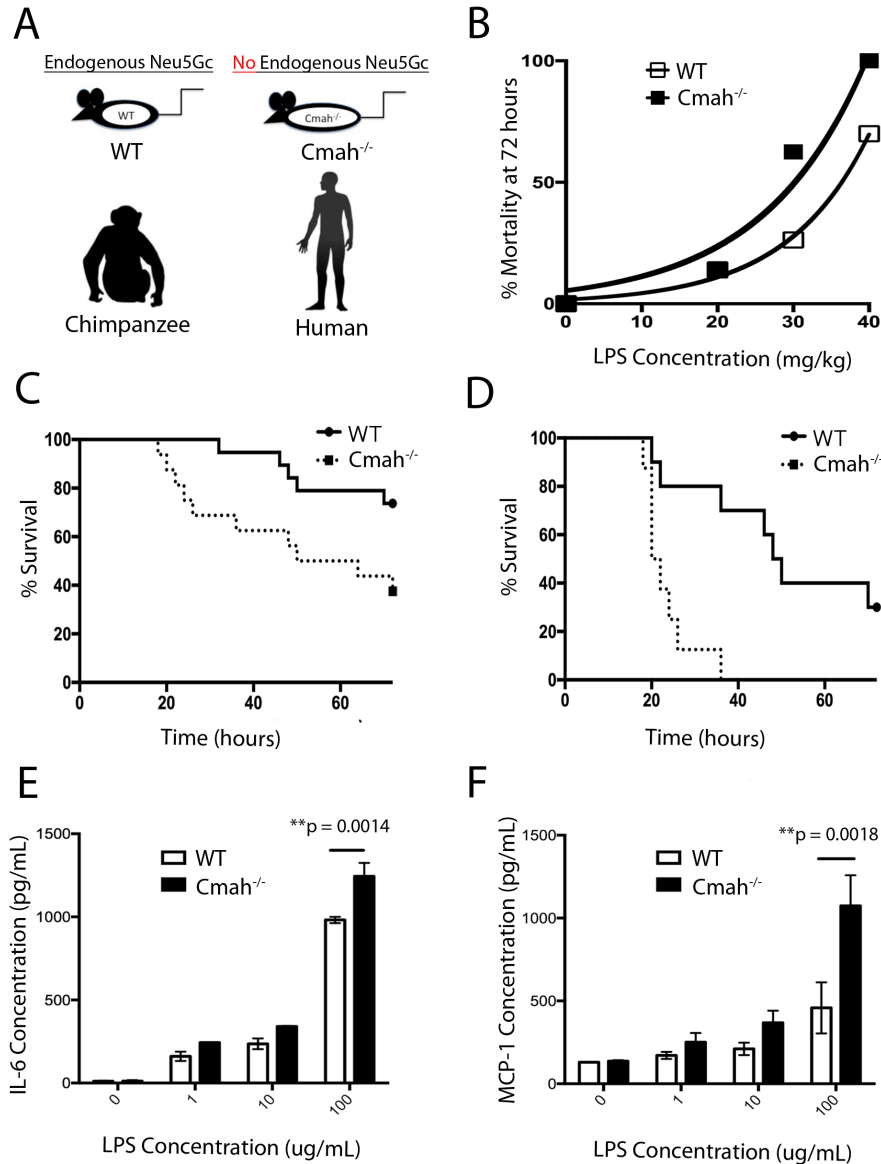
Figure 2-1: Comparison of the Inflammatory Response and Endotoxin Binding in WT and *Cmah^-/-* mice. (A) Humans and *Cmah^-/-* mice lack endogenous Neu5Gc, whereas WT mice and chimpanzees have high Neu5Gc expression. (B) Wild type and *Cmah^-/-* mice were subjected to lethal LPS challenge at different doses (20 mg/kg n=7, 30 mg/kg n=16, 40 mg/kg n=8) and % mortality at 72 hours was reported for each LPS concentration. (C) Wild type (n=19) and *Cmah^-/-* mice (n=16) were subjected to lethal LPS challenge (30 mg/kg) (D) Wild type (n=8) and *Cmah^-/-* mice (n=10) were subjected to lethal LPS challenge (40 mg/kg) and survival was monitored for 72 hours. (E) Bone marrow-derived macrophages (n=3) and (F) Peritoneal macrophages (n=4) from WT and *Cmah^-/-* mice were stimulated for 24 hours with LPS and the secreted cytokines in the supernatant were quantified by cytokine bead assay (BD).
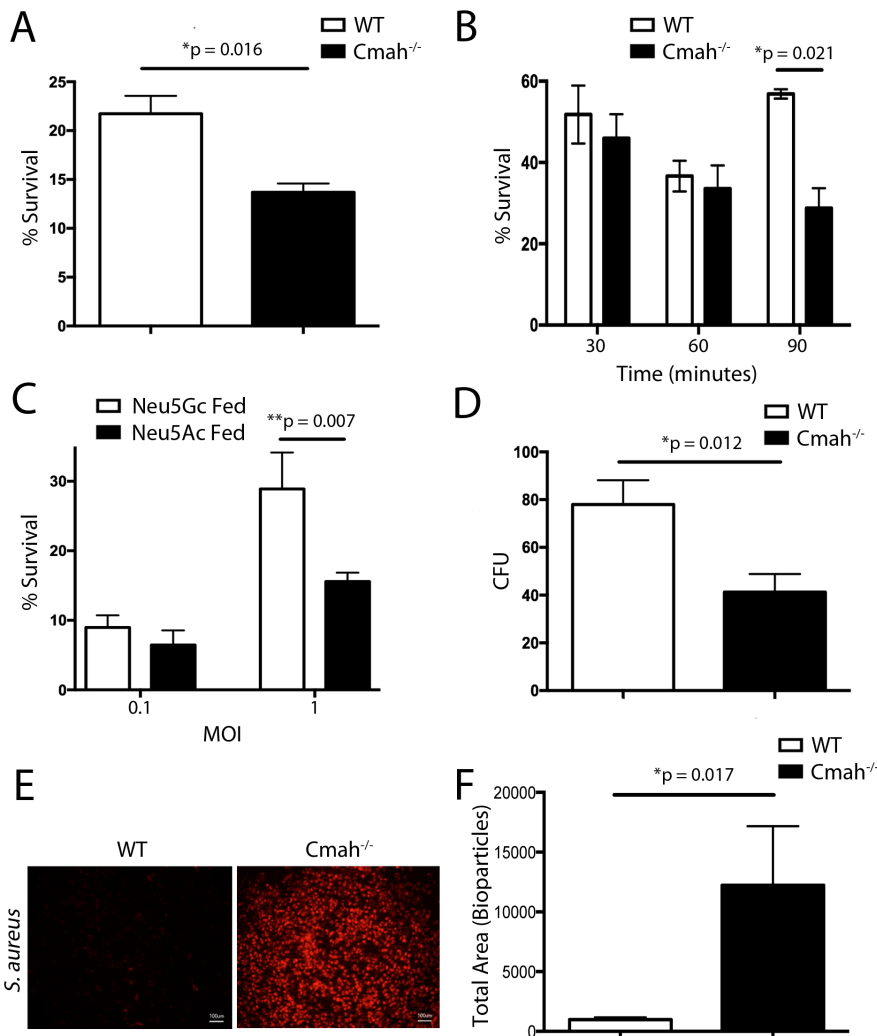
Figure 2-2: Comparison of Bacterial Killing and Phagocytosis in WT and *Cmah^-/- ex vivo* and *in vivo*. (A) Peritoneal macrophages from WT and *Cmah^-/-* mice were stimulated with *E. coli* K12 for 1 hour at an MOI of 1. (B) Whole blood from WT and *Cmah^-/-* mice (n=3) was stimulated with ~4 x 10^5 CFU of *E. coli* K12 and aliquots were plated at 30 min, 60 min, and 90 min. (C) Peritoneal macrophages (n=4) from *Cmah^-/-* mice were fed Neu5Ac or Neu5Gc for 4 days, followed by stimulation with *E. coli* K12 for 1 hour at MOI's of 0.1 and 1. (D) The lungs of WT (n = 9) and *Cmah^-/-*(n=8) mice were infected with 5 x 10^6 CFU of *E. coli* K12 and bacterial survival was assessed by plating an aliquot of the total lung homogenate 24 hours after infection. (E) Peritoneal macrophages from WT and *Cmah^-/-* mice were stimulated with pHrodo Red *S. aureus* bioparticles for 2 hours. (F) Bioparticle fluorescence from WT and *Cmah^-/-* mice was quantified by ImageJ (n=3).
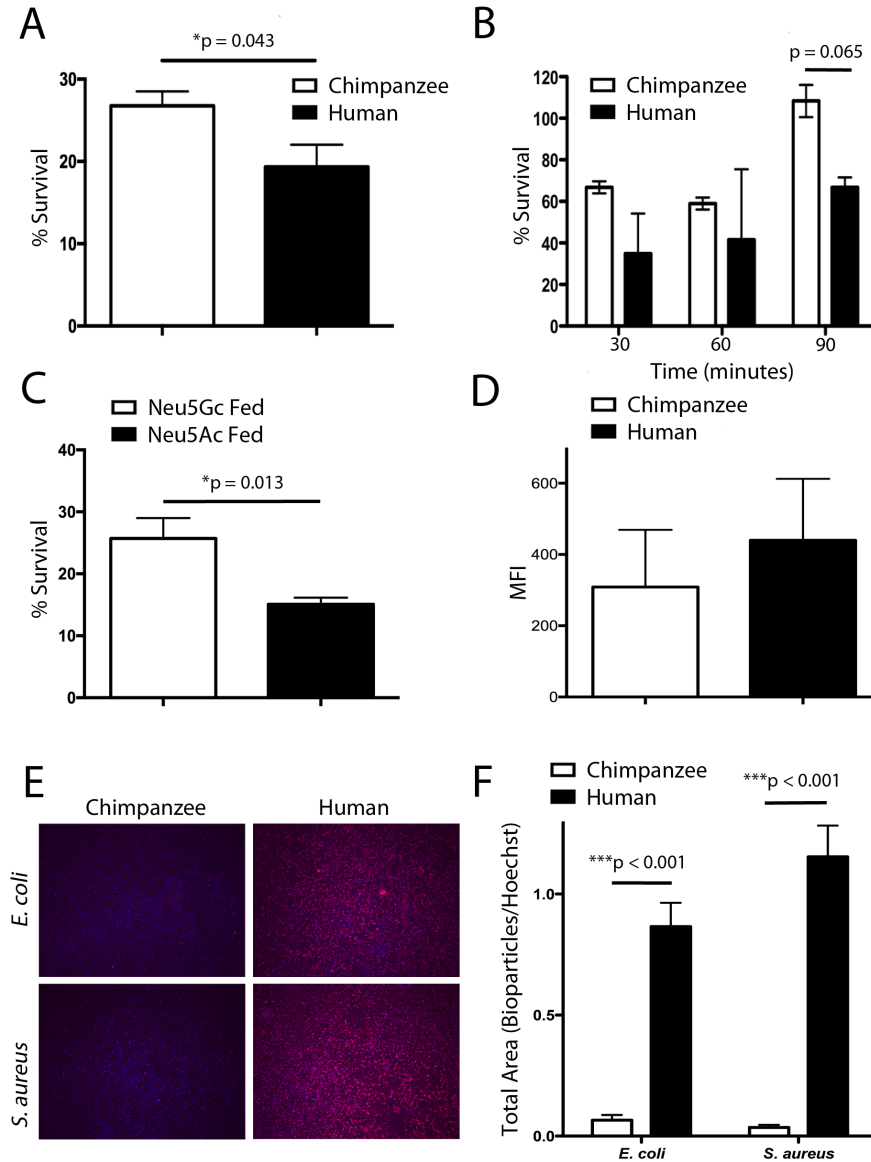
Figure 2-3: A. Comparison of Bacterial Killing and Phagocytosis in Humans and Chimpanzees. (A) Monocyte derived macrophages from humans and chimpanzees (n=6) were stimulated with *E. coli* K12 for 1 hour at an MOI of 1. (B) Whole blood from humans and chimpanzees (n=2) was stimulated with ~4 x 10^5 CFU of *E. coli* K12 and aliquots were plated at 30 min, 60 min, and 90 min. (C) Monocyte derived macrophages from humans (n=7) were fed 2 mM Neu5Ac or Neu5Gc for 4 days, followed by stimulation with *E. coli* K12 for 1 hour at an MOI of 1. (D) Monocyte derived macrophages from 5 humans and 4 chimpanzees were stimulated with 1 ng/ml LPS for 4 hours in the presence of Brefeldin A and intracellular TNFα expression was quantified by flow cytometry. (E) Monocyte derived macrophages from humans and chimpanzees were stimulated with pHrodo Red *E. Coli* or *S. aureus* bioparticles in pooled heat denatured autologous serum for 1 hour. (F) Bioparticle fluorescence from 5 humans and 4 chimpanzees was quantified by ImageJ.
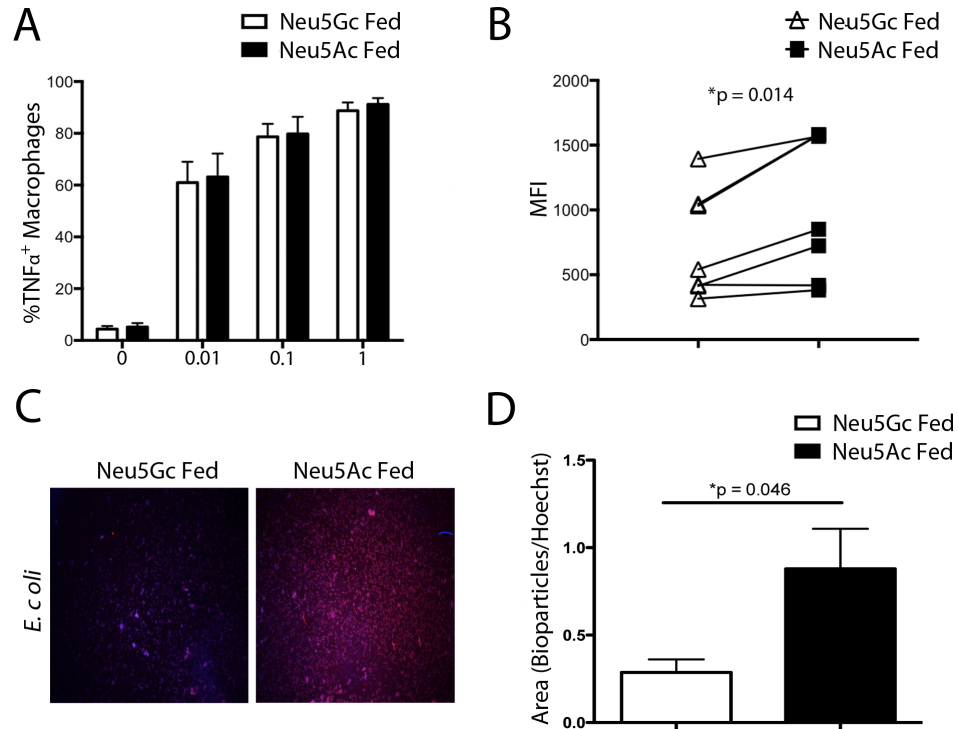
Figure 2-4: Comparison of TNFα production and Phagocytosis in Neu5Gc and Neu5Ac Fed Human Macrophages. (A) Monocyte derived macrophages from humans (n=7) were fed 2 mM Neu5Ac or Neu5Gc for 4 days, followed by stimulation with 0-1 ng/ml LPS in the presence of Brefeldin A for 4 hours and intracellular TNFα expression was quantified by flow cytometry. (B) At one concentration (1 ng/ml), the effect of Neu5Gc feeding was significant. (C) Monocyte derived macrophages were incubated with pHrodo Red *S. aureus* bioparticles in serum free conditions for 2 hours. (D) Bioparticle fluorescence from 4 individuals fed either Neu5Ac or Neu5Gc was quantified by ImageJ.
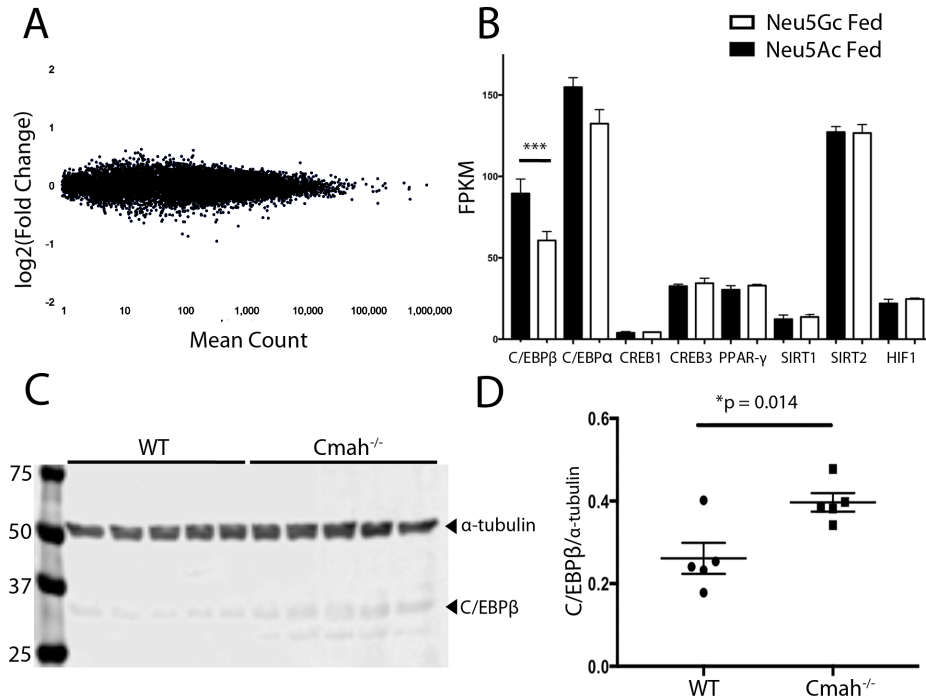
Figure 2-5: C/EBPβ Expression is Suppressed By The Presence of Neu5Gc. (A) MA plot of total RNA-seq results, comparing peritoneal macrophages isolated from Cmah^{-/-} mice (n=3) fed either 2 mM Neu5Ac or 2 mM Neu5Gc for 3 days. (B) C/EBPβ expression was suppressed in macrophages that had been fed Neu5Gc (***q value = 0.000495 by DESeq analysis). (C) Peritoneal macrophages from WT and Cmah^{-/-} mice (n=5) were immunoblotted against α-tubulin and LAP, a 34 kDa isoform of C/EBPβ (background was subtracted in ImageJ with rolling ball radius of 100 pixels). (D) ImageJ quantification and analysis of WT vs. Cmah^{-/-} LAP expression levels normalized to α-tubulin levels.

