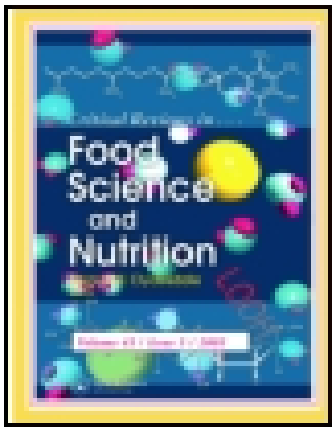

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The Nutritional Role of Free Sialic Acid, a Human Milk Monosaccharide, and Its Application as a Functional Food Ingredient

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THE NUTRITIONAL ROLE OF FREE SIALIC ACID, A HUMAN MILK MONOSACCHARIDE, AND ITS APPLICATION AS A FUNCTIONAL FOOD INGREDIENT

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ABSTRACT

N-Acetyl-D-neuraminic acid (NANA), more commonly known by its trivial name *sialic acid*, is an endogenous human and ubiquitous nutritional monosaccharide. As a bound sugar at the terminal positions of glycans NANA is known to play important roles in many biological events. The data that exist on the occurrence of the free monosaccharide in breast milk and nutrition, however, are less commonly discussed. In most foods of animal origin sialic acid occurs as a mixture of NANA and *N*-glycolyl-D-neuraminic acid (NGNA), a hydroxylated derivative of NANA that is not found in humans. The dietary intake of NGNA has been identified as a risk factor for long-term adverse health effects. Therefore, we present summaries on the

biochemistry, metabolism, bioavailability and the data on NANA and NGNA levels that occur in diverse foods. Finally, we discuss the emerging data demonstrating that free NANA is linked to positive nutritional effects including pronounced anti-oxidative properties. These data and the extremely high safety profile of NANA justify dietary enrichment at levels that correspond to the dietary intake of NANA in infants through breast milk.

KEY WORDS

Sialic acid, Breast-milk, Human Milk Saccharides, Infant Nutrition, Anti-oxidant

ABBREVIATIONS

ADOA = 4-(Acetylamino)-2,4-di-deoxy-D-glycero-D-galacto octonic acid)

CMAH = Cytidine Monophosphate *N*-Acetyl-D-neuraminic acid Hydroxylase

GNE = UDP-GlcNAc 2-Epimerase/ManNAc Kinase

HMM = Human milk monosaccharide

HMO = Human milk oligosaccharide

HMS = Human milk saccharide

KDN = 2-Keto-3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid

ManNAc = *N*-Acetyl-D-mannosamine

D-Neuraminic acid = 5-Amino-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid

NANA = **Neu5Ac** = *N*-Acetyl-D-neuraminic acid

NGNA = **Neu5Gc** = N-Glycolyl-D-neuraminic acid

Sia = Sialic acids

INTRODUCTION

Many saccharides that occur endogenously in humans are involved in biological functions other than simply serving as sources of energy and therefore form a class of potential new functional food ingredients. Of particular interest are the saccharides that are found in human breast milk (termed human milk saccharides, HMS) that possess non-digestible and therefore non-glycemic properties. The mere presence of these saccharides at significant levels in breast milk suggests a biological function since breast milk, which serves as the sole nutrition for the fast growing and vulnerable infant, has evolved under a trade-off optimization process between mother and child throughout evolutionary times (Petherick, 2010, German et al., 2002, Trivers, 1974). Following this reasoning it would make little evolutionary sense for mothers to continue investing in the biosynthesis of such energetically costly saccharides (Pike and Milligan, 2010). The most widely discussed of the non-digestible saccharides of human breast milk are the human milk oligosaccharides (HMO) (Bode, 2012, Malpress and Hytten, 1958). However, human breast milk also contains important non-digestible disaccharides, such as lacto-*N*-biose (LNB) and *N*-acetyl-D-lactosamine (LacNAc), and monosaccharides, such as L-fucose, *N*-acetyl-D-glucosamine (GlcNAc) and sialic acid, all of which are candidates for use as functional food ingredients.

Here, we focus on the potential of the human milk monosaccharide (HMM) sialic acid as a functional food ingredient. Sialic acid is known to occur predominantly as a bound sugar (covalently linked by a glycosidic bond) at the terminal positions of milk oligosaccharides and of conjugated glycans, which are the oligosaccharide structures of gangliosides, glycolipids and glycoproteins that are exposed on the surface of cells. Sialic acid also occurs in its free form in

the human body, including in breast milk, and is present in other nutritional sources. As the terminal sugar of glycans, sialic acid has long been recognized to play important biological roles in phenomena connected to mucus viscosity, proteolytic protection of proteins, cell-cell recognition, reproduction, infection, immunity and cognitive development (Cohen and Varki, 2010, Chen and Varki, 2010, Lewis and Varki, 2009, Varki, 2008, Varki and Varki, 2007, Bianco and Melchioni, 2002, Schauer, 2001, Traving and Schauer, 1998, Kiss and Rougon, 1997, Kelm and Schauer, 1997, Schauer, 1973). The current available scientific and nutritional information on this HMM is summarized in this review and potential roles of the free form are also discussed along with cases of increased need (i.e., when endogenous biosynthesis may be unable to provide optimal amounts of sialic acid). The nutritional and biological importance of sialic acid is further discussed as it relates to the current development of the free form of sialic acid (Choi et al., 2014) for use as a food ingredient for addition to infant formula to provide similar levels as those present in human breast milk.

HISTORICAL NOTES ON DISCOVERY AND NOMENCLATURE

Sialic acid is a monosaccharide with a rather unusual 9-carbon backbone and an α-keto acid functionality that renders it strongly acidic (pKa = 2.6) (Hurd, 1970). The unusual structure of sialic acid when compared to most common sugars is the result of an enzymatically catalyzed aldol condensation that takes place between *N*-acetyl-D-mannosamine (ManNAc) and phosphoenol-D-pyruvate (Figure 1, Panel A). In aqueous solution (and under physiological conditions) sialic acid occurs as a dynamic mixture of various equilibrated tautomeric forms of the compound (open-chain and α/β cyclic anomers) (Klepach et al., 2008).

While ñsialic acidò is the colloquial term used for the sialic acid monosaccharide that exists in the human body, the correct chemical name is *N*-acetyl-D-neuraminic acid (NANA, see below). The term sialic acids (*Sia*) refers instead to a family of monosaccharides comprising more than 60 members that are all derivatives of D-neuraminic acid (Figure 1, Panel B) (Angata and Varki, 2002, Varki and Schauer, 2009, Schauer, 2004). In this sense, the term *sialic acid* is a group designation very much like the term *amino acid*, and the abbreviation *Sia* is commonly used to differentiate the group designation from the colloquial term. However, considering that the trivial name *sialic acid* has been used so persistently over time, it is also used throughout this review to designate NANA, except in instances where differentiation from another form of sialic acid is required.

In the first half of the 20th century sialic acids were the last of the endogenous human monosaccharides to be discovered. Due to their complex structure and their occurrence in several different forms (Figure 1, Panel B) the discovery, isolation, characterization and nomenclature of sialic acids are linked to a complex and fascinating endeavor carried out by several independent research groups over a period of more than 30 years (more information on historical events is provided in table S1 of the supplementary information).

The first indications leading to the discovery of sialic acids were reported in 1927 by Erwin Walz (Walz, 1927), but it was Ernst Klenk who, in the course of his ground-breaking research that would later culminate in the discovery of the brain gangliosides, recognized the significance of the sialic acid that occurs in humans (Klenk, 1935). Indeed, it was Klenk who introduced the name *neuraminic acid* as a result of its isolation from the human brain (Klenk, 1941). Over the

following years this same saccharide was unknowingly isolated from various other sources by independent researchers, but given different names. It was isolated from bovine submaxillary mucin by Gunnar Blix (Blix, 1936), who introduced the name s*ialic acid* (Blix et al., 1952) (derived from saliva), from horse blood erythrocytes by Tamio Yamakawa, who introduced the name *prehemataminic acid* (Yamakawa and Suzuki, 1951), from bovine colostrum by Richard Kuhn, who introduced the name *lactaminic acid* (Kuhn et al., 1954), and from human milk by Paul György (Hoover et al., 1953), who introduced the name *gynaminic acid* (Zilliken et al., 1955). This parallel discovery of sialic acid was soon recognized and in 1957 a general nomenclature for sialic acids was introduced (Blix et al., 1957). This proposed nomenclature has been adopted into the official NOMENCLATURE FOR CARBOHYDRATES issued by the International Union of Pure and Applied Chemistry (IUPAC) (McNaught, 1996).

BIOSYNTHESIS AND METABOLISM OF SIALIC ACID

Anabolism and catabolism

The human body is able to biosynthesize sialic acid endogenously, but there is emerging evidence of cases of increased need (or benefit) for sialic acid beyond the levels that the human body can produce (*vide infra*). The endogenous levels are likely an evolutionary trade-off between the potential maximum benefit and the metabolic expenditure required to biosynthesize sialic acid. To gain a better understanding of these cases of increased need, a look at the endogenous biosynthesis and metabolism of sialic acid is first needed.

The endogenous production of sialic acid occurs in part by enzymatic means. The enzymes responsible for sialic acid biosynthesis (and metabolism) have been reviewed previously (Tanner, 2005, Angata and Varki, 2002, Li and Chen, 2012). Figure 2 provides a general overview of the most important enzymes involved in the *anabolic* and *catabolic* biochemical pathways (Keppler et al., 1999, Bergfeld et al., 2012). Biosynthesis (anabolism) of sialic acid occurs in the cytosol, following which sialic acid is activated to cytidine-5'-monophospho-NANA (CMP-NANA) in the nucleus (Kean et al., 2004). CMP-NANA is then transported out of the nucleus via the CMP-NANA transporter (Eckhardt et al., 1996) and delivered into the Golgi apparatus where a set of tissue- and substrate-specific sialyltransferases (Harduin-Lepers et al., 2001) utilize the CMP-activated sialic acid to sialylate terminal galactose residues of free oligosaccharides (in the mammary gland) or of the oligosaccharide chains of glycoconjugates (leading to the formation of glycoproteins and gangliosides). The key enzyme that catalyzes the rate-limiting step of sialic acid biosynthesis is the bifunctional GNE (UDP-GlcNAc 2-epimerase/ManNAc kinase) enzyme (Hinderlich et al., 2013). This enzyme is also responsible for regulating NANA levels in the human body via a feedback inhibition mechanism involving the allosteric binding of CMP-NANA (Kornfeld et al., 1964) to the enzyme. The mammalian GNE enzyme was fully characterized in 1997 (Hinderlich et al., 1997) and its importance in cell surface sialylation was recognized in 1999 (Keppler et al., 1999). A complete knock-out of the *Gne* gene results in complete loss of endogenous sialic acid production (Hinderlich et al., 2013) and results in early embryonic lethality in mice (Weidemann et al., 2013). The enzyme is broadly expressed at different levels among various tissues in mammals (and other animals), but the highest expression is reported to occur in the liver and the placenta (Reinke et al., 2009).

