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SPECIAL ISSUE

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

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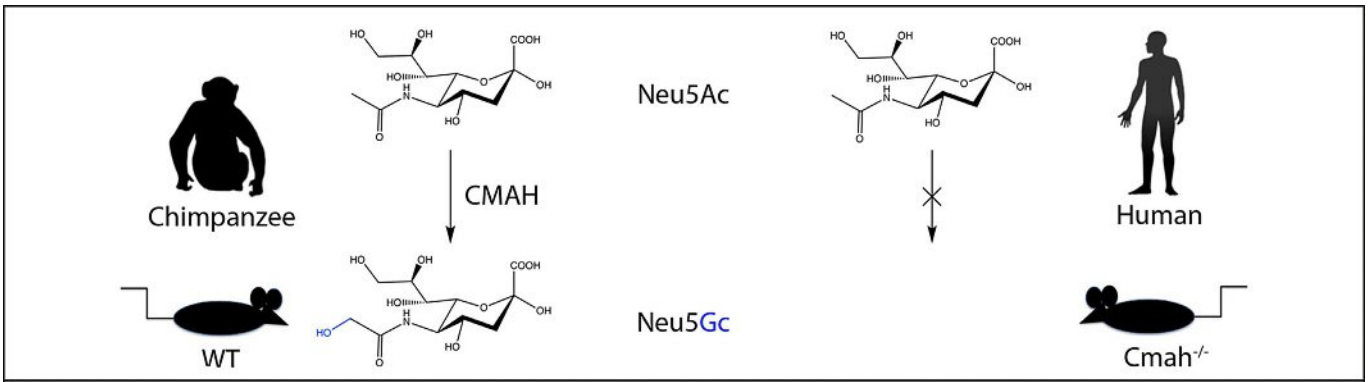


Organ	Cell Type	Phenotype	Proposed Mechanism
Skin	Multiple	Delayed Wound Healing	Unknown
Inner Ear	Multiple	Degeneration, Hearing Loss	Oxidative damage?
Blood/Multiple	B-cell	Anti-Neu5Gc Antibody Production	Nutritional incorporation of commensal bacteria
		BCR Hyperreactivity	Unknown (human)
			Reduced 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

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 through 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 chimpanzee. Although the biophysical and biochemical ramifications of losing tens of millions of Neu5Gc hydroxy groups at most cell surfaces remains poorly understood, we do know that there are multiscale effects functionally relevant to both sides of the host–pathogen interface. Hominin *CMAH* loss might also contribute to understanding human evolution, at the time when our ancestors were starting to use stone tools, 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 by 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 could provide a model for other human-specific processes and pathologies involving sialic acid biology that have yet to be explored.

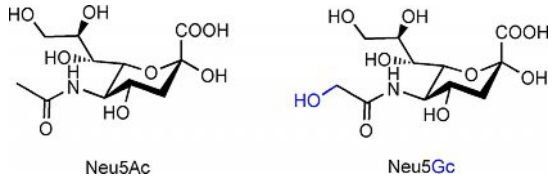
1. Introduction

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 nine-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 $\approx 10^6$–10^8 sialic acids present on each cell of all major tissues, prominently composed of *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc; Scheme 1).[4–9] This review focuses on the human loss of Neu5Gc, which differs from the precursor sialic acid Neu5Ac through the enzymatic addition of a hydroxy group to the *N*-acetyl moiety at C-5.[10,11]

1.2. Discovery of Sialic Acids

In 1935, Ernst Klenk discovered gangliosides in the brain tissue of a patient with Niemann–Pick's disease,[12,13] and in 1941 he described an acid-hydrolyzed carbohydrate component that he named "neuraminic acid".[14] Meanwhile in 1936, Gunnar Blix independently reported that acid hydrolysis combined with fractionation of bovine submaxillary mucin resulted in the formation of crystals of an unknown sugar,[12,15] which he did not name "sialic acid" until 1952.[16] Ultimately it was confirmed that Blix and Klenk were describing the same family of sugars[17] 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.[18] Nonetheless, the two names have persisted to this day.[19] More than 50 types of sialic acids originating from the two "primary sialic acid backbones" (Neu5Ac and Kdn) have now been described in nature,[20,21] but the two most prevalent in mammals are Neu5Ac and Neu5Gc.[10,22]



Scheme 1. The two most common sialic acids in mammals, Neu5Ac and Neu5Gc, which differ by a single hydroxy group, shown in the α-configuration. In cells, CMP-Neu5Ac is converted to CMP-Neu5Gc by the enzyme CMAH, which is pseudogenized in humans.