Supplementary Materials
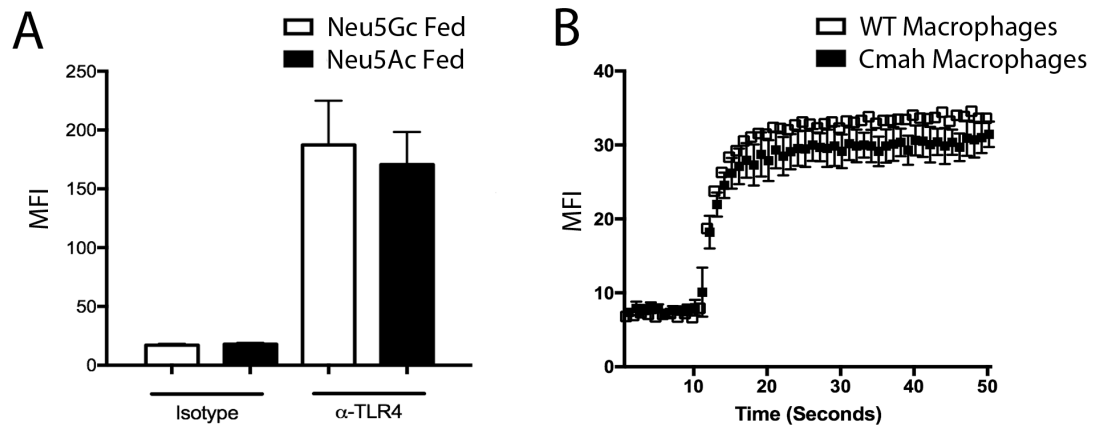
Figure 2-S1: Surface TLR4 Expression and Fluorescent LPS Binding Is Similar in WT and Cmah$^{-/-}$ Macrophages (A) Surface TLR4 expression of peritoneal macrophages isolated from WT and Cmah$^{-/-}$ mice (n=4). (B) Real time measurement of fluorescent LPS binding to macrophages present in the total peritoneal lavage from WT and *Cmah$^{-/-}$* mice (n=4).
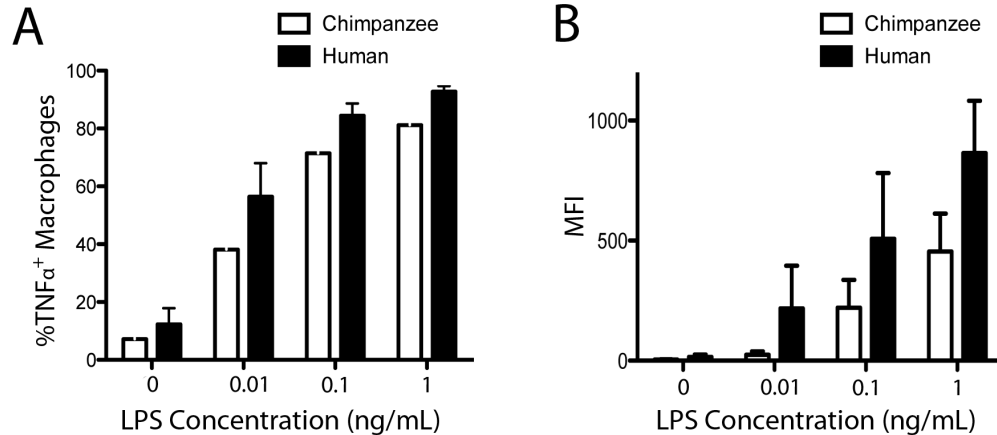
Figure 2-S2: Comparison of the Inflammatory Response in Human and Chimpanzee Monocytes Monocytes from humans and chimpanzees (n=6) were CD14 positively enriched and stimulated with LPS in the presence of Brefeldin A for 4 hours. (A) The %TNFα positive monocytes and (B) the median fluorescence intensity of intracellular TNFα were determined by flow cytometry.
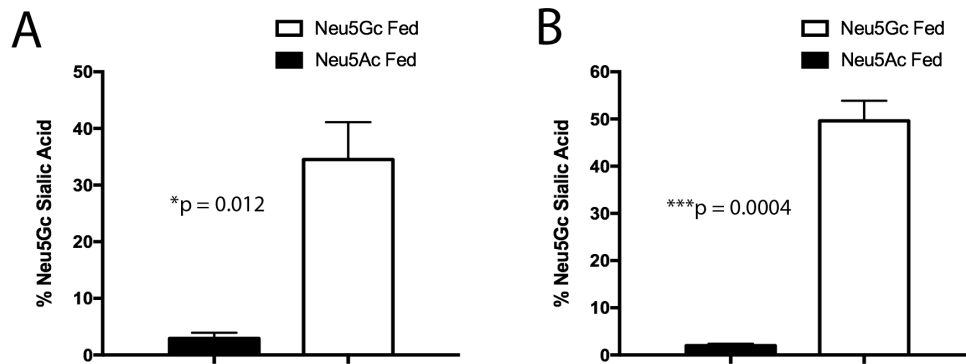
Figure 2-S3: **Mouse and Human Macrophages Incorporate Neu5Gc After Feeding**
(A) Peritoneal macrophages from Cmah$^{-/-}$ mice were fed 2 mM Neu5Gc or Neu5Ac for 3 days (n=5). (B) Monocyte derived macrophages from humans were fed 2 mM Neu5Gc or Neu5Ac for 4 days during differentiation into macrophages. Sialic acid content was determined by DMB-HPLC.