Subsequent metabolism of sialic acid occurs at the monosaccharide level. Thus, prior to entering the catabolic pathway, bound sialic acid (i.e., glycoconjugates containing sialic acid) in the body must first undergo cleavage to release the monosaccharide. The enzymes involved in sialic acid cleavage are the neuraminidases (also known as sialidases) (Monti et al., 2010), which are localized in the lysosome (NEU1), cytosol (NEU2), outer plasma-membranes (NEU3) and intracellular cell compartment membranes (NEU4). NEU3 also occurs in human intestinal mucosa (Ghosh et al., 1968, Den Tandt et al., 1987). Free sialic acid then enters the catabolic pathway. The key enzyme for sialic acid *catabolism* is the acylneuraminate pyruvate-lyase (sialic acid aldolase), which is localized in the cytosol and which splits sialic acid into ManNAc and pyruvate (Schauer et al., 1999). ManNAc is then either recycled by ManNAc kinase within a salvage pathway or is further metabolized to GlcNAc by UDP GlcNAc 2-epimerase. GlcNAc in turn is converted to GlcNAc 6-phosphate by GlcNAc kinase. GlcNAc 6-phosphate is then deacetylated by GlcNAc 6-phosphate deacetylase to glucosamine 6-phosphate, which enters the conventional hexosamine pathway (Bergfeld et al., 2012).

Absence of N-glycolyl-D-neuraminic acid (NGNA) in humans

Closely related to NANA is the sialic acid *N*-glycolyl-D-neuraminic acid (NGNA), which occurs endogenously in almost all mammals, except humans, New World monkeys (Springer et al., 2014) and ferrets (Ng et al., 2014). The reason for which only NANA, and not its hydroxylated derivative NGNA, occurs in the human body is due to the fact that we have lost the ability to convert NANA to NGNA. This biochemical reaction is catalyzed by the enzyme cytidine monophosphate N-acetyl-D-neuraminic acid hydroxylase (CMAH) in all other mammals [with

the notable exceptions of New World monkeys (Springer et al., 2014) and ferrets (Ng et al., 2014)]. The loss in humans is the result of an inactivating mutation of the *CMAH* gene due to a deletion in the first exon, resulting in a frame-shift mutation that leads to the aberrant expression of a non-functional truncated enzyme (Hayakawa et al., 2001). This mutation occurred in the course of human evolution approximately 3.2 million years ago (MYA) and became fixed in the population approximately 2.9 MYA, meaning that all members of this ancestor population had become homozygous for the allele containing the mutation (Hayakawa et al., 2006, Ghaderi et al., 2011). Consequently, humans today have no remaining CMAH enzyme activity. Comprehensive discussions with multidisciplinary insight on the evolutionary implications and consequences of this mutation can be found elsewhere in the scientific literature (Varki et al., 2011, Varki, 2010). It is noteworthy to mention that recent archaic genome sequencing results have independently confirmed that this mutation was indeed shared with our extinct hominin cousins, the Neandertals and Denisovans (Pääbo, 2014).

METABOLIC FATE OF DIETARY SIALIC ACIDS

In addition to the utilization of endogenously produced sialic acid the human body is able to utilize sialic acid from dietary sources. Such dietary sources are either from breast milk or of animal origin and are further discussed later in this review. Here, we provide an overview of the availability, pharmacokinetics, and utilization of dietary sialic acid in the human body. As mentioned previously, sialic acid occurs predominantly in the bound form as the outermost sugar of oligosaccharides and glycoconjugates, including sialylated oligosaccharides, gangliosides, glycoproteins, and mucin glycoproteins (i.e., heavily glycosylated long tandem-repeat peptides).

Sialic acid also occurs in its free form. Thus, the overall metabolic fate of dietary sialic acid will vary depending on the biomolecular source (see Figure 3).

Partial release of dietary free sialic acid

Dietary sialic acid that is present in bound form is partially released into its free form following consumption *via* two distinct mechanisms, one consisting of acid hydrolysis and the other involving enzymatic cleavage. The α-glycosidic bond of sialic acid conjugates is sensitive to acid hydrolysis, and therefore, it is reasonable to assume that a proportion of bound sialic acid is released during gastrointestinal passage as a result of the acidic environment of the gastrointestinal tract. This bond is in fact more sensitive to acid hydrolysis than those of other monosaccharides bound to glycoconjugates present in food (Sonnenburg et al., 2002). A feature that forms the basis of all quantitative analytical methods for sialic acid in which bound sialic acids are hydrolyzed prior to the chromophore formation step (Klenk et al., 1941, Svennerholm, 1957, Hess and Rolde, 1964, Warren, 1959, Crook, 1993, Rohrer, 2000). Consistent with this, a study investigating the stability of sialic acid conjugates of human milk and infant formulas confirmed that levels of total sialic acids are reduced in human milk and infant formula samples following simulated gastrointestinal digestion (Lacomba et al., 2011a). Regrettably, the authors of this study did not report the actual levels of free sialic acid that were released. However, others have determined that the ratio of free to bound sialic acid is strongly shifted towards the free form in infantsô feces (Sabharwal et al., 1991) when compared to the initial ratio in breast milk, which supports that free sialic acid is released from dietary sources in the gastrointestinal tract. At the same time, given the weakly acidic gastric pH of infants (typically between pH 3

and 5.5) (Armand et al., 1996, Maffei and Nóbrega, 1975, Koski and Liukkonen, 1937), the release of free sialic acid from acidic cleavage would be limited. Free sialic acid, however, is also released by enzymatic cleavage through the action of neuraminidases (sialidases), which are present in human breast milk (Wiederschain and Newburg, 2001, Schauer et al., 1976), human intestinal mucosa (Den Tandt et al., 1987, Ghosh et al., 1968) and gut commensal bacterial cultures (Lee et al., 2014, Corfield et al., 1993a), and which therefore contribute to the amount of free sialic acid (and its metabolites) that is released from dietary sources. For instance, the exo-α-sialidase from *Bifidobacterium bifidum* that was recently characterized is an enzyme that is located on the outer cell-surface of the bacterium and cleaves sialic acid from glycoconjugates to make the monosaccharide and the resultant oligosaccharides available to the bacterium (Kiyohara et al., 2011). Additionally, Nöhle and Schauer (1984) have shown that half of an orally administered dose of radiolabeled sialyllactose (a conjugated form of sialic acid) is hydrolyzed to free sialic acid by intestinal sialidase in mice and rats (Nöhle and Schauer, 1984, Dickson and Messer, 1978). Once released, free sialic acid would remain intact as, unlike its activated nucleotide (CMP-NANA), sialic acid itself has been demonstrated to be stable under conditions similar to those of the gastrointestinal tract (Ruano et al., 1999). The general acid stability of free sialic acid has previously been intensively investigated (Karamanos et al., 1990, Kamerling et al., 1975, Takasaki and Kobata, 1974).

Metabolic fate of dietary free sialic acid

Once released, or ingested as a free monosaccharide, NANA becomes available for absorption as is the case with other free monosaccharides. The absorption and subsequent metabolic fate of

NANA in the body has been investigated through pharmacokinetic studies conducted by Witt *et al.* (Witt et al., 1979), and Nöhle and Schauer (Nöhle and Schauer, 1981). The studies were carried out in rats (Witt et al., 1979) and mice (Nöhle and Schauer, 1981) to which free sialic acid was orally administered *via* gavage as a radiolabeled product. The results of these studies demonstrate that NANA is rapidly absorbed, reaching the systemic circulation within 30 minutes to 1 hour of administration, with peak plasma concentrations attained within 1.5 to 4 hours (Witt et al., 1979, Nöhle and Schauer, 1981). The majority of an ingested amount of NANA is absorbed into the body; in mice it was determined that 95 % of the administered dose entered the gastrointestinal tract (within 15 minutes), 90 % of which was absorbed (within 6 hours) (Nöhle and Schauer, 1981). Plasma concentrations decline rapidly after peak levels are attained, following which NANA distributes mainly to the liver and brain (maximum levels of approximately 3 to 4 % of administered dose, respectively), but also distributes to other organs, including the heart, kidneys, spleen and lungs (Witt et al., 1979, Nöhle and Schauer, 1981). Radioactivity was reduced in these organs by 3 hours post-administration in rats, and no sex-dependent variations in uptake or distribution were observed (Witt et al., 1979). The majority (at least 70 to 90 % in mice and rats) of an ingested amount of NANA is excreted unchanged in urine (Witt et al., 1979, Nöhle and Schauer, 1981). Urinary excretion occurs rapidly with detection of the compound in urine as early as 1 hour post-administration (Witt et al., 1979, Nöhle and Schauer, 1981) and with urinary levels increasing linearly with time thereafter (Witt et al., 1979). It is anticipated, however, that a small amount of NANA would undergo catabolic metabolism based on the finding that *N*-acetylneuraminate lyase activity has been detected in the liver, kidney, spleen, brain, and intestinal tissues of mice and that incubation of homogenates

prepared from these tissues with NANA resulted in the formation of metabolites, including ManNAc and pyruvic acid (Nöhle and Schauer, 1981). The metabolites are then available for entry into the sialic acid salvage pathway.

Nöhle *et al.* (Nöhle et al., 1982) conducted a similar pharmacokinetic study in mice by using radiolabeled *N*-glycolyl-D-[2-^{14}C, 9-^{3}H]neuraminic acid (radiolabeled NGNA) and found comparable absorption, distribution and excretion data as with NANA, with the majority of the administered dose excreted unchanged in urine. As with NANA, incubation of NGNA with liver, kidney, spleen, brain, and intestinal tissue homogenates resulted in the formation of ManNGc and pyruvic acid, however, with *N*-acetylneuraminate lyase activity being 10-20 % greater for NANA than for NGNA. This finding supports the assumption that most enzymes involved in the metabolism of sialic acids do not strictly differentiate between NANA and NGNA. Based on the high proportion of free NGNA that was excreted unchanged in the urine and the observation that free NGNA does undergo some catabolic metabolism, the authors speculated that dietary sialic acids may not be available for direct use in the biosynthesis of glycoconjugates (this is discussed in more detail below). In contrast, a large number of animal studies have in fact demonstrated bio-incorporation of sialic acid upon oral administration of the free monosaccharide (Abazia et al., 2003, De Vries and Barondes, 1971, Carey and Hirschberg, 1979, Morgan and Winick, 1980, Morgan and Winick, 1981, Carlson and House, 1986, Colombo et al., 2003, Downing et al., 2001, Wang et al., 2007). In conclusion, while the majority may be excreted unaltered in the urine, the available evidence indicates that a significant proportion of a bolus dose of free sialic acid is incorporated into glycoconjugates.