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

This issue is dedicated to the memory of Professor Werner Reutter, a pioneer in the study of sialic acids. After reporting the famous D-galactosamine study,[23] Reutter and colleagues began making key contributions to the field of sialic acid biology by studying the biosynthesis[24] and half-life of Neu5Ac in normal, cancerous (hepatomas), regenerating, and neonatal livers.[25,26] After discovering that UDP-GlcNAc 2-epimerase (GNE), a key enzyme in sialic acid biosynthesis, was downregulated in hepatomas compared to normal livers,[24] they cloned and characterized its activity and reported it to be a bifunctional enzyme having both UDP-GlcNAc 2′-epimerase and ManNAc kinase activity.[27] *GNE* was subsequently discovered to be the

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gene mutated in inclusion body myopathy 2,[28] the most common hereditary inclusion body myopathy affecting humans.[29]

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.[30] 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.[31]

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 developed the first animal model for an infection affecting over 10% of the human population in Paris at the time: syphilis.[32] Metchnikoff and his colleague Besredka subsequently developed the first non-human primate model for studying typhoid/enteric fever[33] and together, this pioneering work strongly contributed to the modern era of medical primatology.[32] 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 might account for their biological differences".[34] It took more than 20 years for the first clear-cut exception to this hypothesis to be discovery, the selective absence of Neu5Gc in humans,[35] 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.[36,37]

This and subsequently discovered genetic and biomedical differences[38] motivated the initial sequencing of the chimpanzee genome[39] and ongoing sequencing of other non-human primate genomes with the aim of determining genetic components specifically accounting for these differences in phenotypes.[40] 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.[41] Some of those that involve sialic acid biology are discussed further below.

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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.[42] In the final steps of sialic acid biosynthesis, 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 acid.[5,21,43] In relation to glycan biosynthesis in deuterostomes, sialic acids are unique in that they require a monophosphate nucleotide donor (CMP) for activation[44] and must travel to the nucleus in order to be activated into their CMP-conjugated form.[45] Cytosolic CMP-conjugated Neu5Gc is then produced by the hydroxylation of CMP-Neu5Ac by CMAH, the only enzyme known to be able to biosynthesize Neu5Gc from Neu5Ac in any living species.[4,6,8,9,46]

3. Human-Specific Loss of Neu5Gc Expression

Given their high abundance in animal tissues, it is hardly surprising that Neu5Gc and other structural variants of Neu5Ac had already been identified and characterized by the time the term "sialic acid" was officially agreed upon in the 1950s.[18]

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.[9] Although humans had long been 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, particularly on tumors and fetal tissues, were reported by using antibodies.[47–51] It was later shown that Neu5Gc in humans is incorporated from dietary sources and presented on some epithelial and endothelial cell surfaces.[52–55] In 1982, Roland Schauer noted that, despite reports of the presence of Neu5Gc in tissues, Neu5Gc production was missing in humans, possibly antigenic, and potentially contributing to several pathological states,[22] including "serum sickness" in human patients receiving infusions of animal sera.[56] Sixteen years later, two groups independently discovered that humans lack a functional CMAH enzyme and are therefore incapable of endogenous Neu5Gc production.[35–37] Both groups reported a genomic mutation that eliminated a 92 bp exon in CMAH. While one report predicted the existence of a large frame-shifted inactive protein,[36] the other correctly showed that the frame-shift resulted in a small, truncated, inactive protein[37] (see also below). The ramifications of Neu5Gc loss continue to be explored.[54,55,57,58]

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.[59–61] Comparative genomic analysis revealed that chimpanzees, bonobos, gorillas, orangutans, gibbons, baboons, and rhesus monkeys all contain an ancient *AluSq* retroposon[62] (subsequently designated sah*Alu*Sq) ≈350 bp downstream from the human deletion site.[59] 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.[59]

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,[60,63] multiple genomic methods (see below) deduced that hominin CMAH loss likely occurred about 2–3 million years ago (Ma), during the biomechanical and immunological period of transition of early hominins from forests to open savannahs.[64–68] Although technically challenging, biochemical methods were developed to successfully extract sialic acids from some *Homo neanderthalensis* fossils,[60] and the lack of detectable Neu5Gc in these bones confirmed that CMAH inactivation occurred before the last common ancestor between *Homo sapiens* and *H. neanderthalensis* approximately 500 000 years ago.[60] *CMAH* inactivation has since been independently confirmed genetically by analyzing the limited Neanderthal and Denisovan genomic information that has become available.[69] Unfortunately, the same biochemical methods originally used by our group on *H. neanderthalensis* fossils failed to obtain a detectable amount of sialic acid from *Homo erectus* fossils,[70] which likely decayed more rapidly in subtropical or tropical climates.[71] Therefore, three independent genomic methods were employed to approximate hominin CMAH loss.[60,61] First, the timing of human *Alu*-mediated exon deletion based upon *Alu* sequence analysis approximated that CMAH inactivation occurred 2.7 ± 1.1 Ma. Second, molecular clock analysis of the *CMAH* pseudogene (*CMAHP*) based on the substitution rate at nonsynonymous sites versus synonymous sites estimated that CMAH inactivation took place 2.8 Ma.[60] However, these estimations were based upon the divergence of humans and chimpanzees taking place ≈5.3 Ma, which was subsequently estimated to have occurred ≈6 Ma,[72] and the estimation of CMAH inactivation was changed to 3.2 Ma.[61] 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 Ma.[61] In summary, all three genomic approximations performed on *CMAH* placed the initial hominin *CMAH* loss in the era of the australopithecines[64,73] and just prior to the emergence of genus *Homo*.

