Glycom A/S Kogle Allé 4 2970 Hørsholm, Denmark

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OFFICE OF FOOD ADDITIVE SAFETY

23 November 2021

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

Dear Dr. Gaynor:

Re: GRAS Notice of lacto-*N*-neotetraose (LNnT) for Use in Exempt Infant Formula

In accordance with 21 CFR §170 Subpart E consisting of §§ 170.203 through 170.285, Glycom A/S [Kogle Allé 4, 2970 Hørsholm, Denmark], as the notifier, is submitting one hard copy and one electronic copy (on CD), of all data and information supporting the company's conclusion that lacto-*N*-neotetraose (LNnT) produced by derivatives of *Escherichia coli* K-12 DH1 MDO (as per GRN 659), is GRAS on the basis of scientific procedures, for use in exempt term infant formula and is therefore not subject to the premarket approval requirements of the *Federal Food, Drug and Cosmetic Act*. Information setting forth the basis for Glycom's GRAS conclusion are enclosed for review by the agency.

Should you have any questions or concerns regarding this GRAS notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

[redacted]

Christoph H. Röhrig, Ph.D.
Head of Regulatory & Scientific Affairs
Glycom A/S

Glycom A/S is a wholly owned indirect affiliate of DSM Nutritional Products Ltd, a company with registered address at Wurmisweg 576, 4303 Kaiseraugst, Switzerland

GRAS NOTICE OF LACTO-*N*-NEOTETRAOSE (LNnT) FOR USE IN EXEMPT INFANT FORMULA

SUBMITTED TO:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

SUBMITTED BY:
Glycom A/S
Kogle Allé 4
2970 Hørsholm
Denmark

DATE:
23 November 2021

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GRAS Notice of Lacto-*N*-neotetraose (LNnT) for Use in Exempt Infant Formula

TABLE OF CONTENTS

PART 1. § 170.225 SIGNED STATEMENTS AND CERTIFICATION 4
- 1.1 Name and Address of Notifier 4
- 1.2 Common Name of Notified Substance 4
- 1.3 Conditions of Use 4
- 1.4 Basis for GRAS 5

PART 2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT 6
- 2.1 Identity 6
- 2.2 Manufacturing 7
  - 2.2.1 Description of the Production Microorganism 7
  - 2.2.2 Description of the Production Process 7
  - 2.2.3 Quality Control 9
- 2.3 Product Specifications and Batch Analyses 10
  - 2.3.1 Specifications 10
- 2.4 Batch Analysis 11
  - 2.4.1 Manufacturing By-Products, Impurities, and Contaminants 12

PART 3. DIETARY EXPOSURE 14
- 3.1 Background Consumption of LNnT in Human Breast Milk 14
- 3.2 Estimated Intake of LNnT from Proposed Uses 15
  - 3.2.1 Dietary Intake of LNnT by Infant Consumers from Exempt Formula and Background Diet 15
  - 3.2.2 Dietary Intake of LNnT by Infants from Exempt Infant Formula Only 16

PART 4. SELF-LIMITING LEVELS OF USE 17

PART 5. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958 18

PART 6. NARRATIVE AND SAFETY INFORMATION 19
- 6.1 Introduction 19
- 6.2 Metabolic Fate 21
- 6.3 Cow's Milk Protein Allergy (CMPA) and Multiple Food Allergies 21
- 6.4 Risk-Based Safety Assessment 22
- 6.5 Infant Studies 25
  - 6.5.1 Infants with Cow's Milk Protein Allergy (CMPA) 25
  - 6.5.2 Other Infant Studies 27
- 6.6 Other Considerations – Additive Dietary Intakes of LNnT with Other HiMOs 29
- 6.7 General Recognition 30
- 6.8 Conclusion 30