Although from the classical biochemical viewpoint negatively charged and highly hydrophilic molecules such as sialic acid usually do not cross cell membranes, this general assumption does not apply to sialic acid (in line with the above findings). This has been demonstrated in *in vitro* eukaryotic cell studies, in which isotopically labeled free sialic acid was efficiently taken up by the cells, converted to CMP-NANA and incorporated into cell surface sialoglycans (Oetke et al., 2001). The existence of an efficient uptake mechanism for this negatively charged monosaccharide was therefore proposed (Oetke et al., 2001), and later discovered and elucidated by Bardor *et al.* in experiments employing the sialic acid NGNA (Bardor et al., 2005). Considering that the enzymes of the sialic acid metabolic pathway are not able to differentiate between NANA and NGNA, the results of these experiments apply directly to NANA as well. In these experiments, it was demonstrated that free and bound sialic acid is delivered to the cellular lysosome by macropinocytosis, where bound sialic acid is cleaved by neuraminidase and free sialic acid is exported to the cytosol via the sialin transporter (Yarovaya et al., 2005). Within the cytosol, sialic acid is available for entry into the salvage pathway, either by direct transport to the nucleus or via the catabolic intermediate ManNAc. The promiscuity and high efficiency of the sialic acid salvage pathway is highlighted by a new investigational technique called metabolic glycoengineering in which a range of simple derivatives, or metabolic precursors of sialic acid (e.g. ManNAc), are incubated with cells, leading to their bio-incorporation and expression on the cell surface (Du et al., 2009).

The mechanism of bio-incorporation of dietary sialic acids has been further characterized by Banda *et al.* using a *CMAH* $^{-/-}$ mouse model (Banda et al., 2012). The authors reported that the bio-incorporation rate is higher for glycoconjugates of sialic acid than for free sialic acids, likely

because although the latter is rapidly absorbed into the systemic circulation, it is also quickly excreted in urine. The bound form therefore forms a reservoir for sialic acid that is not as quickly excreted as the free form, and animal studies have confirmed this point (Yonekawa et al., 2014). Greater levels of bio-incorporation, however, have been observed in human tumor cell lines and further details on the intra-cellular metabolic pathways for NGNA were outlined in an accompanying publication (Bergfeld et al., 2012). The motivation for these extended studies was on one hand to understand the metabolic mechanisms that underlie the cellular surface expression of the non-human sialic acid NGNA in human tissues, which must originate from dietary sources given the absence of the CMAH enzyme in humans and, on the other hand, to explain why the levels of NGNA in the human body are not steadily enriched by continuous dietary intake. The latter may be explained by the presence of a dynamic equilibrium between Sia bioconjugation and cleavage, in which case the catabolic pathway of NGNA gains relevance as it enables a ñwashing outò of NGNA via the subsequent deacetylation step where GlcNAc 6-phosphate deacetylase removes the N-glycolyl chain (as depicted in the right-hand pathway of Figure 2) (Bergfeld et al., 2012).

Adverse health effects caused by dietary NGNA

The apparent absence of NGNA in the human body was recognized in 1973 (Cabezas, 1973). At the same time there have been frequent reports for its particular enrichment in tumor tissues (Malykh et al., 2001). It was therefore initially assumed that NGNA constitutes a human oncofetal antigen, meaning that it may be transiently expressed during fetal development and re-expressed in instances of tumor progression (Muchmore et al., 1989). It was then discovered that

the absence of NGNA is not linked to developmental down-regulation but that it cannot be biosynthesized in humans (Irie et al., 1998, Chou et al., 1998, Muchmore et al., 1998) and therefore, it was concluded that NGNA must derive from dietary origin (Varki, 2001d). However, it was initially also proposed that NGNA may derive from endogenous glycolyl-CoA via the hydroxypyruvate metabolic pathway (Malykh et al., 2001) but this was later experimentally ruled out (Bergfeld et al., 2012). Furthermore, it was discovered that healthy humans possess serum antibodies against NGNA (Zhu and Hurst, 2002), similar to patients that receive horse serum treatments and who subsequently develop the serum sickness disease caused by the production of antibodies against the NGNA-containing so-called Hanganutziu-Deicher antigens (Higashi et al., 1977, Merrick et al., 1978). The recognition of this molecular event is of great interest as it constitutes the first known biomedical example in which a non-endogenous molecule of dietary origin is absorbed, taken up by cells, is enzymatically incorporated and presented on the cell surface as an integral part of endogenous bioconjugates, but due to its non-human nature, elicits an immune response via antibody production against the bodyôs own cell structures. This phenomenon is expressed by the term ñ*human xeno-auto antigen*ò (Tangvoranuntakul et al., 2003) and more lyrically, but still accurately, described as a ñmolecular Trojan horseò (Talafova and Nahalka, 2012). Immunological testing has revealed that all humans possess circulating antibodies against NGNA (Nguyen et al., 2005) and two alternative mechanisms of antibody formation have been proposed (Taylor et al., 2010, Hedlund et al., 2008), both involving dietary NGNA. The presence of these NGNA-antibodies, and the immune response they mediate, is suspected to cause a subchronic inflammation known as ñxeno-sialitisò (Samraj et al., 2013) and to potentially be involved in the etiology of inflammatory

diseases, such as atherosclerosis (Pham et al., 2009), arthritis and coronary heart disease (Varki, 2010). Furthermore, the epidemiological correlation between increased red meat intake and a high incidence of adverse health effects (Micha et al., 2010) (e.g., breast cancer) (Farvid et al., 2014) has been suggested to be connected to the fact that red meats are a rich dietary source of NGNA (Varki, 2010). There is emerging evidence that the chronic inflammatory status caused by NGNA presentation on cancer cells supports tumor angiogenesis (Hedlund et al., 2008). Yin *et al.* determined that hypoxic culturing of cancer cells induces the expression of sialin, the sialic acid transporter (Yin et al., 2006). In addition, a study by Inoue *et al.* demonstrated that the salvage pathway becomes dominant over sialic acid *de novo* synthesis in cancer cells, which contributes to the understanding of the pronounced enrichment of NGNA in these cells (Inoue et al., 2010). Together, the findings of Yin and Inoue suggest that lysosomal sialin expression may be the key modulator of the salvage pathway. The accumulated evidence describing the involvement of NGNA in human cancer has been recently discussed by Samraj *et al.* (Samraj et al., 2014, Samraj et al., 2015) and provides support to the notion that frequent intakes of larger amounts of NGNA through nutritional sources, like red meat (Samraj et al., 2015), need to be considered a dietary risk factor for cancer.

The biomedical implications connected to the evolutionary loss of NGNA in humans reach further than the potential health risks associated with dietary NGNA. The natural presence of NGNA in the tissues, organs and skin of animal origin presents another, and only recently recognized, immunological barrier for xenotransplantation (Padler-Karavani and Varki, 2011, Scobie et al., 2013, Waldman et al., 2014). Additionally, the presence of NGNA in media (prepared from animal serum) used for cell culturing in the production of biotherapeutic products

(e.g. therapeutic antibodies) presents a source for contaminating such products with NGNA (Diaz et al., 2009, Ghaderi et al., 2010, Ghaderi et al., 2012). Furthermore, there result a number of sialic acid-specific biomedical differences between humans and animals due to the absence of NGNA in humans (Varki et al., 2011), which may lead to differentiated immunological response towards NGNA-containing epitopes. Therefore, NGNA contamination of biotherapeutic drugs (e.g. glycoprotein therapeutics) bears the risk that potential human immunological reactions towards the drug, which should be identified during the preclinical drug development phase, are not adequately recognized in animal-based safety studies. This may explain to some extent the adverse and unanticipated immunological reactions that have arisen during a number of human clinical trials on biotherapeutic antibodies (Wadman, 2006, Nguyen et al., 2006).

ENDOGENOUS LEVELS OF SIALIC ACID IN THE HUMAN BODY

We focus on presenting the data and the general trends extracted from an in-depth analysis of the scientific literature pertaining to the occurrence and levels of sialic acid throughout the human body (more details are provided in the supplementary information). We have omitted a detailed discussion on analytical methods, which have been presented elsewhere (Lacomba et al., 2010, Hurum and Rohrer, 2012, Rohrer, 2000, Svennerholm, 1964, Aminoff, 1961, Warren, 1959, Schauer, 1982, Lamari and Karamanos, 2002, Crook, 1993).

Total sialic acid in the human body

The highest concentrations of total sialic acid (bound and free) in the human body and its fluids are found in breast milk colostrum (879 to 1782 mg/L), feces from breast-fed infants (412 to 1197 mg/kg), the grey matter of the brain (481 to 1067 mg/kg), transitional breast milk (319 to 1196 mg/L), semen (829 mg/L), cerebral myelin (632 mg/kg), blood serum (360 to 1052 mg/kg), mature breast milk (208 to 1122 mg/L), tears (247 to 557 mg/L), the white matter of the brain (275 to 453 mg/kg) and saliva (32 to 253 mg/L) (see Table 1, and Tables S2-S4 of the supplementary information for further details). Lower concentrations of total sialic acid occur in gastric aspirate, the spinal cord, urine, amniotic fluid, feces and cerebrospinal fluid (CSF).

Free sialic acid in the human body and in breast milk

While most studies have focused on total sialic acid concentrations throughout the human body, a number of studies have also reported on the occurrence of free sialic acid in humans. Concentrations of free sialic acid have been quantified in breast milk (colostrum, transitional and mature), brain, urine, saliva, CSF, amniotic fluid and serum (see Table 1 and Tables S2-S4 of the supplementary information for further details). In other tissues of the body it has been estimated that the portion of free sialic acid is approximately 1-2 % of the total sialic acid concentration (Schoop et al., 1969).

The occurrence of free sialic acid in breast milk has only been recognized relatively recently, and thus, some previous reports state the contrary (Lamari and Karamanos, 2002, Nakano et al., 2001, Colombo et al., 2003). This is linked to the fact that earlier analytical methods were

developed to quantify sialic acid of the ganglioside fraction, and thus, failed to differentiate between the bound and free saccharide (Svennerholm, 1957). Many methods required a hydrolysis step, which releases sialic acid from the bound form prior to chromogenic labelling and quantification (Warren, 1959). The reliability of these methods in detecting free sialic acid were therefore questioned in some cases as has been reviewed by Svennerholm (Svennerholm, 1964). In fact, most methods for total sialic acid quantitation to date rely on the quantitative acidic release of sialic acid prior to quantitation of the resulting free saccharide (the glycosidic bond of sialic acids is relatively acid-labile; see above) (Werner and Odin, 1952, Saifer and Gerstenfeld, 1957). An overview on the traditional methods is provided by Crook (Crook, 1993). Today, a number of analytical methods are available with which the bound and free forms can be properly differentiated (Jourdian et al., 1971, Hara et al., 1986, Hara et al., 1987, Hayakawa et al., 1993, Thurl et al., 1996, Wiederschain and Newburg, 2001, Aminoff, 1961, Skoza and Mohos, 1976, Pearce and Major, 1978, Galeotti et al., 2012, van der Ham et al., 2010, Tebani et al., 2011). Through application of these methods, a number of investigators have reported values of free sialic acid in human milk with levels ranging on average between ~ 20 and 59 mg/L (Qiao et al., 2013, Oriquat et al., 2011, Martin-Sosa et al., 2004, Wiederschain and Newburg, 2001, Wang et al., 2001a, Thurl et al., 1996, Hayakawa et al., 1993). The comparably high values reported by Sabharwal *et al.* (129 mg/L) (Sabharwal et al., 1991) and Galeotti *et al.* (179 mg/L) (Galeotti et al., 2012) fall outside the range of these data, which in the latter case may be explained by the lack of NANA-specific calibration of the GC-MS method used. These values are therefore considered as outliers of the calculated values presented in Table 1. The observation, however, by Galeotti *et al.* that milk from secretor-negative and LEWIS-negative

mothers (milk type 4) (Thurl et al., 1997, Kunz and Rudloff, 1993) contains significantly higher levels of free sialic acid than milk from mothers with other phenotypes is remarkable and warrants further investigation.