3.3. General evolutionary implications

The placement of hominin *CMAH* inactivation ≈3 Ma coincides with major evolutionary changes in hominins transitioning from forests to open savannahs, including biomechanical adaptation towards fully striding bipedalism,[68,74] increased consumption of other animals (expansion of prey base),[75,76] increased body and brain size,[77] and the earliest developments of Oldowan stone tool use.[66,76,78,79]

4. Proposed Mechanisms for Selection and Fixation of the 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,[80–83] and many deadly human pathogens such as human influenza,[84–86] *Salmonella typhi*,[87] and *Plasmodium falciparum*[88–90] prefer Neu5Ac over Neu5Gc. Moreover, most of the pathogenic and viral sialic-acid-cleaving enzymes (sialidases/neuraminidases) studied prefer Neu5Ac over Neu5Gc substrates;[91] this could increase susceptibility for many infections.

Conversely, several pathogens have a binding preference for Neu5Gc,[92,93] including *Plasmodium reichenowi*, a close relative

of *P. falciparum* that primarily infects chimpanzees.[90] *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 Ma.[94–96] Subsequent molecular clock analyses indicated that *P. falciparum* is more likely the outcome of a much more recent transfer,[89] from a gorilla to humans.[97] 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 through 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[98] or even expression polymorphisms, which occur in cats and some dogs.[99] Therefore, a second mechanism was proposed for fixation: sexual selection through detection of the 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.[100,101] It was also shown that human serum with high levels of anti-Neu5Gc IgG kills chimpanzee sperm in vitro.[100] 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 led to a rapid fixation of the CMAH loss of function mutation.[100]

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 Ma[94,96] and we have since evolved independently. Also ethical, legal and practical issues limit research on chimpanzees.[102] Therefore, a *Cmah*-null mouse (*Cmah*$^{-/-}$) with the human-like exon deletion was generated to provide a practical model for studying the immediate loss of *CMAH* as it would have happened in hominins ≈3 Ma. *Cmah*$^{-/-}$ mice have several human-like phenotypes (Table 1), including the induction of anti-Neu5Gc antibodies,[103] enhancement of cancer inflammation and progression of Neu5Gc containing tumors,[104–108] enhanced immune clearance of recombinant Neu5Gc containing therapeutics,[109] delayed skin wound healing,[110] enhanced age-related hearing loss,[110,111] altered immune responses,[112–114] sexual selection through Neu5Gc antigenicity,[100,101] altered susceptibility to metabolic disorders,[115–117] altered susceptibility to muscular dystrophy,[118–120] and a xeno-antibody response against the vascular endothelium after nutritional incorporation of Neu5Gc.[121]

6. Biochemical Consequences of CMAH Loss in Humans

6.1. Redox metabolism

CMAH oxidoreductase activity requires Fe^{2+} and a reducing co-factor (NADH or NADPH) for its enzymatic activity.[9] Therefore, disruption of CMAH activity could potentially change redox metabolism,[122] possibly by altering the NAD^+:NADH steady state. Genetic evidence has led to speculation that CMAH loss could indirectly lead to increased oxidative damage,[111] and these mechanisms have been proposed to explain gene expression differences observed during changes in metabolism or age-related hearing loss observed in *Cmah*$^{-/-}$

Table 1. Known host organs and cell types affected by Neu5Gc loss.[a]

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 hyper-reactivity	unknown (human)
			reduced CD22/Siglec G ligand? (mouse)
	T-cell	hyperproliferation	unknown
	monocyte/macrophage	hyper-reactivity	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

[a] See text for discussion and literature citations. n.a.: not applicable.

mice.[110,111,115,116] A detailed biochemical quantification of NAD^+:NADH and ROS levels in relevant *Cmah*$^{-/-}$ tissues is of major interest, but has not yet been performed.

6.2. Metabolic incorporation, recycling, and degradation

Although human pseudogenization of *CMAH* results in a complete loss of enzymatic activity, *CMAHP* is still transcribed, particularly in human stem cells where Neu5Gc uptake was reported to modulate Wnt/β-catenin signaling.[49] 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,[113] 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.[114] Further studies are needed to systematically elucidate the possible mechanisms contributing to the observed phenotypes (Table 2).