PART 7. LIST OF SUPPORTING DATA AND INFORMATION 31

List of Figures and Tables

Figure 6.3-1	Comparison of Estimated Dietary Intake of Milk Protein to Allergy Thresholds Using Risk-Based Assessment	23
Figure 6.4.1-1	Double-Blind Placebo-Controlled Food Challenge Study Flow Chart (Nowak-Wegrzyn *et al.*, 2019a)	26
Table 1.3-1	Summary of the Individual Proposed Food Uses and Use Levels for LNnT in Exempt Infant Formula the U.S.	5
Table 2.2.2-1	Summary of the Overall Manufacturing Process for LNnT	8
Table 2.3.1-1	Product Specifications for LNnT	10
Table 2.4-1	Batch Analysis of LNnT Produced by Fermentation	11
Table 2.4.1.1-1	Detection of Milk Residues in LNnT	13
Table 3.1-1	LNnT Concentration in Human Milk after Full-Term Birth	14
Table 3.3.1-1	Summary of the Estimated Daily Intake of LNnT from Food Uses of LNnT in the U.S. by Population Group (2011-2012 NHANES Data)	15
Table 3.3.1-2	Summary of the Estimated Daily Per Kilogram Body Weight Intake of LNnT from All Proposed Food Uses in the U.S. by Population Group (2011-2012 NHANES Data)	15
Table 3.3.2-1	Summary of the Estimated Daily Intake of LNnT from Infant Formula Only in the U.S. by Population Group (2011-2012 NHANES Data)	16
Table 6.4.1-1	Outcome of Double-Blind Placebo-Controlled Food Challenge to the Test and Control Formula by Group Allocation in the Modified Intention-to-Treat Cohort (Nowak-Wegrzyn *et al.*, 2019a)	27
Table 6.5.2.6-1	Summary of the Interventional Clinical Infant Studies Conducted on LNnT	29

GRAS Notice of Lacto-N-neotetraose (LNnT) for Use in Exempt Infant Formula

Glycom A/S[1] (Glycom), a manufacturer of human-identical milk oligosaccharides, has previously concluded that lacto-*N*-neotetraose (LNnT) is Generally Recognized as Safe for (GRAS) use in non-exempt term infant formula and in select food and beverage products across multiple categories. These conclusions were notified to the offices of the United States Food and Drug Administration (FDA) and filed by the Agency without objection under GRN 659 (U.S. FDA, 2016a). Glycom intends to expand the current GRAS uses of LNnT as described in GRN 659 to also include use in hypoallergenic exempt infant formula for infants with cow's milk protein allergy (CMPA) or multiple food allergies. These formulas may also be appropriate for infants with non-allergenic gut impairment and malabsorptive conditions. Glycom notes that its LNnT is manufactured by using milk-derived lactose as a substrate for LNnT biosynthesis. Accordingly, food uses of LNnT are subject to the allergy labeling requirements of the *Food Allergen Labeling and Consumer Protection Act of 2004* (FALCPA) (U.S. FDA, 2018). As the labeling of LNnT with allergy statements "contains milk" would be conflicting with food uses in exempt hypoallergenic formula for infants with CMPA, Glycom has filed a petition with the FDA in accordance with 21 U.S.C. 343(w)(6) for exemption of LNnT from the allergy labeling requirements of FALCPA. This petition has been filed under FDA docket No. FDA-2021-FL-0655 (FALCPA No. 006) and is currently under review by the Agency (U.S. FDA, 2020a). Where applicable, data and information supporting conclusions that LNnT is absent of detectable milk allergic proteins and would not cause an allergenic response that poses a risk to human health are incorporated by reference to FALCPA No. 006.

[1] Glycom A/S is a wholly owned indirect affiliate of DSM Nutritional Products Ltd, a company with registered address at Wurmisweg 576, 4303 Kaiseraugst, Switzerland.

Part 1. § 170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285 (U.S. FDA, 2021), Glycom A/S (Glycom) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that lacto-*N*-neotetraose (LNnT) as described in GRN 659 (U.S. FDA, 2016a), is not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act* based on Glycom's view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Section 1.3 below. In addition, as a responsible official of Glycom, the undersigned hereby certifies that all data and information presented in this Notice represents a complete, representative, and balanced submission, and considered all unfavorable, as well as favorable, information known to Glycom and pertinent to the evaluation of the safety and GRAS status of LNnT as a food ingredient for addition to exempt infant formula, as described herein.

Signed,

[redacted]

26 Nov 2021

Christoph Röhrig, Ph.D.
Head of HMO Regulatory Affairs
Glycom A/S
Christoph.roehrig@dsm.com

Date

1.1 Name and Address of Notifier

Glycom A/S
Kogle Allé 4
2970 Hørsholm
Denmark
Tel: +45 8830 9500
Fax: +45 4593 3968

1.2 Common Name of Notified Substance

Common Name: Lacto-*N*-neotetraose (LNnT).