It is interesting to note that neuraminidase (Schauer et al., 1976, Wiederschain and Newburg, 2001) and sialyltransferase activity (Bartholomew et al., 1973) have both been detected in human milk, while the enzyme that is responsible for converting free sialic acid to CMP-sialic acid (i.e., CMP-sialic acid synthetase), which is needed for bioconjugation to take place, has not. This enzyme appears to be expressed only in the cell nucleus (Kean et al., 2004, Münster et al., 1998). Equally, NANA aldolase has not been detected in human milk. As a result, sialic acid that is released by the neuraminidase present in human milk accumulates and contributes to the content of free sialic acid in breast milk.

SIALIC ACID IN FOODS OF ANIMAL ORIGIN

Sialic acid also occurs in a wide range of foods of animal origin (see Table 2, and Tables S5-S7 of the supplementary information for further details). However, in most sialic acid-containing foods, it is the ñanimalò form of sialic acid, NGNA, that is present in considerable proportions. This is of high relevance because of the implications of NGNA as a dietary risk factor as described above. Levels of NGNA are highest in red meats, including lamb, goat, beef and pork, with relatively high levels occurring in the milk and milk derived products (cheese, yogurt) of such animals. It is remarkable that the levels of NGNA in cowôs milk colostrum have been reported to be much higher than in mature cowôs milk (32 *vs* 0.9-6.0 %) (Puente and Hueso,

1993), an observation that awaits independent confirmation. Foods of animal origin that do not contain NGNA include mainly fish and products derived from birds (orders Galliformes, Anseriformes and Apodiformes). While not typically considered a food source, reptiles also lack NGNA (Schauer et al., 2009), likely as a result of an independent evolutionary loss in the Sauropsida lineage of the gene encoding the CMAH enzyme, which is responsible for converting NANA to NGNA (Davies and Varki, 2013, Varki and Gagneux, 2012). This is similar to the evolutionary loss of the CMAH enzyme in New World monkeys (the platyrrhines) (Springer et al., 2014). The foods with the highest sialic acid content presently known are edible birdôs nests (EBN) (Marcone, 2005), which exclusively contain NANA at levels varying between 7 and 12 w/w % (Strecker et al., 1992, Wieruszeski et al., 1987, Kathan and Weeks, 1969, Howe et al., 1961, Yang et al., 2014b). The high sialic acid content of EBN is attributed to dried swiftletôs saliva, which is rich in highly sialylated mucin glycoproteins (Strecker et al., 1992). EBN are considered a culinary delicacy in several Asian countries, foremost China, where they are highly esteemed for their health effects and reach high prices for authentic products (Marcone, 2005, Ogura, 2011, Wu et al., 2010).

Information on the concentrations of free sialic acids in foods is currently limited. The foods for which data are available are cowôs milk and infant formulas (see Table 2); these data indicate that the concentrations of free sialic acid in mature cowôs milk and cowôs colostrum are only slightly lower than that of human breast milk, but that the levels in infant formula are 10-100 times below the levels present in human breast milk (see Table 3). This suggests that supplementation of infant formula with free sialic acid would more closely match the composition of breast milk, which clearly may be connected to beneficial health effects.

CASES OF INCREASED NEED OF SIALIC ACID

As mentioned earlier, while sialic acid is biosynthesized by the human body, there are a number of cases of increased need for sialic acid when it may be recommended to support endogenous synthesis with dietary enrichment.

Gestation and lactation

Most striking is the remarkably high concentration of sialic acid in human breast milk, which is strongly dependent on the lactational period with the highest values occurring in colostrum (Table 3). It has also been reported that preterm milk contains significantly higher levels of sialic acid than term milk (Wang et al., 2001a). In addition, it is relevant to mention that infant formula typically contains significantly lower levels of sialic acid than breast milk (see Table 3). In line with this observation is that the feces of breast-fed infants contain much higher levels of sialic acid than feces of formula-fed infants (Kohler et al., 2002). Higher sialic acid levels have also been noted in the brains and saliva of breast-fed infants compared to formula-fed infants (Wang et al., 2003a, Wang et al., 2001u, Tram et al., 1997). These findings suggest that the breast-fed infant is able to utilize sialic acid in the dietary salvage pathway. It is therefore proposed that supplementary dietary sialic acid would benefit formula-fed infants in meeting the sialic acid intake and the body sialic acid levels normally achieved in breast-fed infants.

Importantly, due to the absence of the CMAH enzyme in humans, breast milk is virtually devoid of any NGNA admixture, and fortifying infant formulas with sialic acid sources of animal origin appears not appropriate in the light of potential adverse health effects of NGNA. To date, there

has been only one reported exception of a mother whoôs milk contained NGNA (2 mg/L, 0.7 % of total sialic acid) (Lacomba et al., 2011a). This appears to be the first reported case of metabolic incorporation of dietary NGNA into human breast milk and raises the following questions: How strong is GNE expression in the mammary gland? And does the salvage pathway translate efficiently into breast milk? For instance, would consumption of red meat increase the levels of NGNA in breast milk glycoconjugates? This is a question that could be tested. Ghaderi *et al.* have tested the reverse mechanism *in vitro*, namely the concept of metabolic competition to deplete the NGNA content of glycoproteins, which are expressed in NGNA-rich cell cultures. Addition of NANA to the cell culture medium was successful in reducing (outcompeting) NGNA content (Ghaderi et al., 2012, Ghaderi et al., 2010). There are, however, no data yet available on whether such metabolic competition occurs in humans through the dietary route.

It has also been determined that the sialic acid content in the saliva of pregnant women decreases with gestation and quickly returns to normal levels postpartum (D'Alessandro et al., 1989), suggesting that the endogenous synthesis of sialic acid in pregnant women may be limited and/or dependent on nutritional status. The increased demand for sialic acid during pregnancy is further supported by a case of GNE myopathy (see below) in which rapid deterioration of the patientôs muscular strength was observed during pregnancy (Sim et al., 2013).

Ageing

It has been reported that the sialic acid content of the human brain initially increases from infancy to throughout some of adulthood (maximum between 20 and 40), but then begins to

decrease slowly at > 60 years of age, with the most pronounced reduction occurring at > 90 years of age (Svennerholm et al., 1994, Yu and Ledeen, 1970). The elderly population may therefore present an additional case of need for dietary sialic acid intake with the purpose of increasing sialic acid levels in the body to those normally occurring in younger adults.

GNE myopathy

An applied demonstration that the dietary salvage pathway for free sialic acid is effective in humans is provided by nutritional intervention studies in patients with GNE myopathy. GNE myopathy (also known as *hereditary inclusion body myopathy*, HIBM, or *distal myopathy with rimmed vacuoles*, DMRV) (Huizing et al., 2014) is a rare, progressive inherited neuromuscular disease with early adult onset (Mori-Yoshimura et al., 2014). The condition was only described as late as in 1981 in Japan (Nonaka et al., 1981) and 1984 in the Iranian-Jewish population (Argov and Yarom, 1984) due to the rarity of the disease; there are likely less than 2,000 patients affected in the developed world. The complex set of various genetic mutations leading to the disease have been mapped and it was revealed that all of the mutations affect the gene that encodes for GNE, the key enzyme in the sialic acid synthesis pathway (see Figure 2) (Eisenberg et al., 2001). It was recognized that most mutations causing GNE myopathy *reduce* GNE enzyme activity rather than fully eliminating it (Martin and Freeze, 2003). Alternatively, some GNE enzyme activity may remain due to expression from the other allele. This conclusion is supported by the comparably mild progression of the disease in humans, while in mice, the loss of GNE function results in embryonic lethality (Schwarzkopf et al., 2002, Topham et al., 2008). GNE null mutations on both alleles have not yet been identified in humans (Hinderlich et al., 2013).

Interestingly, a set of other mutations of the same gene exists in which the result is a loss of feedback inhibition on GNE by CMP-NANA, which in turn results in a ñbiochemically invertedò disease known as sialuria and which shows a completely different phenotype (Martin and Freeze, 2003, Montreuil et al., 1968). In this extremely rare disease, sialic acid biosynthesis is uncontrolled, resulting in the urinary excretion of multi-gram quantities of free sialic acid per day (Hinderlich et al., 2013). In one case, excretion of 11-36 g of sialic acid per liter of urine was reported (Kamerling et al., 1975). In contrast, GNE myopathy leads to a hypo-sialylated phenotype. The slow onset of disease progression is thought to be due to compensation of the reduction in endogenous sialic acid biosynthesis via salvage pathways (Hennet, 2009). This explanation appears reasonable in light of the sialic acid levels that occur in breast milk and in foods (see Tables 1-3). In fact, Banda *et al.* have reported that incorporation of sialic acid via salvage pathways is more efficient for conjugated forms of sialic acid due to the comparably rapid urinary excretion of free sialic acid (Banda et al., 2012). However, from the same work it has become apparent that it is not recommended to compensate diminished NANA levels through intake of dietary sources of animal origin that are high in NGNA content (Banda et al., 2012, Varki, 2010).

A number of approaches have been investigated in an attempt to develop effective therapies for GNE myopathy (Malicdan et al., 2009, Jay et al., 2009, Malicdan et al., 2010, Nemunaitis et al., 2011, Malicdan et al., 2012, Niethamer et al., 2012, Nishino et al., 2014, Yonekawa et al., 2014). Therapies based on dietary supplementation of free sialic acid (Nishino et al., 2014) have demonstrated to increase uptake and incorporation of sialic acid to such an extent that these types of therapies have become an investigated nutritional treatment option as revealed by two

pharmacokinetic phase I studies performed in Japan (ClinicalTrials.gov; NCT01236898) (Mori-Yoshimura et al., 2012) and the USA (ClinicalTrials.gov; NCT01359319) (Kakkis et al., 2012). According to an article published online on Globe Newswire on April 30, 2014, a small-scale Phase II clinical trial (ClinicalTrials.gov; NCT01517880) has furnished encouraging results with dietary supplemental intake of free sialic acid at a dose of 6 grams per day (Ultragenyx, 2014), and a longer follow-up study is to address the outcome of a greater supplemental dose of 12 g per day (ClinicalTrials.gov; NCT01830972). Importantly, no adverse effects were reported to be associated with the high amounts of nutritional free sialic acid that are required to achieve positive effects in GNE myopathy patients (6-12 g/day). Such exposure levels (estimated to be ~ 100ï 300 mg/kg BW/day) are far above the typical dietary intake levels of free sialic acid from food (~ 10–20 mg/kg BW/day), and hence, the studies highlight the safety and tolerability of dietary free sialic acid.