Free exogenous sialic acids can be taken up by cells through macropinocytosis and transported into the cytosol by the lysosomal transporter sialin,[123] which can be upregulated in hypoxic conditions including in cancer tissue.[124] 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.[125] 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 through the transporter sialin.[123]

NEU1-mediated sialic acid catabolism and transportation occur frequently, in order to maintain the cell steady state. Furthermore, NEU1 has long been considered the only clinically relevant neuraminidase, as loss of its expression and/or function results in the lysosomal storage and neurodegenerative disorder sialidosis.[126] However, there are three other vertebrate sialidases primarily involved in sialic acid catabolism (NEU2–NEU4) that are less abundantly expressed but play significant roles in many biological functions. NEU2 is cytosolic and highly expressed in muscle, where it is also found in the nucleoplasm.[127] NEU3 is associated with the plasma membrane and primarily targets cell-surface gangliosides. NEU4[128] is associated with intracellular membranes such as the endoplasmic reticulum and mitochondria.[129–131] Sialic acids are constantly being cleaved by these host sialidases and reutilized in new glycoconjugates before degradation; this might explain why the rate of sialic acid turnover is particularly slow in some tissues, such as brain.[132] The half-life in normal liver, where glycans are primarily protein bound,[19] is ≈33 h;[22,25,133] however, the half-life in brain, where glycans are primarily lipid bound,[19] varies greatly between 4 and 45 days.[134] Pulsing with Neu5Gc in a human B-cell lymphoma cell line yielded a similar half-life (≈4 days) to what has been observed in brain tissue.[135]

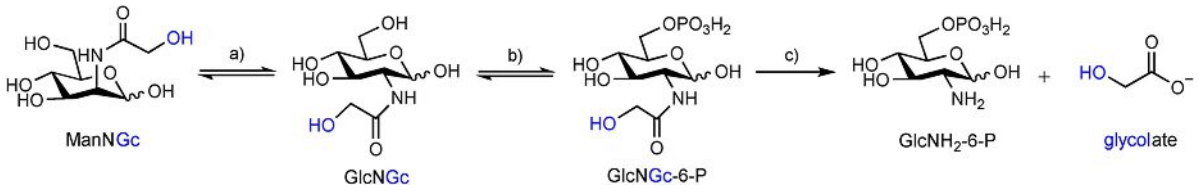
Although Neu5Ac and Neu5Gc are handled similarly by enzymes of the hexosamine biosynthetic pathway, terminal degradation of Neu5Gc produces glycolate, whereas degradation of Neu5Ac produces acetate[135,136] (Scheme 2). Thus, once CMAH has converted Neu5Ac to Neu5Gc, the acetyl-to-glycolyl conversion 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 so as to maintain a steady state, it's not clear if intracellular acetate, which is quickly converted to acetyl-CoA,[137] drives metabolism in a different direction than glycolate, which is converted to oxalate[138] or glyoxylate.[136] Beyond limited gene-expression studies,[111,116] the true ramifications of these altered metabolic fates during the constant degradation to maintain steady state have not been fully explored.

7. Consequences of CMAH Loss in Humans for Cell Biology

Sialic acids have a multitude of functions on cell surfaces, such as repulsing other cells,[139] protecting from proteases,[140] and modulating certain cell-signaling pathways.[54,141–143] Some

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

Mechanism	Ramifications
Loss of millions of cell surface hydroxy 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 under 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)



Scheme 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 pyruvate lyase, which forms ManNAc or ManNGc. GlcNAc-2-epimerase (a) potentially modifies ManNAc or ManNGc into GlcNAc or GlcNGc, which could then potentially be phosphorylated at position 6 by GlcNAc kinase (b). Thereafter, the *N*-acetyl or *N*-glycolyl group could be irreversibly removed from GlcNGc-6-P by the GlcNAc-6-P deacetylase (c), which would result in $GlcNH_2$-6-P and either acetate or glycolate, which might have different metabolic fates. Modified from ref. [135], Copyright 2012: American Society for Biochemistry and Molecular Biology.

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 have been extensively characterized,[19] very little is known about whether the structural differences between Neu5Ac and Neu5Gc could alter these processes.[144] Theoretically, the loss of Neu5Gc and subsequent loss of millions to tens of millions of hydroxy 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 "ganglioside patches"[142] or lipid-raft compartments, small changes in interactions involving Neu5Ac versus Neu5Gc are potentially magnified in concentrated compartments or through multivalent interactions.[145] Changes in the partition coefficient of a drug alters its diffusion rate across membranes,[146] therefore changes in membrane hydrophobicity (through the loss of Neu5Gc) could affect the diffusion of hydrophobic molecules across membranes. Although drug permeability has been studied extensively between species,[147] 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)[148] has yet to be tested.