Table 2-S1: Genes Are Significantly Differentially Expressed between *Cmah*^{-/-} macrophages fed equal concentrations of either Neu5Ac or Neu5Gc

Gene	Mean Count	log2(Fold Change)	Standard Error log2 (Fold Change)	q value
Jund	2250	-0.558	0.0553	6.01E-20
Scand1	322	-0.954	0.108	5.75E-15
Crlf2	2200	-0.354	0.0628	0.0000627
Bri3	726	-0.694	0.124	0.0000627
Cebpb	1840	-0.548	0.106	0.000495
Bcl7c	109	-0.869	0.171	0.000495
Csf2ra	3780	-0.3	0.0593	0.000495
Fam20c	9130	-0.144	0.0284	0.000495
Map7d1	4550	-0.179	0.0355	0.000495
Ptms	3880	-0.243	0.0477	0.000495
Hnrnpa0	1190	-0.358	0.0744	0.00148
2310036O22Rik	1140	-0.301	0.0629	0.0016
H2afj	310	-0.624	0.136	0.00389
Dctn1	1970	-0.247	0.0549	0.00545
Sdf2l1	718	-0.316	0.0706	0.00574
Rnaseh2c	358	-0.413	0.0949	0.00904
Trim8	858	-0.297	0.0682	0.00904
Ehmt2	1850	-0.216	0.0507	0.0127
Ppp1r9b	2830	-0.309	0.0731	0.0133
Dhrsx	323	-0.586	0.139	0.0145
Slc15a3	21500	-0.195	0.0469	0.0165
B4galnt1	2200	-0.218	0.0532	0.0207
Heatr1	709	0.277	0.0678	0.0207
Zfp771	88	-0.83	0.204	0.0221
Atp13a2	3810	-0.278	0.0687	0.0227
Rps2	1580	-0.25	0.0619	0.0227
Ssbp4	961	-0.256	0.0641	0.0257
Zfp36l2	4670	-0.285	0.072	0.029
Kdm6b	409	-0.459	0.117	0.0333
Irf2bp2	1440	-0.279	0.0716	0.0352
Rnf44	1780	-0.248	0.0641	0.0398
Keap1	814	-0.276	0.0719	0.0422
Iscu	481	-0.313	0.0822	0.0447
Plxnd1	4120	-0.255	0.0667	0.0447
Tmsb4x	43100	0.101	0.0267	0.0455

Genes are displayed in order of significance (q value, determined by DESeq analysis).

The text of Chapter 2, in full, is a reprint of the material as it appears in the article of the same title by Okerblom J, Schwarz F, Olson J, Fletes W, Ali SR, Martin PT, Glass CK, Nizet V, and Varki A in the Journal of Immunology, 2017. The dissertation author was the primary author of this article.

Chapter 3

***CMAH* Inactivation Increases Skeletal Muscle Respiration and**

Endurance: Implications for the Running Phenotype of *Homo*

Abstract (250 words)

Humans are exceptional long-distance runners compared to other primates, a feature that likely emerged in genus *Homo* ~2 million years ago, and mainly attributed to anatomical and physiological adaptations, including striding bipedalism and heat dissipation. We studied the oxygen metabolism and exercise capacity of *Cmah*$^{-/-}$ mice, emulating *Alu*-mediated inactivation of *CMAH* ~2-3 million years ago, which may have contributed to speciation of the genus *Homo*. *CMAH* encodes production of Neu5Gc, a major cell surface sialic acid that differs by a single oxygen atom from the precursor Neu5Ac, which is thus increased in humans. *Cmah*$^{-/-}$ mice voluntarily exercise further and run faster after adaptation to exercise. They also manifest a human-like increase in endurance during treadmill testing. Gastrocnemius complex time to fatigue measured *in situ* was more than 2-fold higher compared to WT. E*x vivo*, soleus and diaphragm maximum force and fatigability was comparable. Capillarity to muscle fiber ratio is significantly higher in *Cmah*$^{-/-}$ vs. WT soleus. Metabolites in the pentose phosphate pathway and amino acid metabolism are enriched in exercise-trained Cmah$^{-/-}$ soleus compared to WT. Oxidative phosphorylation of soleus and diaphragm muscle fiber bundles measured *ex vivo* was also significantly higher in Cmah null vs. WT mice. C/EBPδ, a transcription factor involved in regulation of metabolic and inflammatory pathways is also differentially expressed in *Cmah*$^{-/-}$ vs. WT muscle. Loss of *CMAH* in Homo likely allowed greater maximum aerobic capacity, independent of biomechanical or dermatological evolution, contributing a selective advantage when *Homo* transitioned towards persistence hunting and greater consumption of meat.

Introduction

Early phases of the emergence of genus *Homo* coincided with a period of major transition from forests to the open savannahs 2-3 millions of years ago (Mya), which was coupled with many postcranial biomechanical adaptations towards the facilitation of taking longer strides and becoming very strong long distance runners[117, 118, 446]. As descendants of this lineage, we humans today rank among the top endurance runners in the animal kingdom, along with horses, dogs, ostriches etc.[447]. Notably, no other extant primate lineage has achieved this distinction[448, 449].