It is of interest to note that a similar mutational loss has also occurred with a well-known class of essential nutrients, the vitamins. Humans, some other primates and guinea pigs have lost the ability to biosynthesize vitamin C (L-ascorbic acid) due to loss of the enzyme L-gulonolactone oxidase (Chatterjee, 1973), but are able to compensate for this loss through dietary intake. In the case of GNE myopathy, however, greater amounts of the nutrient are required for compensation and such amounts are difficult to obtain from conventional foods, otherwise the loss would likely go unnoticed (as was the case with the vitamins). Other similarities between ascorbic and sialic acid exist; both ascorbic and sialic acid are small and highly water soluble nutrients, both are strongly acidic (Hurd, 1970) and both are effective anti-oxidants (see next section). In fact, Richard Kuhn, a 1938 chemistry Nobel prize winner for his works on carotenoids and vitamins

(Shampo and Kyle, 2000, Baer, 1969), considered the milk saccharides as belonging to the ñvitamins of milkò (Kuhn, 1952). Richard Kuhn discovered the human milk oligosaccharides together with Paul György (György et al., 1952), including sialyllactose (Kuhn and Brossmer, 1959) and sialic acid [which he called lactaminic acid (Kuhn and Brossmer, 1957)], and was the first to elucidate the correct structure of sialic acid in 1962 (Kuhn and Baschang, 1962b, Kuhn and Brossmer, 1962).

SIALIC ACID AS AN ANTI-OXIDANT

Free sialic acid

In 2004, Iijima *et al*. reported that free sialic acid consumes toxic hydrogen peroxide (H_2O_2) under physiological conditions (Iijima et al., 2004) by means of a reaction involving a radical mechanism that had been described earlier for simpler α-ketocarboxylic acids (Jefford et al., 1976). Detailed and mechanistic studies on this chemical reaction have been reported recently (Neyra et al., 2014). Subsequent studies by Iijima confirmed that the reaction requires the free reducing end of the sugar (the open α-ketocarboxylic acid functionality, see Figure 4) to form a decarboxylated metabolite known as 4-(acetylamino)-2,4-di-deoxy-D-glycero-D-galacto octonic acid (ADOA) (Iijima et al., 2007). It was established that the reaction can also occur between free sialic acid and biologically relevant lipid hydroperoxides (Iijima et al., 2009). Lipid hydroperoxides cause oxidative stress via reactive oxygen species (ROS) and are involved in inflammatory processes, such as arteriosclerosis (Iijima et al., 2009). The physiological

relevance of these proof-of-concept studies is demonstrated by studies in Wistar rats, where free sialic acid rescued lipopolysaccharide (LPS)-induced renal failure and reduced LPS-induced liver dysfunction via mechanisms that neutralize ROS-mediated oxidative damage (Yang et al., 2014a, Ho et al., 2009).

Bound sialic acid

Bound sialic acid also possesses anti-oxidative properties but through a mechanism separate from that of free sialic acid (Eguchi et al., 2005). In this case, other types of ROS, including the superoxide anion, remove bound sialic acid from its terminal position on cell-surface glycans, thereby depleting cell-surface sialylation. This reaction was shown to reduce sialyl lewis x-mediated cell adhesion in cell culture experiments (Eguchi et al., 2005) and was confirmed to be of high relevance to disease progression in a rat model (Yasuda et al., 2006). Whether the released sialic acid was in the form of free sialic acid and/or reduced sialic acid produced through the actions of ROS as described above (see Figure 4) was not addressed in these studies. It has also been demonstrated that sialic acid moieties of mucins are essential hydroxyl radical scavengers (Ogasawara et al., 2007). This protective effect was demonstrated for bovine submaxillary gland mucin, which was shown to lose its anti-oxidative properties after sialidase (neuraminidase) treatment (Ogasawara et al., 2007). In this study, neither free sialic acid nor the subsequent metabolite of free sialic acid oxidation, ADOA, could be detected by LC-MS analysis. The authors speculated that other, yet unknown, metabolites are the products of the anti-oxidative action of bound sialic acid (Tanaka et al., 1997).

The anti-oxidative properties of sialic acid suggest a potential beneficial role for sialic acid in diseases where oxidative stress plays an important role in the etiology of the disease such as in type 2 diabetes mellitus (Renju et al., 2012), cancer (Manju et al., 2002) and aging (Mehdi et al., 2012). In fact, erythrocyte cell-surface sialic acid levels decrease significantly with age (Mehdi et al., 2012) and decreased sialic acid residues are a well-established signal for apoptotic cell clearance (Meesmann et al., 2010). An independent cell study confirmed that treatment of cells with free sialic acid replenished diminished cell-surface sialylation caused by oxidative stress-induced desialylation, and the authors recommended to investigate sialic acid as a treatment in *in vivo* studies (Pawluczyk et al., 2014). Additionally, sialylation levels have been consistently recognized as a marker for muscle aging and nutritional supplementation with sialic acid has been suggested to prevent muscle aging (Hanisch et al., 2013). In line with the anti-oxidant role of sialic acid are studies on *in vitro* models of GNE myopathy that have revealed that the impaired antioxidant capacity of hypo-sialylated muscular myotubes is strongly associated with more severe phenotypes of the disease (Cho et al., 2012, Cho et al., 2013). In one of these studies, the increases in intracellular oxidants and cell death were reduced by the addition of free sialic acid to the culture medium, a finding that was proposed to be due to improved antioxidant capacity (Cho et al., 2013).

All these findings support that free and bound sialic acid play important roles as anti-oxidative agents in human biology.

SIALIC ACID AS A POTENTIAL PREBIOTIC MONOSACCHARIDE

The prebiotic activity of free sialic acid can currently be regarded as speculative, but some emerging evidence has become available. It was mentioned previously that bacterial neuraminidases (e.g. from *Bifidobacterium bifidum*) are present in the human gut (Lee et al., 2014, Corfield et al., 1993a, Kiyohara et al., 2011). These neraminidases release sialic acid, rendering the monosaccharide and the resultant oligosaccharides available for utilization either by the same bacterium or by other gut commensals. On this basis, a potential selective prebiotic activity on *bifidobacteria* species has been proposed for sialic acid (Lee et al., 2014). A recent investigation has confirmed this concept with an infant gut commensal strain of *B. breve* that is anticipated to be connected to positive health effects and that possesses a gene cluster for uptake and metabolism of free sialic acid. Such mechanisms enable this strain to utilize free sialic acid released by other gut commensals, including utilization as an exclusive carbon-source for growth. Additional bacterial species and strains are known to utilize free sialic acid (Lee et al., 2014), but the potential effects on the whole microbiome are difficult to predict based on single bacterial cultures. Further work is warranted to better assess the potential of free sialic acid to have beneficial health effects via its impact on the composition of the gut microbiota.

SIALIC ACID AND COGNITIVE DEVELOPMENT

The interest in sialic acid as a nutrient to support cognitive development has its roots in the discovery of sialic acid from human brain tissue (Klenk, 1941, Klenk, 1935), where it occurs at particularly high levels in the grey matter (Klenk et al., 1941). It is well established that sialic

acid plays a central role in brain function and development, specifically in the posttranslational modification of the neural cell adhesion molecule (NCAM), which results in the formation of polysialic acid (polySia) (Muehlenhoff et al., 2013, Fryer and Hockfield, 1996). There has been intensive research on the possible molecular mechanisms involved in mediating the effects of sialic acid (Wang, 2012, Murrey and Hsieh-Wilson, 2008) and the impact of dietary sialic acid on cognitive development (Wang, 2009, McJarrow et al., 2009, Karim and Wang, 2006, Wang and Brand-Miller, 2003). But little attention has been paid to the fact that the various nutritional sources of sialic acid (free, oligosaccharide, glycoprotein, gangliosides) will all possess distinct bioavailability and bio-incorporation profiles. To date, there have been no human clinical studies performed investigating such effects. Consequently, the available evidence can be grouped into three principal categories: (1) *indirect* evidence linking human intake with cognitive performance, (2) animal studies investigating cognitive performance upon exposure to sialic acid and (3) bioanalytical studies investigating accumulation of sialic acid in the brain upon administration of sialic acid. A short summary of the observed trends is provided below.

Indirect evidence for the role of sialic acid in cognitive development

An increasing number of studies have reported on the beneficial effects of breast-feeding on the cognitive development of the infant (Kovar et al., 1984, Lucas et al., 1992, Gale and Martyn, 1996, Agostoni et al., 2009). While initially contested for confounding factors, the data of several studies have recently been subject to meta-analyses that have specifically addressed these concerns (Anderson et al., 1999, Geoff et al., 2006, Horta et al., 2007, Brion et al., 2011). As a result, the general notion that breast-feeding has a positive impact on cognitive development is

widely recognized today (Iacovou and Sevilla-Sanz, 2010). Thus, the high sialic acid content of human breast milk as compared to infant formula along with its role in brain development suggests that sialic acid in breast milk has an impact on infant cognitive development. The fact that sialic acid concentrations are relatively high in breast milk also suggests that brain growth creates a greater need for sialic acid than what endogenous biosynthesis in the infant can provide. This is supported by a study from Wang *et al*. (2003) who measured sialic acid in brain samples from infants (1-38 weeks) that died of sudden infant death and showed that the sialic acid content was higher in the brains of breast-fed infants than those of formula-fed infants (Wang et al., 2003a, Wang et al., 2003d) (see also Table S2 of supplementary information); as shown in Table 3, infant formulas are reported to contain significantly less sialic acid than human milk. Another study from Wang *et al.* (1998) reported higher sialic acid concentrations in the human brain than in the brains of 7 other mammalian species studied (Wang et al., 1998). Other studies provide a less clear cut result, but indicate that breast-feeding animals (mammals) typically have a higher sialic acid content in their brain than other vertebrate species (Ueno et al., 1978, Reglero et al., 1980, Ando et al., 1978, Yu and Ledeen, 1970).

Regardless of the total amounts of sialic acid in the brain, it has been observed that in all vertebrates studied the amount of NGNA in the brain is extremely low when compared to other tissues studied (only trace levels of NGNA are found in the brain) (Davies and Varki, 2013). Thus, while NGNA is the major sialic acid in many non-human mammals, its biosynthesis is limited to extra-neural tissues, presenting ñ*a rather rare example of a gene that is widely expressed in many tissues and yet selectively down regulated only in the brain*ò (Varki, 2001a). Varki postulated the hypothesis that ñ*there must be a reason why the mammalian brain has*

*restricted its NGNA content for > 100 million years of evolution*ò (Varki, 2001a), and provided the first experimental evidence demonstrating that NGNA incorporation into NCAM may reduce neuronal plasticity due to the resistance of NGNA chains to neuraminidase cleavage (Davies and Varki, 2013, Davies et al., 2012). This resistance in turn is the result of the conformation adopted by NGNA chains that differs from that of NANA chains (Davies and Varki, 2013, Davies et al., 2012). Such resistance to neuraminidase cleavage likely has a negative impact on brain plasticity, a neuronal process that requires the dynamic equilibria of polysialic acid chain extension and truncation. It is apparent that the mutational loss of NGNA in humans efficiently eliminates any traces of NGNA in the brain that may reduce plasticity. It is noteworthy that the estimated timing of the mutational loss of the *CMAH* gene in humans predates the phase of rapid brain expansion in ancient hominin species (Chou et al., 2002, Schoenemann, 2006) and the emergence of the genus *Homo* (Wood and Collard, 1999, Wood, 2014). Furthermore, the initial loss of NGNA expression was followed by a number of resulting adaptations in sialic acid biology (Varki and Gagneux, 2012), which mainly affected the immunologically important receptor class of the sialic acid-binding immunoglobulin lectins (Siglecs) (Crocker et al., 2007, Brinkman-Van der Linden et al., 2000, Crocker et al., 1998). For instance, the Siglec-11 and Siglec-16 mutations were estimated to have occurred ~ 1.1 million years ago (MYA) (Wang et al., 2012a), the Siglec-13 and Siglec-17 mutations ~ 0.4 MYA (Wang et al., 2012c) and the CD33-related Siglec suppression on T-cells ~ 0.1-0.2 MYA (Nguyen et al., 2006). Most of these adaptations are linked to the immunological aspects of sialic acids (Padler-Karavani et al., 2013, Wang et al., 2012c) and need to be understood in the context of a high number of evolutionary adaptations occurring since the divergence of the human line from our closest living ancestors

(O'Bleness et al., 2012). However, some adaptations such as that of Siglec-11 which is mainly expressed in the brain, a feature exclusive to humans (Wang et al., 2012a), likely represent an important contribution to evolutionary human cognitive development (O'Bleness et al., 2012, Varki, 2001a, Varki et al., 2011). While this may have appeared to be mere speculation only a few years ago, the rapid progress of full genome sequencing technologies (Hayden, 2014), including sophisticated techniques to reconstruct ancient genome sequences (Shapiro and Hofreiter, 2014), has already provided insight into this hypothesis, warranting further investigation (Pääbo, 2014).