7.2. Changes in Siglec binding

Changes in the Neu5Gc/Neu5Ac ratio could potentially alter cell reactivity through changes in the binding of sialic acid ligands to that are complementary receptors, the Siglecs. Siglecs are immunoglobulin 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.[149] This phenomenon is also exploited through Neu5Ac sialic acid mimicry by multiple invading pathogens.[81,82,150] Siglecs are rapidly evolving and highly variable across species,[151] thus making it difficult to model human Siglec biology in mice, which have no functional equivalents to human Siglec-5, Siglec-6, Siglec-7, Siglec-11, Siglec-XII, Siglec-13, or Siglec-14.[152] Some human Siglecs have also evolved particularly rapidly, such as human Siglec-9, which binds both Neu5Ac and Neu5Gc relatively equally whereas chimpanzee and gorilla Siglec-9 strongly prefers to bind Neu5Gc.[153] Furthermore, some Siglecs, such as sialoadhesin (Siglec-1)[154] and MAG (Siglec-4)[155] 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.[153] In mice, CD22 (Siglec-2) has a strong preference for Neu5Gc. As CD22 is highly expressed on B cells (and to a lesser degree on T cells[156]), loss of inhibitory signaling through the loss of Neu5Gc ligands has been proposed as the mechanism for the B-cell hyper-reactivity observed in *Cmah*$^{-/-}$ mice.[112,113,157]

7.3. Changes in neuraminidase susceptibility

Although glycosphingolipids (particularly gangliosides) account for ≈80% of the total glycan mass in the brain,[19] neuronal plasticity and development is heavily regulated by very long polysialic acids (PSA) that are primarily (≈95%) conjugated to Neural cell adhesion molecule (NCAM) to form NCAM-PSA[158] and catabolized by host neuraminidases.[159] Despite its presence in most mammalian tissues, Neu5Gc is present in the brain endothelium but absent from the neuronal brain tissue of all animals tested.[160] One proposed mechanism for this phenomenon is a NEU1 preference for the α2–8Neu5Ac linkages common in brain polysialic acids[160–162] over the α2–8Neu5Gc linkages commonly found in fish eggs.[163] A recent study has shown that Neu5Gc overexpression in the nervous system has multiple detrimental effects, including the loss of the MAG ligand, impaired CNS mylination, increased PNS degeneration, impaired locomotor activity, and impaired memory.[162]

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 under a multitude of different signaling conditions, including the activation of receptor tyrosine kinases and TLRs.[164–166] As most microbial sialidases have a preference for Neu5Ac or Neu5Gc and host NEU1 prefers α2–8-linked Neu5Ac over Neu5Gc, it could be that NEU1 has a preference for the α2–3 or α2–6 Neu5Ac versus 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.[49,113,114]

7.4. Altered cell-surface sialic acid 9-O-acetylation

Another common modification of Neu5Ac is 9-O-acetylation, which can inhibit the surface recognition of sialic acids by some Siglecs (e.g., CD22) and certain pathogens, while also preferentially binding other pathogens, such as influenza C.[167] Compared to chimpanzees, humans were found to contain higher levels of cell-surface 9-O-acetylation;[168] this phenomenon is similarly observed in *Cmah*$^{-/-}$ versus WT mice.[110] Although 9-*O*-acetylation is found to disrupt CD22 (Siglec-2) binding in vitro,[169] genetic deletion in mice leads to the development of auto-antibodies,[157] and more work is needed to determine how secondary changes in surface O-acetylation through CMAH loss could contribute to human inflammation and autoimmunity.[170]

7.5 Alterations in cell signaling

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.[171,172] Recent studies have shown the removal or reintroduction of Neu5Gc to be capable of modulating adaptive and innate immune cell responses in both humans and mice.[112,113,129,130,143,165,166,173–175] Specific examples of the role of sialic acid in hyper-reactivity are discussed below.

7.5.1 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-phytohemagglutinin (PHA), *Staphylococcus aureus* super antigen, and a superagonist α-CD28 Ab, as well as in mixed leukocyte reactions (MLRs).[174,176] The same phenomenon was observed in *Cmah*$^{-/-}$ mice compared to WT controls.[113] 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, under conditions under which there is no known difference in Siglec expression or Neu5Gc preference.[113] Several unanswered questions about the influence of Neu5Gc on T-cell function remain, some of which are further discussed in regards to HIV below.

7.5.2. 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) is of particular importance to anergic B cells to help prevent autoimmunity; this has been reviewed extensively elsewhere.[172,177] It has been reported that *Cmah*$^{-/-}$ mice display BCR hyper-reactivity in vivo,[112] which is partially attributed to loss of the CD22 ligand. Furthermore, BCR hyper-reactivity can also occur in human B cells,[174] which express a Siglec-2 that does not have a preference for Neu5Ac over Neu5Gc ligands in vitro.[153] Thus, CD22 preference alone might not explain the hyper-reactivity observed in B cells, particularly because hyper-reactivity is also observed in human and mouse T cells.[113,175]