The genus *Homo* likely evolved from the *Australopithecus* lineage[450], which has now arguably become associated with the earliest development of stone tools (*K. platyops*[142] and *A. afarensis*[116, 132], ~3.3 mya). The development of these tools likely occurred as ancient hominins increased their consumption of other animals, and the stones were likely used for breaking open bones and consuming energy-rich bone marrow[140, 438, 451]. The biomechanical adaptations towards efficient running in *Homo erectus* (~1.8 mya) then apparently facilitated active killing of other animals. However, while some improvements in stone tools occurred[452], there is a >1 million gap before the earliest evidence of stone spear heads (~0.5 mya)[453] or wooden thrusting spears[454]. How *Homo* achieved its hunting prowess remains an unsolved mystery, and a likely explanation appears to be persistence hunting[455, 456], which would have required endurance running.

The exceptional aerobic capacity of humans during conditions such as persistence hunting has been attributed to the major alterations in skeletal biomechanics, loss of fur,

and the development of a vast network of eccrine glands that emerged in the old world ape lineage, which facilitated a more rapid and more efficient loss of heat compared to other mammals that primarily lose heat through apocrine gland sweating and/or panting[118, 446]. However, other mutually non-exclusive explanations can be considered, and the molecular events underlying this transition also remain unclear.

About 2-3 Mya, the Alu:Alu fusion-mediated loss of CMP-Neu5Ac hydroxylase (CMAH) function became fixed within the entire human lineage, possibly due to the selective pressures of an ancient Neu5Gc-recognizing pathogen, coupled with an anti-Neu5Gc antibody-mediated reduction in fertility[178, 457]. The ramifications of this major change in cell surface biochemistry continue to be explored in mouse models[458] and there is evidence for a multitude of significant phenotypic effects, including a human-like increase in sensitivity to certain muscular dystrophy pathologies[195, 196]. During these muscle studies, no major differences in the force frequency relationship ex vivo were shown between WT and Cmah null muscle itself. However, independent gene expression studies pointed towards alterations in redox biology[188, 193] and in several transcription factors linked to metabolism and inflammation, such as CREB1, C/EBPβ, and C/EBPα [195].

CMAH is a hydroxylase/mono-oxygenase enzyme that utilizes a variety of co-factors, including cytochrome b5/b5 reductase, iron, oxygen, and NADH during the hydroxylation of the *N*-acetyl moiety of the sialic acid *N*-acetylneuraminic acid (Neu5Ac), converting it to the hydroxylated form, *N*-glycolylneuraminic acid (Neu5Gc)[21, 22]. For this reason, alterations in redox biology due to Cmah loss have been proposed as a molecular mechanism for the age-related hearing loss that occurs in *Cmah*-

/- mice[188], but only very limited investigations into any physiological effects of alterations in redox metabolism, substrate utilization, or oxygen delivery have been performed [188, 192, 193, 459].

The loss of surface Neu5Gc, which results in the loss of millions of hydroxyl groups on all cell surfaces, could also potentially alter the hydrophobicity of membranes, which could in turn alter the interactions between red blood cells (RBCs) and endothelial cells within capillaries[460, 461]or perhaps modulate the diffusibility of hydrophobic molecules, such as oxygen[238]. Regardless of whether this notion is correct, oxygen delivery and utilization is particularly important in conditions of maximum aerobic capacity, such as during the conditions of persistence hunting [462, 463]. In this regard, alterations in both oxygen substrate utilization and in oxygen delivery (capillary density) have been shown to directly affect maximum aerobic capacity and muscular endurance in multiple mammalian models [464–468].

Considering multiple clues linking CMAH loss to muscle function and no known effect on skeletal biomechanics or the ability to lose heat through differences in fur and sweat glands, we became motivated to physiologically investigate the effects of Cmah loss on exercise endurance in *Cmah-/-* vs. WT mice.

Materials and Methods

Animals and tissue collection. All animal experiments were conducted under approved protocols and according to the regulations and guidelines of the Institutional Animal Care and Use Committee at the University of California, San Diego. All WT and *Cmah-/-* animals tested for this study originated from a single founder cage of

heterozygous *Cmah* C57BL/6N mice. All terminal tissue collection took place on euthanized mice that were fasted from 9:00 AM to 1:00 PM, with tissue collection beginning at 1:00 PM.

Treadmill Exercise. All mice were familiarized on a treadmill (model no. CL-4, Omnitech, Columbus, OH) for 10 minutes at a speed of 10 m/min for two consecutive days. After walking the mice for 10 minutes at 10 m/min, treadmill speed was then ramped up to 20 m/min over the course of 10 min at a speed of 1m/min per minute. Mice were then ramped to 25 m/min for 20 min, 30 m/min for 5 min, 35 m/min for 5 min, 40 m/min for 40 min, and then increased at 1 m/min per minute until exhaustion. For each running test, exhaustion was defined as the point at which the mouse was no longer able to maintain normal running position on the treadmill and/or sitting on a shock grid (≤ 0.1 milliamps) at the rear of the treadmill for 4 consecutive seconds.

Voluntary Exercise. Mice were allowed free access to a running wheel connected to a digital recorder for 15 days. Distance run and average speed were recorded on a daily basis at 9:00 AM. After 15 days of running, running wheels and food were removed at 9:00 AM, which was 4 h before terminal tissue and serum collection began.

Serum Chemistry Profiling. Chemistry tests are carried out at the UC San Diego Health Clinical Laboratory, La Jolla, which uses the Cobas 8000 automated chemistry analyzer (Roche) with a general coefficient of variance of <5%.

In situ Fatigability. Mice were anesthetized with isoflurane, and maintained on a heated surgery table to maintain temperature (37 ± 1) throughout the procedures. Oxygen saturation was monitored with a Mouse PulseOx (STARR Life Sciences) and maintained

at above 90% in all groups before experimentation. The sciatic nerve was then exposed and connected to electrodes. The gastrocnemius complex was then separated from the bone, and the tendons connected by a suture to a force transducer and electrically stimulated with a Grass S88X Stimulator (Astro-Med, West Warwick, RI) to contract with a single pulse (8 volts, 200-ms duration electrical stimulation) to set L_o. To measure fatigue, the gastrocnemius complex was stimulated with repeated trains (8 volts, 0.25 trains per second (tps), 80 Hz) until the force generated fell to 60% of the initial force output (time to fatigue).

Ex vivo Muscle Testing. Mice were anesthetized with isoflurane and the soleus[466] and diaphragm[469]} muscles were dissected. For each test, the muscle was mounted in a chamber filled with Kreb's solution at 32°C (126 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, and 25 mM $NaHCO_3$ at pH 7.2) and continuously bubbled with oxygen (95% O_2-5% CO_2). One tendon was tied with silk thread to a force transducer, and the other tendon was tied to an adjustable tube at the opposite end of the chamber, allowing muscle length to be changed incrementally to set the optimal muscle length (L_o). Muscles were stimulated with an S48 stimulator (Grass Technologies, Quincy, MA) at supramaximal voltage using platinum electrodes placed on either side of the muscle. Following 15 min of equilibration, the muscles were tested for contractile function and fatigue resistance. To assess contractile function, the force frequency relationship was established for each muscle. Maximum isometric tetanic force was measured by recording force output at stimulation frequencies of 1, 15, 30, 50, 80, 100, 150, and 250 Hz (500-ms train duration, 0.2-ms pulse duration) for soleus and diaphragm. The muscles were stimulated to contract once every 1.5 min to prevent

fatigue. Stability of the muscle was checked by periodically stimulating the muscle at maximal frequency throughout the course of the experiment. To measure fatigue, isometric tetanic contractions were elicited with 500-ms train duration and 0.2-ms pulse duration at 80 Hz (soleus) or 40 Hz (diaphragm). Stimulation frequency was increased every 2 min (soleus) in a progressive manner (from a contraction every 8s to 4s to 3s to 2s to 1 s). Time to fatigue was measured at the time it took each muscle to reach 60% of the maximal developed force for that muscle. When all testing was finished, L_o was measured using a reticle with a surgical microscope (Zeiss OPMI, Thornwood, NY), tendons were removed, and the muscles were blotted and weighed. Cross-sectional area was calculated, as previously described [470], and the specific force was expressed in Newtons per square centimeter.

High Resolution Respirometry. Following dissection, tissues were placed immediately in preservation solution at 4°C until measurement could be made (~30 min to 4 hr after euthanasia). Preservation medium (BIOPS) contained 10 mM Ca^{2+}EGTA buffer, 20 mM imidazole, 50 mM K^+-4-morpholineothanesulfonic acid (MES), 0.5 mM dithiothreitol, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine and a pH of 7.1. Tissue samples (~1 mg) were weighed using a microbalance and transferred into a calibrated respirometer (Oxygraph 2k, OROBOROS INSTRUMENTS, Innsbruck, AT) containing 2 ml of media in each chamber. Respirometry was performed in duplicate at 37°C in stirred media (MiR05) containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, and 1 g/l BSA essentially fatty acid free, adjusted to pH 7.1. $[O_2]$ in the media was kept between 300–500 pmol/ml.

A simplified substrate-uncoupler-inhibitor-titration (SUIT) protocol was utilized to assess maximum ADP-stimulated oxidative phosphorylation (OXPHOS)[471], including 10 mM glutamate and 2 mM malate to support electron entry through complex I (GM; 'LEAK' state), 5 mM ADP to stimulate oxidative phosphorylation, 10 mM succinate to maximize convergent electron flux at the Q-junction, and 10 μM cytochrome-c to test for outer mitochondrial membrane integrity as a quality control (>10% cytochrome C response was excluded).

Skeletal muscle morphology and immunohistochemistry. Capillaries and fibers in 10-μm cryosections were detected using the Capillary Lead-ATPase method [472]. Images were captured using a Hamamatsu Nanozoomer Slide Scanning System. Total capillary numbers, total fiber numbers, and total muscle areas were calculated by ImageJ.