Animal studies investigating cognitive performance upon exposure to Sialic acid

A limited number of animal studies have investigated the effect of administered sialic acid (from various sources) on cognitive performance. Although, in their totality, these studies show an overall tendency towards improved cognitive performance, they inherently highlight the principal limitations of such short-term nutritional intervention tests, including the following: statistical significance has to be attained in a time-limited dietary intervention study, the typical comparison has been between an intervention group versus a *healthy* control group both with inherent significant individual variation at baseline, and due to the costs involved, the studies employ rather small group sizes. Under these restrictions, only very strong effects would reach statistical significance and it is therefore not realistic to expect conclusive results from such small-scale and short-term animal studies. The results of the available animal studies are summarized in the supplementary information (Table S9).

Bioanalytical studies investigating accumulation of sialic acid in the brain upon administration of sialic acid

Given the difficulties of demonstrating enhanced cognitive performance upon short term dietary intervention many researchers have focused on investigating more generally the bioavailability and brain incorporation of administered sialic acid to support a possible mechanism of action (see also section Metabolic fate of dietary free NANA). The results of these studies demonstrate that dietary sialic acid is bioavailable and partly incorporated into the brain, however, the extent of bioavailability and incorporation depends on the form of sialic acid administered and the currently available data is limited to animal studies (see Table S10 of the supplementary information).

SOURCES OF HIGH-PURITY SIALIC ACID

Most common and conveniently available nutritional sources of sialic acid, other than breast milk, suffer from the disadvantage of being typically contaminated with significant levels of the animal form of sialic acid, NGNA (see Table 2). Hence, they are not ideal sources for the enrichment of foods with sialic acid or as raw materials for the isolation of pure NANA. A possible exception to this general rule seems to be provided by bovine κ-caseinglycomacropeptide (CGMP), which contains comparatively high levels of sialic acid. CGMP is composed of a glycopeptide mixture of various peptide isomers and glycoforms, but which has not been reported to contain any NGNA to date (Guerrero et al., 2015, Daali et al., 2001).

Alternatively, and more economic from a molecular perspective, pure NANA can be manufactured by (I) chemical synthesis alone (Cornforth et al., 1958, Kuhn and Baschang, 1962a, Stallforth et al., 2012), (II) enzymatic condensation of pyruvate with *N*-acetyl-D-mannosamine (ManNAc) (Brunetti et al., 1962, Comb and Roseman, 1960), (III) enzymatic synthesis starting from a mixture of ManNAc and *N*-acetyl-D-glucosamine (GlcNAc) (Mahmoudian et al., 1997), (IV) a coupled enzymatic reaction starting from GlcNAc (Kim et al., 1988, Kragl et al., 1991, Maru et al., 1998, Maru et al., 2002), or (V) whole-cell biocatalysis using genetically modified microorganisms (Ishikawa and Koizumi, 2010, Zhang et al., 2010, Han et al., 2011, Lin et al., 2013). Regardless of the principal production process applied, extremely high-purity NANA can then only be obtained if robust crystallization procedures are applied that provide a crystalline ingredient that, other than also containing crystal-bound water, strictly contains pure NANA (Schroven et al., 2013, Vrasidas et al., 2012, Flippen, 1973).

CONCLUSION

Due to its tremendous importance to human nutrition, biology and evolution, sialic acid is likely one of the most remarkable biomolecules studied to date, and due to its prominent occurrence in breast milk, it has been rightfully termed a human milk monosaccharide. However, the occurrence of the free form of sialic acid in human breast milk and its potential nutritional roles have long been overlooked. Free sialic acid has been demonstrated to be bioavailable and to be incorporated into the body, yet apparently with lower efficiency than bioconjugates of sialic acid. Data from animal studies using radiolabeled free sialic acid have also shown that a fraction of dietary sialic acid is incorporated into the brain; however, it remains a challenging task to

demonstrate the same in humans where application of radiolabelled molecules is not an ethical option. An important nutritional property of free sialic acid is its anti-oxidative property, which protects particular body compartments from reactive oxygen species, such as hydroperoxides, and is potentially beneficially involved in such processes as inflammation and ageing. Free sialic acid also has potential impact on the gut microbiota. Due to its proven utilization by *bifidobacteria*, sialic acid has been proposed to play a prebiotic role and the first supporting data for this role is currently emerging. Together, the nutritional and biological importance and unquestionable safety of sialic acid forms the basis for which the free form is currently being developed as a food ingredient (Choi et al., 2014) for addition to infant formula for the purpose of providing similar levels of free sialic acid as those present in human breast milk. After more than 6 years of intensive research and development, free sialic acid is becoming available now as an economic and high purity food ingredient that guarantees the absence of any NGNA contamination. In contrast, manufacturing economic bioconjugates of NANA that equally fulfill the criteria of NGNA-absence remains for the time being a challenging task, but further development is warranted in the future.

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CHR is employee of Glycom A/S a company developing the human high-purity form of sialic acid (NANA) for food applications including infant formula. SSHC and NB are employees of Intertek Scientific & Regulatory Consultancy providing Scientific and Regulatory consultancy to Glycom A/S. This review was mainly written by CHR with support and collaboration of the other authors.

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Table 1. Total and free sialic acid (NANA) concentrations in human tissues and fluids.

Tissue / Fluid	**Total Sia** mg/kg	**Free NANA** mg/kg	**Free NANA** of total NANA	**Total NGNA** of total Sia	**References**
Breast milk (colostrum)	**1,240** ± **229**	46	~ 3-4 %	nd	(Martin-Sosa et al., 2003, Oriquat et al., 2011, Martin-Sosa et al., 2004, Wang et al., 2001a, Brand-Miller et al., 1994, Carlson, 1985, Heine et al., 1993)
Feces (breast-fed infant)	**930** ± **705**	n/a	n/a	nd	(Kohler et al., 2002)
Brain (grey matter)	**892** ± **168**	41	~ 4-5 %	nd	(Wang et al., 1998, Wang et al., 2003a, Svennerholm et al., 1994, Ueno et al., 1978, Yu and Ledeen, 1970)
Breast milk (transitional)	**881** ± **273**	27	~ 3-4 %	nd	(Martin-Sosa et al., 2003, Neeser et al., 1991, Oriquat et al., 2011, Martin-Sosa et al., 2004, Wang et al., 2001a, Sabharwal et al., 1991, Heine et al., 1993)
Semen	**829.0**	n/a	n/a	nd	(Warren, 1959)
Brain (cerebral myelin)	**632.0**	n/a	n/a	nd	(Ueno et al., 1978)
Serum	**609** ± **120**	0.6	~ 0.1 %	nd	(Hayakawa et al., 1993, Hara et al., 1987, Siskos and Spyridaki, 1999, Schauer, 1982, Haverkamp et al., 1976, Chrostek et al., 2014, Crook, 1993, Hara et al., 1986, Lorentz et al., 1986, Wang et al., 2014)
Breast milk (mature)	**505** ± **251**	22	~ 2-4 %	nd	(Li and Fan, 2014, Martin-Sosa et al., 2003, Neeser et al., 1991, Qiao et al., 2013, Oriquat et al., 2011, Martin-Sosa et al., 2004, Wang et al., 2001a, Thurl et al., 1996, Hayakawa et al., 1993, Lacomba et al., 2011a, Brand-Miller et al., 1994, Carlson, 1985, Wiederschain and Newburg, 2001)
Tears	**433.0**	n/a	n/a	nd	(Kuizenga et al., 1990)
Brain (white	**396** ±	n/a	n/a	nd	(Svennerholm et al., 1994, Ueno et al.,

matter)	**59**				1978)
Saliva	**122** ± **36**	11	~ 10-15 %	nd	(Hayakawa et al., 1993, Wang et al., 2001u, Siskos and Spyridaki, 1999, Tram et al., 1997, D'Alessandro et al., 1989, Haverkamp et al., 1976)
Gastric aspirate	**93.0**	n/a	n/a	nd	(Corfield et al., 1993c)
Brain (spinal cord)	**87.0**	n/a	n/a	nd	(Ueno et al., 1978)
Urine	**78** ± **13**	14	~ 15-20 %	nd	(Chen et al., 2014, Hayakawa et al., 1993, Siskos and Spyridaki, 1999, Romppanen and Mononen, 1995, Tebani et al., 2011) (Hayakawa et al., 1993, Siskos and Spyridaki, 1999, Romppanen and Mononen, 1995)
Amniotic fluid	**69.0**	1.1	~ 2 %	nd	(Hayakawa et al., 1993)
Feces	**42.**	n/a	n/a	nd	(Sabharwal et al., 1991, Kohler et al., 2002)
Cerebrospinal fluid	**12.3** ± **3**	4.3	~ 35 %	nd	(Warren, 1959, Hayakawa et al., 1993, van der Ham et al., 2010, Lorentz et al., 1986)

Sia = NANA + NGNA; **n/a** = data is not available; **nd** = not detected.

Table 2. Total and free sialic acid concentrations in foods of animal origin and content of NGNA as a percentage of total sialic acid concentration.