7.5.3. Toll-like receptor 4 (TLR4) activation and bacterial killing: Because *Cmah*$^{-/-}$ mice experience delayed wound healing,[110] greater inflammation in some models of muscular dystrophy,[118] and increased growth of transplanted human tumor cells,[104] 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. The results were consistent with those of previous studies that established that humans and chimpanzees respond at the same order of magnitude to endotoxin.[178] A small increase in the 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 than their WT and chimpanzee counterparts.[114] Thus, it can be speculated that human CMAH loss might have been beneficial for clearing minor infections, but could be potentially deleterious in 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,[179] 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.[180] It has been reported that *Cmah*$^{-/-}$ mice might experience an altered glucose metabolism at baseline[116] and after consumption of a high fat diet.[115,117] Impairment of glucose metabolism after a high-fat diet in *Cmah*$^{-/-}$ mice was attributed to pancreatic β-cell failure rather than insulin resistance, as determined from the reduced pancreatic islet area.[115] Subsequently, WT and *Cmah*$^{-/-}$ true littermate controls were independently examined under different conditions.[117] Although a detailed quantification was not reported, a reduction in both pancreatic islet size and β-cell number was independently observed in *Cmah*$^{-/-}$ mice compared to WT controls.[117] Thus, *Cmah*$^{-/-}$ might have smaller pancreatic islets and reduced β-cells; this is interesting because human pancreatic islets are smaller than monkeys'[181] and contain fewer β-cells (and more α-cells) compared to rodents' and most non-human primates'.[182] A deeper investigation (including a detailed quantification of WT versus *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 susceptibility to diabetes mellitus remains to be clearly determined.

8.2. Delayed wound healing

It has been reported that *Cmah*$^{-/-}$ experience delayed skin-wound healing,[110] 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.

8.3 Age-related hearing loss

By nine months of age, *Cmah*$^{-/-}$ mice display reduced hearing sensitivity across all frequencies, increased outer-hair-cell degeneration throughout the cochlea, and collapse of the outer organ of Corti compared to WT controls.[110] This phenotype has been independently confirmed,[111] but the biochemical mechanisms involved have yet to be characterized or described.

8.4. Gut microbiome

The human body harbors trillions of microbes in the gastrointestinal (GI) tract[183] that are proposed to influence virtually every aspect of human health, including immune cell function,[184] metabolic disorders,[185] neurodegeneration,[186] and cancer.[187] Many gut microbes, including pathogenic types, have developed the ability to use host sialic acids as an energy source in multiple ways, either through cleavage and/or scavenging with subsequent differential transport capabilities.[83,188] Although the sialic acid synthesis and neuraminidase preferences of many bacteria have been studied in relation to the host–pathogen interface, whether these individual microbes prefer Neu5Gc over Neu5Ac as an energy source in culture or within the intestine remains an open question to be explored in WT and *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, rather it has been attributed to its cell-surface presentation by common commensal bacteria (e.g., *Haemophilus influenza)* after consumption of Neu5Gc after birth,[52,103] 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).[54,55,58,189] 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).[108,190] When Neu5Gc-rich food is consumed, it is absorbed and either eliminated in urine or metabolically incorporated into some tissues.[52,191] The display of this foreign antigen induces an immune response through antibody- and complement-mediated xeno-autoantigen immunity. Chronic inflammation induced in this way was recently shown to increase the propensity for carcinoma formation in the *Cmah*$^{-/-}$ mouse.[108] In this regard, Neu5Gc has been reported for decades as a potential antigen in multiple cancer pathologies including lung,[51] liver,[51,192] colon,[47,51,193] kidney,[194] breast,[50,52,195] skin,[195–197] ovary,[197] and throat[198] cancers as well as malignant lymphoma.[51] 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.[103] *Cmah*$^{-/-}$ mouse anti-Neu5Gc antibody production has become an important model for the study of Neu5Gc antigenicity in humans.[54,55,58,189] Anti-Neu5Gc antibodies have been directly shown to enhance tumor growth in *Cmah*$^{-/-}$ mice by promoting cancer-associated inflammation[104,108] and anti-Neu5Gc antibodies have been identified as potential serum biomarkers for tumors in humans.[107] The possibility that higher levels of antibodies might be tumoricidal needs further study, as anti-Neu5Gc passively transferred into mice bearing a syngeneic MC-38 colon adenocarcinoma display a hormetic relationship between tumor growth and antibody dose.[105,106] The potential effects of anti-Neu5Gc antibodies on the vascular endothelium have been modeled in vitro but have yet to be described in vivo.[121] Neu5Gc aggregates have also been found in dystrophic human and *Cmah*$^{-/-}$ mouse muscle tissue,[120] but the implications of this are not yet fully understood.