Electron Microscopy (TEM) preparation of Muscle Tissue

Tissues were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15M Sodium Cacodylate buffer (SC buffer pH 7.4), treated with 1% osmium in 0.15M sodium cacodylate for 1-2 hr. on ice, washed with 0.15M SC buffer followed by rinsing in ddH2O on ice, incubated in 2% UA for 1 to 2 hrs at 4°C, dehydrated in ethanol, embedded in durcupan, sectioned by diamond knife, and post stained with uranyl acetate and lead. Images were captured on FEI Spirit Tecnai TEM at 80KV with Eagle 4kx4k camera.

Metabolomics Performed by West Coast Metabolomics Center, UC Davis Genome Center−Metabolomics, University of California Davis, 451 Health Sciences Drive, Davis, California 95616, United States Instrument: Gerstel CIS4 –with dual MPS Injector/ Agilent 6890 GC- Pegasus III TOF MS. Injector conditions: Agilent 6890 GC

is equipped with a Gerstel automatic liner exchange system (ALEX) that includes a multipurpose sample (MPS2) dual rail, and a Gerstel CIS cold injection system (Gerstel, Muehlheim, Germany) with temperature program as follows: 50°C to 275°C final temperature at a rate of 12 °C/s and hold for 3 minutes. Injection volume is 0.5 μl with 10 μl/s injection speed on a splitless injector with purge time of 25 seconds. Liner (Gerstel #011711-010-00) is changed after every 10 samples, (using the Maestro1 Gerstel software vs. 1.1.4.18). Before and after each injection, the 10 μl injection syringe is washed three times with 10 μl ethyl acetate. Gas Chromatography conditions: A 30 m long, 0.25 mm i.d. Rtx-5Sil MS column (0.25 μm 95% dimethyl 5% diphenyl polysiloxane film) with additional 10 m integrated guard column is used (Restek, Bellefonte PA). 99.9999% pure Helium with built-in purifier (Airgas, Radnor PA) is set at constant flow of 1 ml/min. The oven temperature is held constant at 50°C for 1 min and then ramped at 20°C/min to 330°C at which it is held constant for 5 min. Mass spectrometer settings: A Leco Pegasus IV time of flight mass spectrometer is controlled by the Leco ChromaTOF software vs. 2.32 (St. Joseph, MI). The transfer line temperature between gas chromatograph and mass spectrometer is set to 280°C. Electron impact ionization at 70V is employed with an ion source temperature of 250°C. Acquisition rate is 17 spectra/second, with a scan mass range of 85-500 Da.

Antibodies. C/EBPδ Antibody #2318 (Cell Signaling, MA, USA) was used for western blot.

Statistical Analysis. Error data represents standard errors of the means (SEM) of the results. When comparing WT vs. Cmah^{-/-}, statistical analysis was performed using two-way analysis of variance (ANOVA) followed by the Tukey's multiple comparisons

test or Student's paired two-tailed t test when appropriate. Unless otherwise specified in the figure legend, * (P < 0.05), ** (P < 0.01), *** (P < 0.001), and **** (P < 0.0001) represent statistical significance.

Results

Mice with a human-like Cmah deficiency perform better during forced exercise and voluntary exercise testing. WT and *Cmah*^{-/-} mice were subjected to an endurance ramp up treadmill exercise protocol. Separate groups were tested either at baseline (Figure 3-1 A) or after 30 days of voluntary exercise with treadmill training once a week (Figure 3-1 B). In multiple independent tests, *Cmah*^{-/-} mice displayed a greater running endurance compared to WT controls, both at baseline and after adaptation to exercise. A separate study also measured exercise parameters (speed and distance) and serum biochemistry profiles of WT and *Cmah*^{-/-} mice during 15 days of voluntary exercise. After adaptation to exercise, *Cmah*^{-/-} ran at a significantly faster pace (Figure 3-2 A) and longer distance (Figure 3-2 B) compared to WT controls. Although Cmah null mice outperformed WT mice, a serum chemistry panel performed after day 15 of exercise revealed that multiple transaminases, which indicate muscle damage during intense exercise [473, 474], were increased only in the serum of exercise-trained WT mouse, but not exercise-trained Cmah null mice (Figure 3-2 C-D). Lactate was also slightly elevated in the soleus of WT exercise-trained mice but not *Cmah*^{-/-} exercise-trained mice (Table 3-1). These results indicate that WT muscle may be exercising at a greater intensity or stress, despite a reduced speed and distance compared to *Cmah*^{-/-}.

Gastrocnemius complex from *Cmah^-/-* mice display a greater resistance to fatigue *in situ* but not *ex vivo*, which could be related to a difference in capillarity. To directly measure the fatigue resistance of WT vs. *Cmah^-/-* mice with intact vascular and nervous systems, we electrically stimulated the gastrocnemius complex to contract *in situ* in anesthetized mice. *Cmah^-/-* gastrocnemius complex reached fatigue (60% of initial force) later (7.1 min ± 0.4) than the control group (3.2 min ± 0.3) (Figure 3A-B). We next evaluated the force-frequency (Figure 3-3 C-E) and time to fatigue (Figure 3-3 F) in an isolated peripheral muscle (soleus) and respiratory muscle (diaphragm). No change in the maximal force (soleus p=0.4, diaphragm p=0.8) or time to fatigue (soleus p=0.9, diaphragm p=0.4) was detected between the experimental groups. However, histological analysis showed that the capillary to fiber ratio of the soleus is higher in non-exercise trained *Cmah^-/-* mice than WT controls (Figure 3-4 A). There was a trend for a higher capillary density (p= 0.1) (Figure 3-4 B) but not mean fiber area (p= 0.6) in Cmah null soleus compared to WT (Figure 3-4 C). Mitochondrial volume densities in WT and *Cmah^-/-* tibialis anterior muscles were not significantly different (p= 0.4, Figure 3-4 D).

Pentose phosphate pathway and amino acid metabolism are enriched in *Cmah^-/-* exercise trained mouse locomotor muscle. To determine any difference in the major metabolic pathways, the soleus muscles from exercised and non-exercised WT and *Cmah^-/-* mice were subjected to GC-TOF untargeted metabolomics analysis. The top 100 metabolite expression differences are visually illustrated by a heat map (Figure 3-5 A). Metabolic pathway enrichment analysis comparing exercise-trained WT and *Cmah^-/-* mice revealed enrichment of metabolites that comprise the pentose phosphate pathway and amino acid metabolism (Figure 3-5 B). Targeted evaluation of specific metabolites

illustrates a greater upregulation of ribulose-5-phosphate, ribose, xylulose, and multiple amino acids in *Cmah*^{-/-} exercise-trained muscle compared to WT exercised trained muscle*,* particularly proline, phenylalanine, and the branched chain amino acids leucine and isoleucine. Aspartate, threonine, asparagine, and tyrosine were also upregulated in *Cmah*^{-/-} exercise-trained soleus but not in WT exercise-trained soleus in the same conditions. Several other amino acids were also upregulated in both WT and *Cmah*^{-/-} exercised tissues as a general effect of exercise shared between the two groups, although most were trending at a higher upregulation in *Cmah*^{-/-} exercised muscle vs. WT exercised muscle. Lactate levels were also slightly elevated in WT exercise-trained muscle but not in *Cmah*^{-/-} exercise-trained muscle (Table 3-1).

Locomotor and respiratory fibers from *Cmah*^{-/-} mice show a greater mitochondrial efficiency. To assess the coupled respiratory capacity of WT and *Cmah*^{-/-} muscle, oxygen consumption (O_2 flux) was measured in permeabilized fiber bundles derived from soleus (Figure 3-6 B) and diaphragm (Figure 3-6 A) in the presence of complex I substrates (glutamate and malate), state III (saturating ADP conditions), and complex I + II substrates while in state III (glutamate + malate + ADP + succinate) with subsequent cytochrome C addition as a quality control. We found that in saturating conditions of ADP and mitochondrial complex I +II substrates, *Cmah*^{-/-} muscle had a greater O_2 flux (oxygen consumption per second normalized to muscle fiber bundle mass) compared to WT controls (Figure 3-6 A-B), indicating a higher maximum ADP-stimulated oxidative phosphorylation (OXPHOS) capacity in *Cmah*^{-/-} mouse muscles compared to WT controls.

Differential Expression of the Transcription Factor C/EBPδ

Based on gastrocnemius gene expression data reported previously [195], we sought to investigate whether the transcription factor C/EBPδ protein expression was upregulated in the soleus muscles of *Cmah*^{-/-} mice and confirmed that C/EBPδ protein expression is indeed upregulated in *Cmah*^{-/-} soleus muscle compared to WT controls (Figure 3-7 A-B).

Discussion

Hominin evolution is like a bush, with many stems arising in many places, making it difficult to truly determine the exact origins of Homo sapiens, the only species to have successfully reached the top of the bush [475]. Hominin *CMAH* loss coincides with the major biomechanical and environmental changes that took place while transitioning from the forests to the open savannahs[108, 109, 114, 127] and this may have facilitated an increase in the capabilities of ancient hominins to perform persistence hunting. For this reason, the exact timing of *CMAH* loss in the fossil record is of major interest and a biochemical method to indirectly determine the sialic acid composition of ancient hominin fossils by measuring the composition of a highly stable Neu5Gc metabolite has recently been developed (Bergfeld et. al., in review).