Food	**Total Sia** mg/kg	**Free NANA** mg/kg	**Free NANA** of total NANA	**Total NGNA** of total Sia	**References**
Swiftlet edible birdôs nest	**95,100.0**	n/a	n/a	nd	(Yang et al., 2014b)
Crucian carp eggs	**4,574.2**	n/a	n/a	nd	(Chen et al., 2014)
Whitefish caviar	**3,193.8**	**459.0**	16.7 %	13.0 %	(Samraj et al., 2015)
Cow Cheddar cheese	**1,824.4**	**27.2**	1.5 %	2.0 %	(Samraj et al., 2015)
Salmon caviar	**1,763.2**	**148.8**	12.1 %	29.0 %	(Samraj et al., 2015)
Tilapia	**671.8**	**99.9**	14.9 %	nd	(Samraj et al., 2015)
Mahi-mahi (dorado)	**616.1**	**116.6**	18.9 %	nd	(Samraj et al., 2015)
Chicken Egg yolk	**592 ± 124**	**0.6**	0.1 %	nd	(Wang, 2009, Li and Fan, 2014, Chen et al., 2014, Nakano et al., 1994, Warren, 1959)
Salmon (wild-caught)	**565.7**	**105.5**	18.6 %	nd	(Samraj et al., 2015)
Cow colostrum	**549 ± 294**	**31**	5.6 %	0.9-32.0 %	(Martin-Sosa et al., 2003, Puente et al., 1992, McJarrow and van Amelsfort-Schoonbeek, 2004, Martin et al., 2001, Puente and Hueso, 1993)
Cow Mozarella cheese	**515.0**	**15.5**	3.1 %	4.0 %	(Samraj et al., 2015)
Cow Jack cheese	**438.5**	**9.6**	2.3 %	4.0 %	(Samraj et al., 2015)
Thresher	**333.4**	**33.4**	10.0 %	nd	(Samraj et al., 2015)

Food	**Total Sia** mg/kg	**Free NANA** mg/kg	**Free NANA** of total NANA	**Total NGNA** of total Sia	**References**
shark					
Bison meat	**309.1**	**25.4**	12.3 %	32.0 %	(Samraj et al., 2015)
Buffalo Milk	**266.0**	n/a	n/a	2.3 %	(Karunanithi et al., 2013)
Chicken Egg white	**251** ± **83**	**0.2**	0.1 %	nd	(Wang, 2009, Li and Fan, 2014, Chen et al., 2014, Nakano et al., 1994, Warren, 1959, Samraj et al., 2015)
Swordfish	**247.8**	**30.0**	12.1 %	nd	(Samraj et al., 2015)
Cow Cheese	**205** ± **66**	n/a	n/a	1.1-6.9 %	(Li and Fan, 2014, Karunanithi et al., 2013, Chen et al., 2014, Tangvoranuntakul et al., 2003)
Cow Yogurt	**182** ± **80**	n/a	n/a	2.1-3-1 %	(Karunanithi et al., 2013, Chen et al., 2014, Spichtig et al., 2010)
Cow Milk	**173** ± **67**	**27.6**	16.0 %	0.9-6.0 %	(Li and Fan, 2014, Karunanithi et al., 2013, Chen et al., 2014, Spichtig et al., 2010, Tangvoranuntakul et al., 2003, Martin-Sosa et al., 2003, Puente et al., 1996, Puente et al., 1992, Neeser et al., 1991, Morrissey, 1973, Martin et al., 2007, Martin et al., 2001, Puente and Hueso, 1993, McJarrow and van Amelsfort-Schoonbeek, 2004, Samraj et al., 2015)
Sheep Milk	**169.0**	n/a	n/a	92.0 %	(Spichtig et al., 2010, Morrissey, 1973)
Goat Milk	**168** ± **74**	n/a	n/a	57.0 %	(Spichtig et al., 2010, Puente et al., 1996, Puente et al., 1994, Morrissey, 1973)
Lamb (meat)	**166** ± **111**	**26.6**	20.9 %	18-36 %	(Wang, 2009, Li and Fan, 2014, Chen et al., 2014, Tangvoranuntakul et al., 2003, Zeng and Wang, 2007, Samraj et al., 2015)
Pork (meat)	**146** ± **88**	**38.8**	33.2 %	9-26 %	(Wang, 2009, Li and Fan, 2014, Chen et al., 2014, Tangvoranuntakul et al., 2003, Samraj et al., 2015)
Goat Cheese	**143** ± **55**	**9.7**	12.8 %	42-53 %	(Karunanithi et al., 2013, Tangvoranuntakul et al., 2003, Samraj et al., 2015)
Beef (meat)	**138** ±	**18.9**	23.3 %	22-43 %	(Wang, 2009, Li and Fan, 2014, Chen et al.,

Food	Total Sia mg/kg	Free NANA mg/kg	Free NANA of total NANA	Total NGNA of total Sia	References
	112				2014, Tangvoranuntakul et al., 2003, Zeng and Wang, 2007, Samraj et al., 2015)
Cod fish	**106.0**	n/a	n/a	nd	(Li and Fan, 2014, Tangvoranuntakul et al., 2003)
Duck	**96 ± 76**	n/a	n/a	nd	(Li and Fan, 2014, Chen et al., 2014, Tangvoranuntakul et al., 2003)
Tuna	**84 ± 55**	**44.9**	53.5 %	nd	(Li and Fan, 2014, Tangvoranuntakul et al., 2003, Samraj et al., 2015)
Chicken (white meat)	**76 ± 51**	**6.5**	8.6 %	nd	(Wang, 2009, Li and Fan, 2014, Chen et al., 2014, Tangvoranuntakul et al., 2003, Samraj et al., 2015)
Golden pomfret fish	**75.0**	n/a	n/a	nd	(Chen et al., 2014)
Salmon (farm raised)	**65 ± 38**	**17**	26.6 %	0-3.4 %	(Wang, 2009, Li and Fan, 2014, Tangvoranuntakul et al., 2003, Samraj et al., 2015)
Crucian carp	**55.0**	n/a	n/a	2.7 %	(Chen et al., 2014)
Sardines	**51.0**	**13.6**	26.7 %	nd	(Samraj et al., 2015)
Grass carp	**46.0**	n/a	n/a	1.0 %	(Chen et al., 2014)
Turkey	**29 ± 15**	**7.7**	26.6 %	nd	(Wang, 2009, Tangvoranuntakul et al., 2003, Samraj et al., 2015)
Rainbow trout	**27.5**	**9.9**	36.0 %	nd	(Samraj et al., 2015)
Squid	**19.0**	**4.6**	24.2 %	1-8 %	(Chen et al., 2014, Samraj et al., 2015)
Prawn	**9.0**	n/a	n/a	12.2 %	(Wang, 2009)
Octopus	**3.0**	n/a	n/a	nd	(Chen et al., 2014)
Shrimp	**2.0**	n/a	n/a	nd	(Chen et al., 2014, Samraj et al., 2015)
Crab	**1.0**	n/a	n/a	nd	(Chen et al., 2014)
Clams, abalone	**0.0**	n/a	n/a	nd	(Chen et al., 2014, Samraj et al., 2015)

Sia = NANA + NGNA; **n/a** = data is not available; **nd** = not detected.

Table 3. Total and free sialic acid concentrations in infant and follow-on formulas in comparison to breast milk sialic acid (NANA) concentrations.

Food	**Total Sia** mg/kg	**Free NANA** mg/kg	**Free NANA** of total NANA	**Total NGNA** of total Sia	**References**
Breast milk (colostrum)	**1240** ± **229**	**46**	~ 3-4 %	0 %	(Martin-Sosa et al., 2003, Oriquat et al., 2011, Martin-Sosa et al., 2004, Wang et al., 2001a, Brand-Miller et al., 1994, Carlson, 1985, Heine et al., 1993)
Breast milk (transitional)	**881** ± **273**	**27**	~ 3-4 %	0 %	(Martin-Sosa et al., 2003, Neeser et al., 1991, Oriquat et al., 2011, Martin-Sosa et al., 2004, Wang et al., 2001a, Sabharwal et al., 1991, Heine et al., 1993)
Cow colostrum	**549** ± **194**	**31**	~ 2-7 %	0.9-32 %	(Martin-Sosa et al., 2003, Puente et al., 1992, McJarrow and van Amelsfort-Schoonbeek, 2004, Martin et al., 2001, Puente and Hueso, 1993)
Breast milk (mature)	**505** ± **251**	**22**	~ 2-4 %	0 %	(Li and Fan, 2014, Martin-Sosa et al., 2003, Neeser et al., 1991, Qiao et al., 2013, Oriquat et al., 2011, Martin-Sosa et al., 2004, Wang et al., 2001a, Thurl et al., 1996, Hayakawa et al., 1993, Lacomba et al., 2011a, Brand-Miller et al., 1994, Carlson, 1985, Wiederschain and Newburg, 2001)
Buffalo Milk	**266.0**	n/a	n/a	2.3 %	(Karunanithi et al., 2013)
Preterm formula*	**196** ± **31**	**3**	~ 1.6 %	n/a	(Wang et al., 2001a, Heine et al., 1993)
Infant formula*	**178** ± **60**	**0.3**	~ 0.3 %	1.8 - 9.3 %	(Spichtig et al., 2010, Neeser et al., 1991, Wang et al., 2001a, Lacomba et al., 2011a, Neelima et al., 2012, Hurum and Rohrer, 2012, Lacomba et al., 2011g, Sanchez-Diaz et al., 1997, Martin et al., 2007, Sørensen, 2010)
Cow Milk	**173** ± **67**	**18**	~ 4-12 %	0.9-6.0 %	(Li and Fan, 2014, Karunanithi et al., 2013, Chen et al., 2014, Spichtig et al., 2010,

Food	**Total Sia** mg/kg	**Free NANA** mg/kg	**Free NANA** of total NANA	**Total NGNA** of total Sia	**References**
					Tangvoranuntakul et al., 2003, Martin-Sosa et al., 2003, Puente et al., 1996, Puente et al., 1992, Neeser et al., 1991, Morrissey, 1973, Martin et al., 2007, Martin et al., 2001, Puente and Hueso, 1993, McJarrow and van Amelsfort-Schoonbeek, 2004)
Sheep Milk	**169**	n/a	n/a	92 %	(Spichtig et al., 2010, Morrissey, 1973)
Goat Milk	**168** ± **52**	n/a	n/a	57 %	(Spichtig et al., 2010, Puente et al., 1996, Puente et al., 1994, Morrissey, 1973)
Follow-on formula*	**165** ± **52**	**1.9**	~ 1.4 %	2.5-5.0 %	(Wang et al., 2001a, Lacomba et al., 2011a, Lacomba et al., 2011g, Sanchez-Diaz et al., 1997, Martin et al., 2007, Heine et al., 1993)
Growing up milk*	**143** ±**8**	n/a	n/a	2.4 %	(Spichtig et al., 2010)

Sia = NANA + NGNA; **n/a** = data is not available; **nd** = not detected.

*Values are calculated on reconstituted powder at 130 g/L.