9.2 Infectious diseases

Infectious diseases remain a major cause of death, disability, and suffering for hundreds of millions of people throughout the world.[199] Many pathogens and toxins bind specific linkages of sialic acids,[200] and some major human-specific pathogens have been found to prefer Neu5Ac over Neu5Gc linkages. Multiple infectious disease pathologies are further complicated by an apparently 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 *P. falciparum*. 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 chimpanzee RBCs and that Neu5Gc feeding could suppress the EBA-175 binding to a human erythroleukemia line.[90] In contrast, EBA-175 from the chimpanzee parasite *P. reichenowi* strongly prefers Neu5Gc, and this difference in binding preference could account for the difference in species infectivity observed between *P. falciparum* and P. *reichenowi* for humans and chimpanzees. Furthermore, primary RBCs from New World monkeys, which also lack cell-surface Neu5Gc, also showed a similar susceptibility to EBA-175 binding to human RBCs.[90] Taken together, these data illustrate a human Neu5Ac binding specificity for *P. falciparum* EBA-175 protein. Whether or not *P. falciparum* has binding preferences for WT versus *Cmah*$^{-/-}$ mouse RBCs has yet to be 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.[201] Historically, the 1918 flu pandemic killed more people than the entire First World War.[202] 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,[203] and its host specificity is dependent upon its sialic acid linkage preference.[84] For example, human influenza type A hemagglutinin preferentially binds α2–6-linked sialic acids (Neu5Ac), whereas avian influenza hemagglutinin preferentially binds α2–3-linked sialic acid.[204,205] Alterations in hemagglutinin binding specificity from α2–3 to α2–6 or from Neu5Ac to Neu5Gc can be achieved through minor amino acid substitutions.[206,207] Influenza Neu5Ac or Neu5Gc binding preferences are also species dependent. For example, the horse trachea expresses ≈90% Neu5Gc, and some equine influenza types prefer Neu5Gc for invasion and replication.[208] Swine tracheae are ≈50% Neu5Gc, and swine influenza types can vary between Neu5Ac or Neu5Gc preference.[209] Interestingly, some human influenza strains are still capable of binding Neu5Gc,[206] yet Neu5Gc feeding has also been found to suppress human epithelial cell infectivity in influenza type A strains with Neu5Gc binding capability.[210] 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 the airborne transmission of human influenza.[86]

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.[211] Although chimpanzees suffer from an AIDS-like SIVcpz immunopathology,[212] HIV progression to AIDS occurs more frequently and is more severe in humans compared to chimpanzees.[41,213,214] The causative mechanisms for this have never been unequivocally determined. HIV, the HIV envelope proteins gp120 and gp41, and the HIV gag protein p24 elicit a strong proliferative response in chimpanzee lymphocytes, even after years of HIV infection.[213] Conversely, human lymphocyte proliferative responses to HIV are relatively impaired compared to chimpanzee lymphocytes[215] and this has been proposed as a mechanism of human AIDS susceptibility.[213] Others have proposed that human AIDS is attributed to a greater susceptibility of lymphocytes to apoptosis,[216,217] potentially through differential expression of Siglecs.[176,217] Although Neu5Gc feeding alone is capable of altering T-cell proliferation after TCR activation,[113] whether or not Neu5Gc feeding can alter the susceptibility to apoptosis in these systems is of interest, but has not yet been systematically explored. It is also notable that Siglec-1, which initiates formation of the virus-containing compartment and enhances macrophage-to-T cell transmission of HIV-1,[218] has a very strong preference for recognizing Neu5Ac over Neu5Gc. Siglec-1 also shows increased positivity and altered distribution in humans compared with chimpanzees.[153]

9.2.3 Susceptibility to bacterial infections

9.2.3.1. *Streptococcus pneumoniae*: *S. pneumoniae* infections cause ≈11% of all deaths among children up to 5 years old[219] and are the major cause of community-acquired pneumonia in the elderly.[220] Like many other sialic-acid-utilizing pathogens, *S. pneumoniae* expresses a neuraminidase (nanA) and a sialic acid transporter (SatABC) that together are capable of harvesting and taking up sialic acids from host mucins and other glycoconjugates in the nasopharynx. Interestingly, *S. pneumoniae* TIGR4 (serotype 4) has evolved to respond preferentially to Neu5Ac over Neu5Gc under conditions of low glucose (e.g., nasopharynx) with a positive feedback loop, upregulating both nanA and htrA, which protects from oxidative stress. This phenomenon might ultimately explain why *Cmah*$^{-/-}$ mice experience a faster pneumococcal disease progression after intranasal but not intravenous challenge.[221]

9.2.3.2. Typhoid fever: *Salmonella enterica* serovar typhi (*S. typhi*) is a 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.[222] Although *S. typhi* is not capable of infecting mice, a mouse model that preferentially binds Neu5Ac was developed by injection with typhoid toxin.[223] Thus, typhoid toxin can produce typhoid fever symptoms in WT mice, but not in transgenic mice that overexpress *Cmah* (≈98% Neu5Gc on all tissues).[87] Although this does not explain why humans and chimpanzees develop typhoid infection after consumption of *S. typhi,* it might help explain why humans experience a more severe form of typhoid fever than chimpanzees.[33]

9.2.3.3. SubAB toxins: Shiga toxigenic *Escherichia coli* (STEC) is a common food-borne pathogen[224] that can cause serious diseases, including bloody diarrhea, and sometimes hemolytic-uremic syndrome (HUS). STEC secretes a SubAB toxin that was found to preferentially bind Neu5Gc in vitro and ex vivo. Although the 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 to a lack of competitive inhibition of serum proteins in *Cmah*$^{-/-}$ mice.[93] Regardless, owing to its Neu5Gc binding preference, humans who consume red meats rich in Neu5Gc might incorporate it into their gut epithelium and this can allow for toxicity of a subsequent SubAB-positive infection.[225]