Emulating human *CMAH* loss by knockout of *Cmah* in mice illustrates a molecular mechanism for an increased capability for the utilization of oxygen, most evident by an increase in endurance running performance, muscle fatigability *in situ*, and muscle respiration *ex vivo*. Importantly, these differences are completely independent of differences in biomechanics or sweat glands strongly associated with the success of humans for long distance running compared to other vertebrates. At least part of this difference in oxygen utilization is due to a difference in baseline skeletal muscle

capillarity in the soleus, which is a highly oxidative muscle compared to most muscles in a mouse, but more closely resembles the fiber type distribution of human muscles[476, 477]. Increasing capillarity would increase nutrient and oxygen delivery, which is vital during periods of prolonged endurance *in vivo* or resistance to fatigue *in situ*[468, 478–480]. A comparable performance of muscle fatigability *ex vivo* further strengthens the evidence for an essential nutrient and oxygen delivery component contributing to the differences in muscle fatigability measured *in situ* and *in vivo* and some components such as RBC/endothelial interactions involving the glycocalyx are a possibility[238, 460, 461]. But differences observed in the ADP-stimulated OXPHOS of saponin-permeabilized muscle fiber bundles raises the possibility that *Cmah*^{-/-} muscle might be unable to perform at maximum oxidative capacity when measuring fatigability *ex vivo,* where there are known limitations in the capability to deliver oxygen to the entire tissue in saturating conditions[481].

Because *Cmah*^{-/-} mice are more susceptible to multiple models of muscular dystrophy, we also investigated the expression levels of the transcription factor C/EBPδ, which connects changes associated with metabolism[482] to the inflammatory response[483, 484] and muscle wasting[485]. We have also previously reported that the expression of another C/EBP family member (C/EBPβ) could be modulated by simply feeding Neu5Gc to macrophages *ex vivo* [191] and that this transcription factor is differentially expressed in WT vs. *Cmah*^{-/-} macrophages. Thus, we now provide further evidence that C/EBP family members are becoming differentially expressed in multiple tissue types in *Cmah*^{-/-} mice. Although much further work is needed to determine what factors are contributing to this transcription factor differences, our current work suggests that there were likely complex

effects of *Cmah* loss on vascular and skeletal muscle physiology, apparently providing a greater capability for long distance running *in vivo*, resistance to muscle fatigability *in situ, and* greater ADP-stimulated OXPHOS in skeletal muscle *ex vivo* despite no measurable difference in fatigue resistance *ex vivo*. Upregulation of the transcription factor C/EBPδ may also help explain why the *Cmah*-null state alters disease severity in mouse models of human disease associated with inflammation and muscle wasting, such as muscular dystrophy.