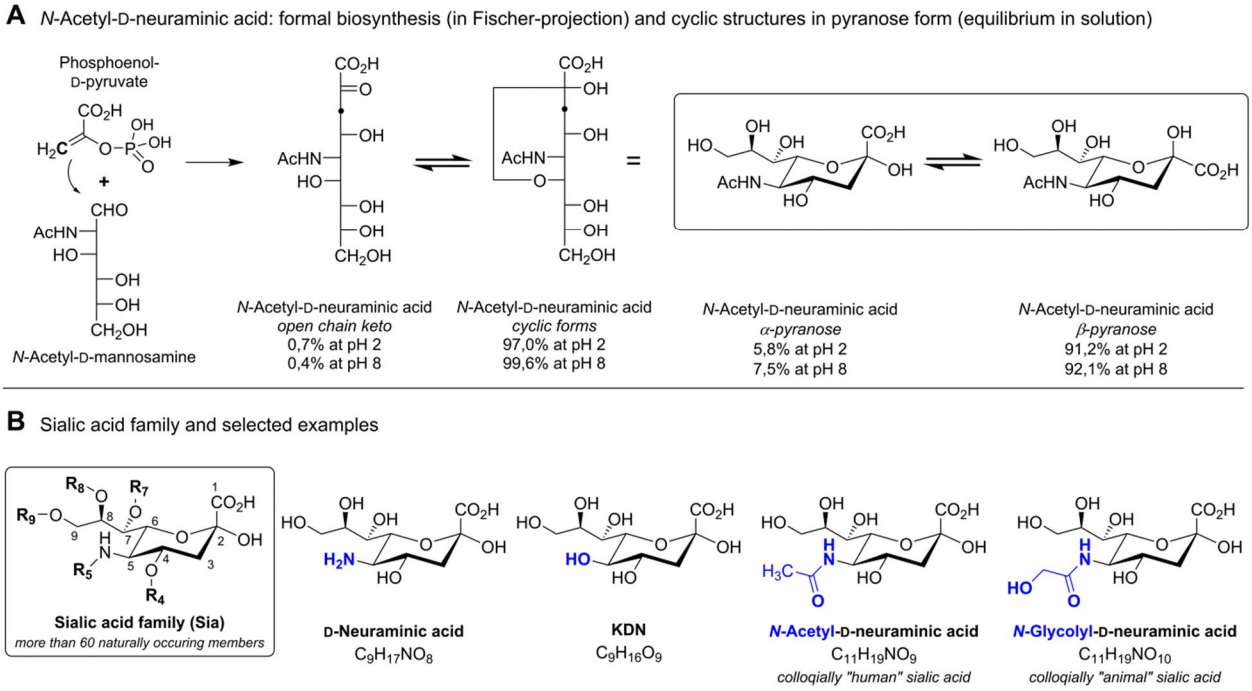

Figure 1. *N*-Acetyl-D-neuraminic acid and related members of the Sia family. A) Biosynthesis of *N*-Acetyl-D-neuraminic acid and chemical structure. B) Chemical structure of sialic acids and selected examples.

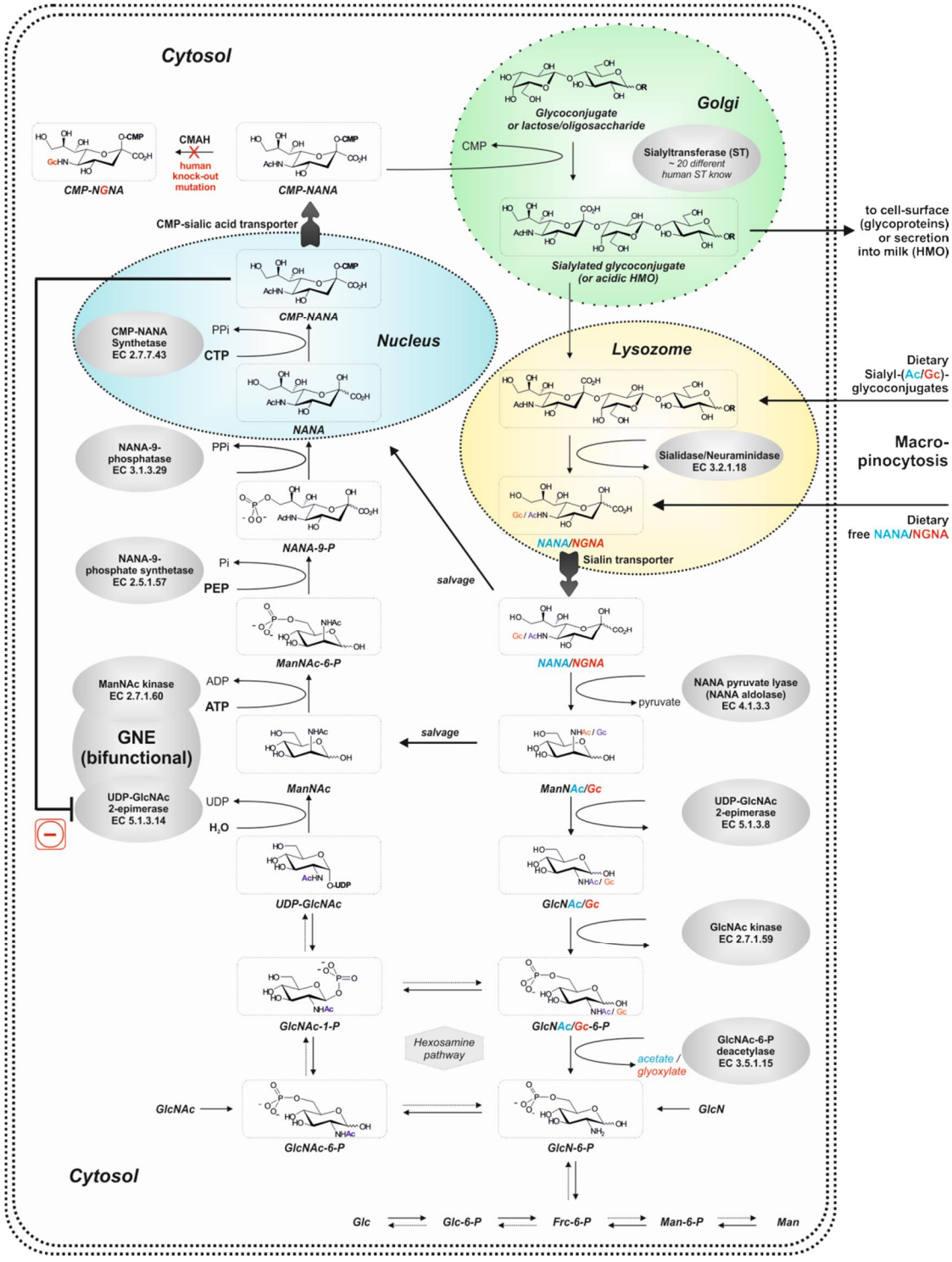

Figure 2. Enzymes of mammalian sialic acid anabolism and catabolism. The left-hand pathway depicts the anabolism (biosynthesis) of sialic acid and the right-hand pathway illustrates the catabolism of sialic acid.

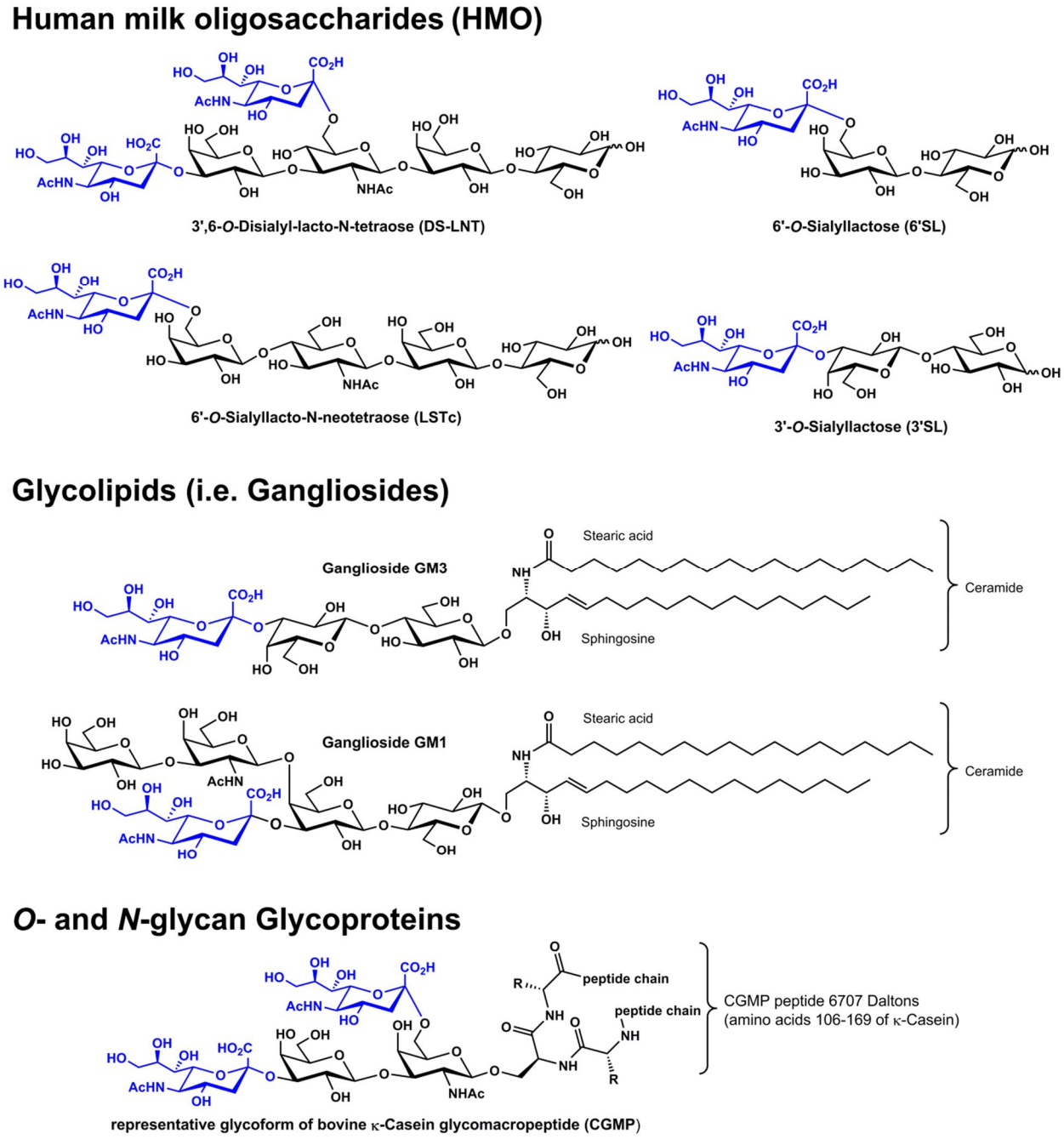

Figure 3. Prominent examples of biomolecular sources of sialic acid.

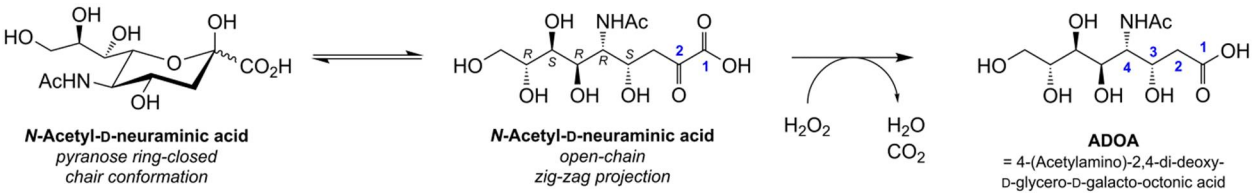

Figure 4. Chemical reaction forming the basis for the anti-oxidative properties of free sialic acid.