9.3. Muscular Dystrophy

In humans, Duchenne muscular dystrophy (DMD) is the most common and most severe muscular dystrophy affecting children.[226,227] 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.[227,228] But until recently, a critical barrier to progress in the field has been the stark difference in the severity of the muscular dystrophy observed between mice

and humans,[229] with mice showing minimal phenotypes. However, *Cmah*$^{-/-}$ mice experience a profound increase in DMD and limb-girdle muscular dystrophy 2D (LGMD2D) severity, most notably in DMD life expectancy.[118–120] *Cmah*$^{-/-}$/mdx mice experience greater muscle weakness, greater skeletal muscle fibrosis, greater immune cell recruitment to both the cardiac and skeletal muscle tissue, more inflammatory cytokine production in skeletal muscle, and decreased survival compared to WT/mdx controls.[118–120] Further work is needed to determine whether this difference should be attributed to an intrinsic difference in muscle physiology, the adaptive immune system, and/or the innate immune system. This could be tested by HSA-Cre (muscle), CD4-Cre (T-cells of the adaptive immune system), or CD14-Cre (innate immune system) expression of Cmah in *Cmah*$^{-/-}$/mdx mice, but such experiments have yet to be reported. Besides an apparent increase in Neu5Gc versus Neu5Ac affinity for α-laminin in vitro,[118] the underlying mechanisms responsible for this phenomenon are completely unknown, and it is possible that "xenosialitis" could be an aggravating factor.[120] Understanding these mechanisms could reveal new therapeutic targets that are possibly beneficial to the human lineage. They could also help us to understand what has set human muscle tissue apart from mouse and chimpanzee muscle tissues, which are both relatively high in Neu5Gc.[120]

10. Evolutionary Implications of Human CMAH Loss

Although sexual and microbial selection might have led to the fixation of *CMAHP*, subsequent changes in systemic inflammation and metabolism could have benefited ancient hominins transitioning towards exposure to new pathogen regimes during the transition from forests to open savannahs, to an increased consumption of other animals, and the earliest developments of stone tool use.[64–66,79,114,230] The definite evolutionary cost of CMAH loss is at least twofold: first, the inability to modulate the ratio of Neu5Gc and Neu5Ac in 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.[231]

11. Other Medical Implications of CMAH Loss in Humans

11.1. Xenotransplantation

Humans, apes, and Old World monkeys also lack a terminal α-galactose (α-gal) residue, which is a major antigen (along with Neu5Gc) causing hyperacute rejection after xenotransplantation in humans or decreased half-life in animal-based transplantations.[232] To address this, pigs lacking α-gal[233] or both α-gal and Neu5Gc[234] were created so as to decrease the immunogenicity of pig xenografts—with some success.[235] 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.[236] Ongoing studies are seeking to systematically improve potential sources of tissue for xenotransplantation.[237]

11.2. Stem cells and recombinant proteins

Nutritionally, there is a major difference in immunogenicity between Neu5Gc and α-gal that occurs during catabolism, during which α-gal becomes free galactose (and is further utilized normally), but free Neu5Gc is incorporated and presented on host cell surfaces as the same bound foreign antigen.[58,238] 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; this potentially causes rapid clearance from circulation[109] and/or triggers an antibody-mediated inflammatory response.[49,109,239]

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 (walruses, sea lions and seals), mustelids (ferrets), and platyrrhines (New World monkeys) have all been shown to have independently lost or inactivated CMAH and the endogenous production of Neu5Gc.[86,240] Similarly to humans,[205] ferrets express high levels of α2–6-linked Sias in their airway epithelium, which, along with Neu5Gc loss, explains their successful use as a model for human influenza infections.[86] Interestingly, New World monkeys are the only other primates known to lack Neu5Gc and are the standard model for *P. falciparum* infection in vivo.[90,241] Further field studies are necessary to determine whether New World monkeys are potentially a reservoir for human *P. falciparum* infection in the wild.[242]

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 to be at play. Importantly, these mechanisms could have implications for the treatment of diseases specifically affecting humans, such as muscular dystrophy, that are difficult to model in rodents. Exogenously, many deadly human pathogens have a Neu5Ac preference and possibly a linkage-specific preference contributing to their species-specific infectivity, as is the case with influenza. Furthermore, many pathogens, commensal bacteria, and/or symbiotic bacteria, such as *S. pneumoniae*, might have an optimal metabolic preference for Neu5Ac over Neu5Gc. Many of these and other questions about human sialic acid biology remain unexplored or unreported. The hope is that these studies will highlight the

great importance of sialic acids and spur further investigations towards the development of therapeutics.

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Conflict of Interest

The authors declare no conflict of interest.

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