Overall, it is clear that no single mechanism can fully account for the increase in exercise and endurance capacity observed in Cmah null mice. Indeed, it appears that multiple mechanisms were likely involved. Given the timing of the mutation and the potential relevance of its fixation to the emergence of the genus Homo, it is reasonable to speculate that this mutation may have facilitated the emergence of the unusual phenotype of our likely ancestral lineage.
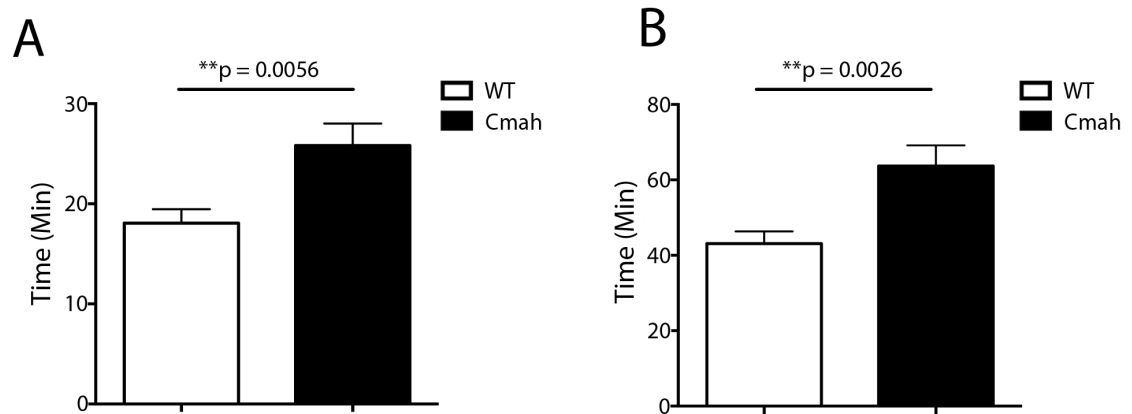
Figure 3-1: Forced treadmill running performance in WT and Cmah$^{-/-}$ mice (A) Untrained endurance times (time to exhaustion) in treadmill running performance (n=31 WT and 36 *Cmah$^{-/-}$*) and (B) Exercise-trained endurance times (n=18 WT and *Cmah$^{-/-}$*).
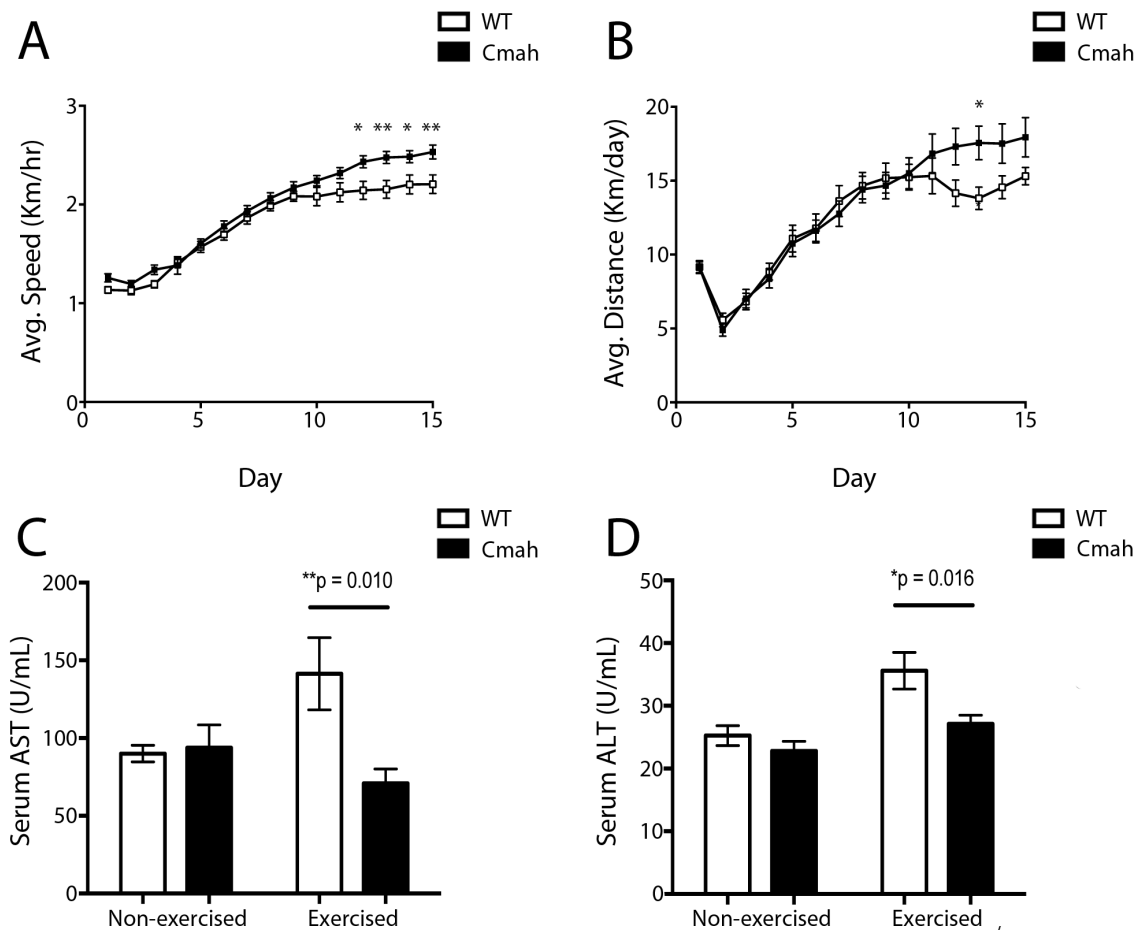
Figure 3-2: Voluntary exercise speed and distance in WT and *Cmah*^-/- mice (A) Average speed and (B) average distance travelled (n=18 WT and 16 *Cmah*^-/-) during voluntary exercise with running wheels for two weeks. Serum **(C)** AST and **(D)** ALT transaminase concentrations in non-exercised (n=4 WT and 5 *Cmah*^-/-) or 15 day voluntarily exercised mice (n=5 WT and 8 *Cmah*^-/-).
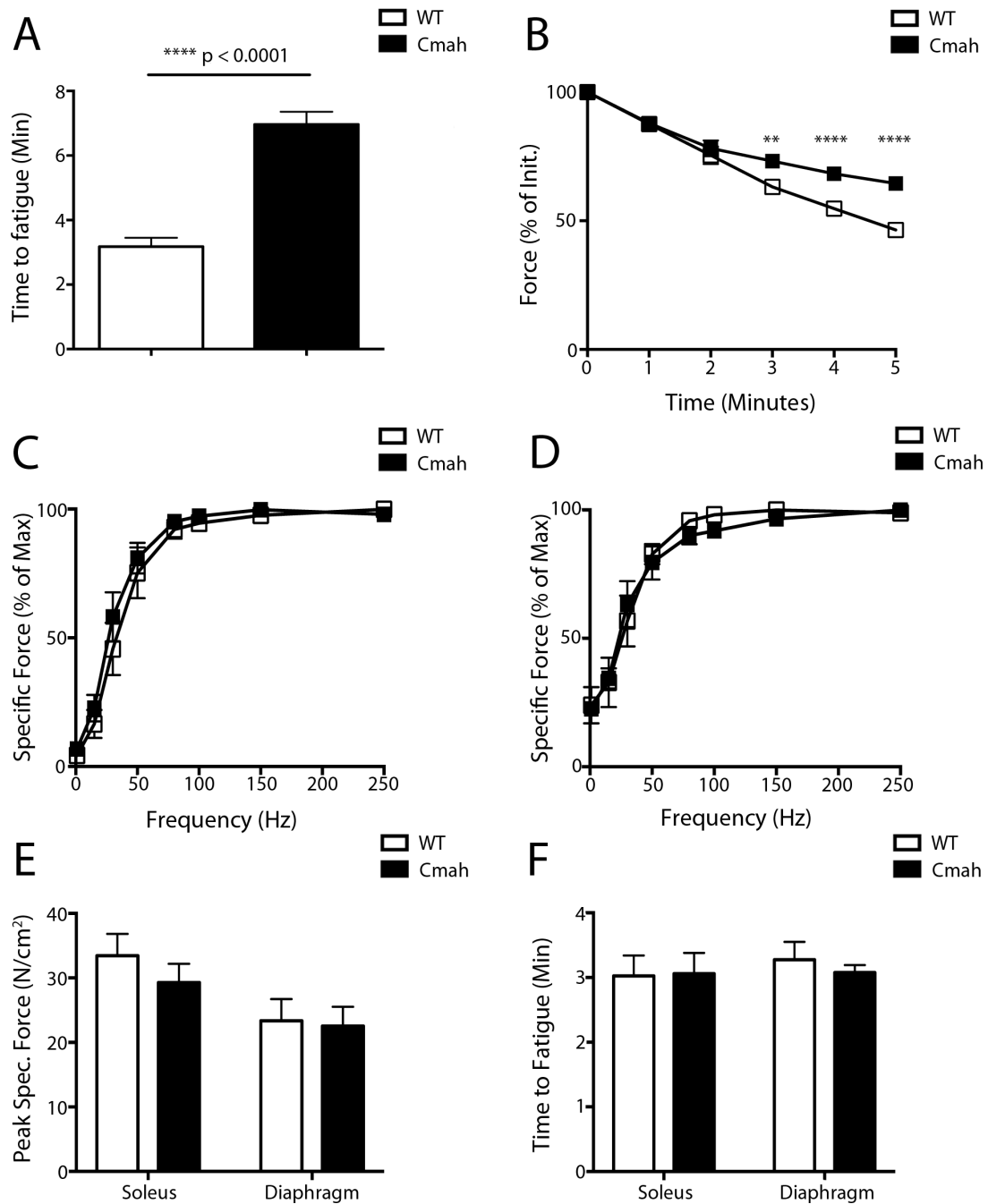
Figure 3-3: Peripheral and respiratory muscle contractile function in WT and *Cmah*^{-/-} **mice. (A)** Time to fatigue (60% of initial) of gastrocnemius complex electrically stimulated via the sciatic nerve (n=6). **(B)** Force production normalized to % of initial contraction during the first 5 minutes (n=6) of tetanic contractions. **(C)** Force frequency relationship of soleus (n=4) and **(D)** diaphragm (n=4) muscles. **(E)** Peak specific forces in soleus and diaphragm during force frequency testing and **(F)** time to fatigue (60% of initial) during repetitive tetanic contractions in soleus (80 Hz) and diaphragm (40Hz).
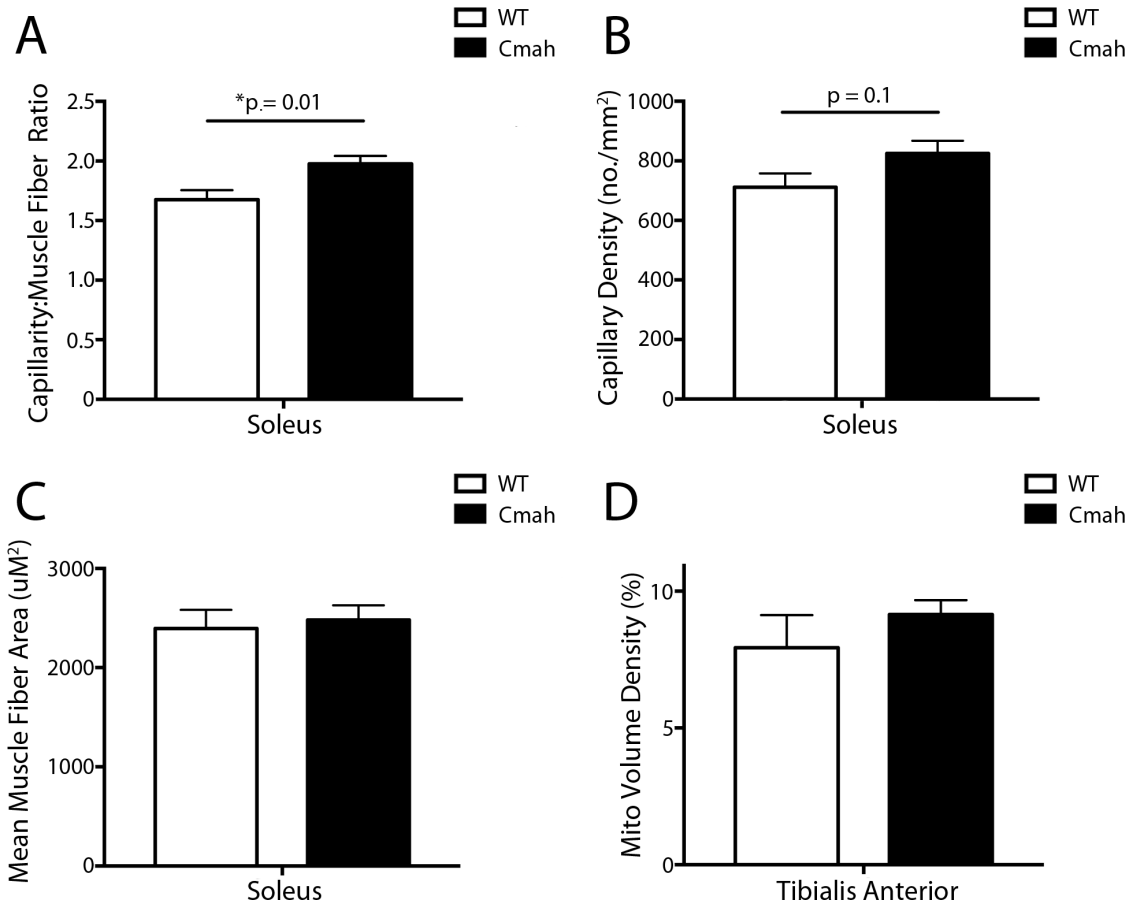
Figure 3-4: Image analysis of skeletal muscle vascularity and mitochondrial density in WT and *Cmah*^{-/-} mice. (A) Muscle capillary-to-fiber ratio was measured by lead ATPase stain in the soleus (n=6). **(B)** Number of capillaries divided by the total muscle fiber area was calculated to report capillary density (n=6). **(C)** Total muscle fiber area divided by the number of muscle fibers was calculated to report mean muscle fiber area (n=6). **(D)** Mitochondrial volume density was also measured by transmission electron microscopy of the tibialis anterior (n=3).
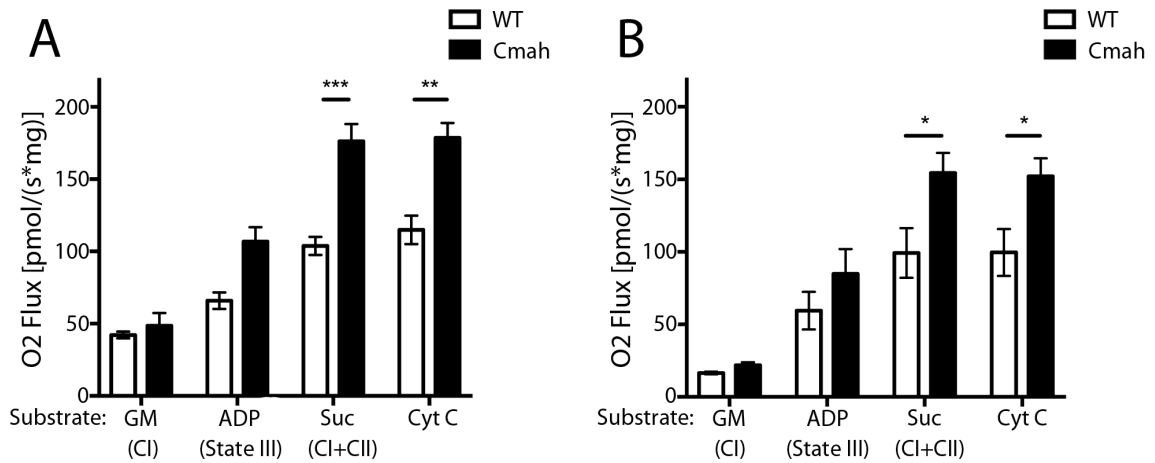
Figure 3-5: Comparison of muscle fiber bundle respiration in WT and Cmah$^{-/-}$ mice.
(A) Diaphragm (n=6) and **(B)** soleus (n=6) saponin-permeabilized muscle fiber bundles were exposed to saturating conditions of the following substrates: glutamate and malate (GM) to measure complex I respiration, ADP to measure state III respiration, complex II substrate succinate (Suc) to measure complex I and complex II respiration combined, and Cytochrome C (Cyt C) as a quality control to ensure mitochondrial outer membrane integrity.
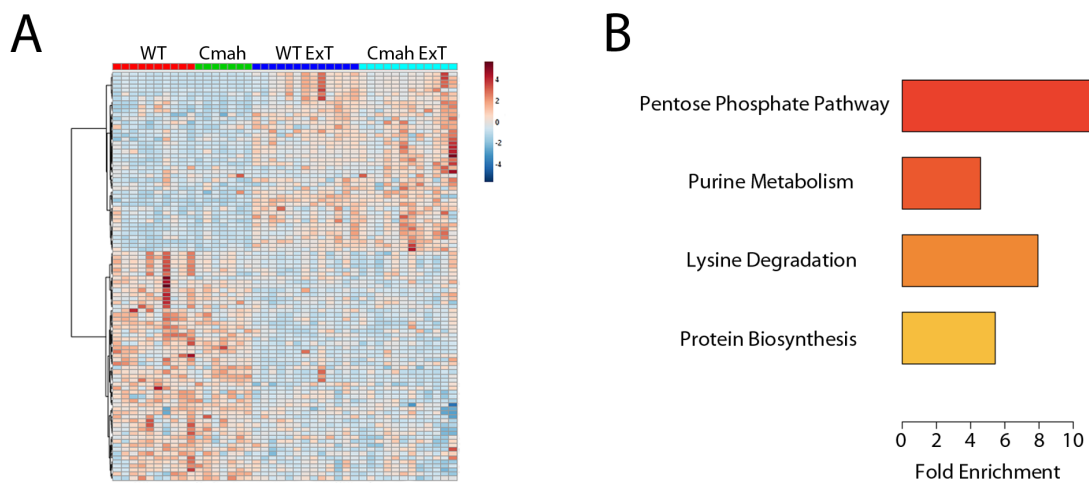
Figure 3-6: Metabolite profiles and pathway enrichment in WT vs. *Cmah*$^{-/-}$ mice. (A) Heat map for visualization of 100 metabolite expression profiles in non-exercised (n=10 WT and 7 *Cmah*$^{-/-}$) and exercise trained (ExT) (n=13 WT and 12 *Cmah*$^{-/-}$) mice. **(B)** Metabolite Set Enrichment Analysis (MSEA)[486] comparing *Cmah*$^{-/-}$ vs. WT exercise trained mice (n=13 WT and 12 *Cmah*$^{-/-}$).

Table 3-1: Targeted Metabolic Pathway Analysis

Pathway	WT	WT ExT	KO	KO ExT
Lactate	**330 ± 38**	**445 ± 21^**	378 ± 25	380 ± 25
Citric Acid Cycle				
Citrate	<u>5.9 ± 2.2</u>	<u>11.8 ± 1*</u>	**2.7 ± 0.8**	**14.2 ± 1.5****
Succinate	0.3 ± 0.02	0.3 ± 0.04	0.3 ± 0.04	0.3 ± 0.08
Fumarate	12.3 ± 0.8	14.5 ± 0.7	13.9 ± 0.9	16.1 ± 1.7
Malate	14.2 ± 1.3	19.9 ± 1.2	15.6 ± 1.4	19.7± 1.2
Pentose Phosphate Pathway				
Ribose	1.0 ± 0.2	**0.8 ± 0.1**	0.8 ± 0.1	**1.24 ± 0.01^**
Xylulose	0.2 ± 0.04	**0.1 ± 0.01**	0.1 ± 0.02	**0.2 ± 0.02^**
Oxidative Phase				
Ribulose-5-Phosphate	0.7 ± 0.1	**0.6 ± 0.05**	0.6 ± 0.05	**1.0 ± 0.1***
Non-Oxidative Phase				
Ribose-5-Phosphate	2.3 ± 0.4	2.1 ± 0.2	2.6 ± 0.1	2.8 ± 0.5
Amino acid metabolism				
Proline	0.5 ± 0.04	**0.9 ± 0.04**	0.6 ± 0.1	**1.54 ± 0.2***
Oxoproline	173 ± 45	161 ± 5	125 ± 7	254 ± 64
Phenylalanine	0.8 ± 0.1	**2.0 ± 0.2**	0.7 ± 0.1	**2.7 ± 0.3****
Isoleucine	4.8 ± 0.5	**7.7 ± 0.4**	4.7 ± 0.4	**11.0 ± 0.9***
Leucine	11.4 ± 0.1	**15.5 ± 0.7**	11.0 ± 0.7	**20.8 ± 2.1***
Valine	<u>10.5 ± 0.4</u>	<u>16.4 ± 0.6*</u>	**10.6 ± 0.6**	**18.9 ± 1.4****
Glutamine	1.1 ± 0.4	1.4 ± 0.3	3.0 ± 2.5	1.3 ± 0.3
Glutamate	44.7 ± 9.4	62.5 ± 4.6	42.9 ± 3.7	57.1 ± 7.9
Aspartate	14.9 ± 2.6	47.2 ± 6.4	**14.4 ± 3.7**	**64.0 ± 9.9****
Threonine	5.0 ± 0.7	7.3 ± 0.4	**4.3 ± 0.8**	**10.3 ± 1.4***
Methionine	<u>0.5 ± 0.1</u>	<u>1.1 ± 0.1*</u>	**0.5 ± 0.1**	**1.5 ± 0.2****
Cysteine	<u>0.2 ± 0.02</u>	<u>0.3 ± 0.03*</u>	**0.2 ± 0.02**	**0.4 ± 0.04***
Asparagine	0.1 ± 0.01	0.1 ± 0.01	**0.1 ± 0.01**	**0.2 ± 0.02***
Serine	<u>6.8 ± 0.9</u>	<u>13.7 ± 0.7*</u>	**6.9 ± 0.5**	**17.7 ± 2.9***
Lysine	2.3 ± 0.4	3.2 ± 0.4	2.3 ± 0.6	3.8 ± 0.5
Tyrosine	4.4 ± 0.5	5.6 ± 0.4	**3.8 ± 0.4**	**6.3 ± 0.7***

All samples are 10^3 A.U. ^p = 0.07, *p < 0.05, **p < 0.001 based on 2-way ANOVA w/ multiple comparisons made in each row between samples in bold or between samples underlined
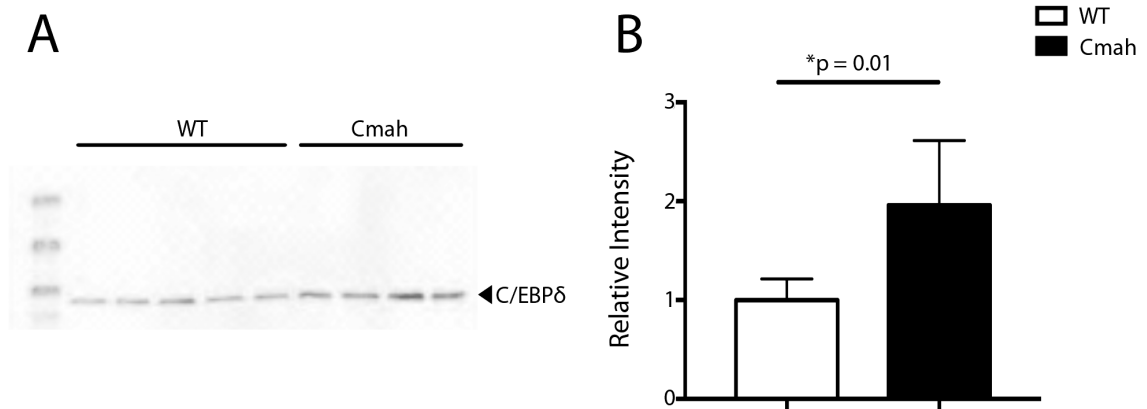
Figure 3-7: Comparison of C/EBPδ expression in WT vs. Cmah⁻/⁻ muscle. (A) Immunoblot of soleus muscle homogenates (40μg) for C/EBPδ (n=5 WT and 4 Cmah⁻/⁻) **(B)** Relative intensity quantification of C/EBPδ.

The text of Chapter 3, in part, is currently being prepared for submission for publication by Okerblom J, Fletes W, Lee J, Patel H, Schenk S, Varki A, and Breen E. The dissertation author was the primary author of this material.

Chapter 4

Conclusions and Future Directions

The work presented in this thesis explored various implications of the human evolutionary loss of *CMAH* and Neu5Gc expression, primarily in a mouse model for Cmah loss (*Cmah*$^{-/-}$ mice). Loss of Cmah in mice increases innate inflammation, which has advantages for bacterial killing but is likely deleterious in situations of overwhelming infection and endotoxic shock. In the future, it would be important to challenge *Cmah*$^{-/-}$ mice with multiple clinically-relevant models for infection to determine if the differences seen with the non-pathogenic *E. coli* K12 bacterial strain are strain specific or broadly relevant. It would also be interesting to explore the actual contribution of Cmah null mouse hyperinflammation to the observed increase in severity phenotype associated with *Cmah*$^{-/-}$/mdx mice. This could be accomplished by utilizing a CD14-targeted Cre-Lox-inducible system for *Cmah* expression in *Cmah*$^{-/-}$/mdx mice with all of the proper controls.

Loss of Cmah also increases running endurance capabilities, which could have benefited early hominins in persistence hunting. Mechanistically, current evidence points to a difference in vascularity. But differences observed with high resolution respirometry also hint at a greater mitochondrial efficiency. To determine what specific systems are responsible for differences in fatigability observed, we are now developing HSA (skeletal muscle) and PDGF (~endothelial cell) Cre-Lox inducible systems for Cmah expression.

We are also planning to perform serial block scanning electron microscopy (SEM) 3D reconstructions of soleus and gastrocnemius muscle to determine if there are differences in mitochondrial networking (particularly since we do not observe any gross differences in mitochondrial volume density) and to further investigate the observed differences in muscle vascularity.

Another important component of oxygen delivery is cardiac output. Despite its obvious importance, we have not yet investigated or discussed any potential role for cardiac physiology in the observed fatigability phenotypes, primarily because of the differences observed in gastrocnemius complex fatigability *in situ*, where the heart is not overwhelmed. Notably, cardiac vascularity has been reported to be higher in human hearts compared to chimpanzee hearts, where different pathological processes cause heart disease between the two species. In the future, I hope to test how these alterations in innate inflammation and vascularity could be affecting cardiac physiology in *Cmah*$^{-/-}$ mice, particularly in regards to conditions of oxygen deprivation and oxidative stress, such as during ischemia/reperfusion injury.

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