Trade Name: GlyCare™ LNnT 9000 HA

1.3 Conditions of Use

LNnT is intended to be added to exempt term infant formula targeted to infants with cow's milk protein allergy (CMPA) or multiple food allergies. These formulas may also be appropriate for infants with non-allergenic gut impairment and malabsorptive conditions. Uses of this ingredient in exempt infant formula (*i.e.*, infants up to 12 months) will provide a use level of LNnT of 600 mg/L in the exempt formula (see Table 1.3-1). The maximum use levels are proposed on the basis of providing similar levels of LNnT, on a body weight basis, as those consumed by breastfed infants (see Section 3.1). Example products to which LNnT may be added include extensively hydrolyzed infant formula (EHF) for infants with CMPA such as Gerber

Extensive (Nestlé), which is lactose free and contains probiotics and medium chain triglycerides (MCT). Addition of LNnT to amino acid-based formula such as Alfamino (Nestlé) would represent products targeted to infants not responding to EHF or for infants with moderate to severe CMPA, including those with anaphylaxis, food protein-induced enterocolitis syndrome (FPIES), multiple food protein allergy of infancy (non-IgE-mediated), or eosinophilic esophagitis.

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for LNnT in Exempt Infant Formula the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2021)	Proposed Food Use	Target Population	RACC[a] (g or mL)	Proposed Maximum Use Level[b] (g/RACC)	Proposed Maximum Use Level[b] (g/kg or g/L)
Exempt Term Infant Formulas	Extensively hydrolyzed formula (EHF) (*e.g.*, Gerber Extensive)	Cow's milk protein allergy (CMPA) Cow's milk protein intolerance Cow's milk-induced food protein-induced enterocolitis syndrome (FPIES) Soy protein sensitivity Fat malabsorption	100 mL[c]	0.06	0.6
	Amino acid-based formula (*e.g.*, Alfamino)	CMPA – Symptoms that persist after use of an EHF Multiple food allergies Eosinophilic GI disorders FPIES Short-bowel syndrome (SBS) Malabsorption	100 mL[c]	0.06	0.6

CFR = *Code of Federal Regulations*; GI = gastrointestinal; LNnT = lacto-*N*-neotetraose; RACC = Reference Amounts Customarily Consumed per Eating Occasion; U.S. = United States.
[a] RACC based on values established in 21 CFR §101.12 (U.S. FDA, 2021). When a range of values is reported for a proposed food use, particular foods within that food use may differ with respect to their RACC.
[b] Use level expressed on a LNnT basis in the final food, as consumed.
[c] RACC not available; 100 mL employed as an approximation.

1.4 Basis for GRAS

Pursuant to 21 CFR §170.30 (a)(b) of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2021), Glycom has concluded, on the basis of scientific procedures, that LNnT is GRAS for addition to exempt term infant formula, as described in Table 1.3-1.

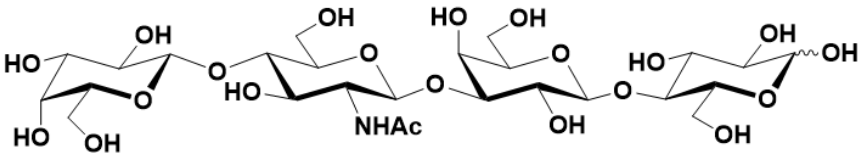
Part 2. Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity

LNnT is a naturally occurring tetrasaccharide found in mammalian milk with the highest concentrations present in *human* milk, and is therefore referred to as a *human* milk oligosaccharide (HMO). LNnT is a linear tetrasaccharide consisting of D-galactose, *N*-acetyl-D-glucosamine, D-galactose and D-glucose. Glycom has confirmed, based on ^{1}H- and ^{13}C-NMR-, mass spectrometry (MS), and high-performance liquid chromatography (HPLC) data, that the LNnT manufactured by Glycom is chemically and structurally fully identical to the LNnT that is present in human breast milk.

Common Name:	Lacto-*N*-neotetraose
Common Abbreviation:	LNnT
Trade Name:	GlyCare™ LNnT 9000 HA
International Union of Pure and Applied Chemistry (IUPAC) Name:	β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucose
Alternative Denotations:	**O**-β-D-Galactopyranosyl-(1→4)-**O**-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl-(1→3)-**O**-β-D-galactopyranosyl-(1→4)-D-glucose; β-D-Gal*p*-(1-4)-β-D-GlcNAc*p*-(1-3)-β-D-Gal*p*-(1-4)-D-Glc
Chemical Abstracts Service (CAS) Registry Number:	13007-32-4
Chemical Formula:	$C_{26}H_{45}NO_{21}$
Molecular Weight:	707.63

Structural Formula:



2.2 Manufacturing

2.2.1 Description of the Production Microorganism

LNnT is produced by a derivative of *Escherichia coli* K-12 DH1 MDO, a platform strain from which other human-identical milk oligosaccharide (HiMO) production strains have been derived including several GRAS ingredients such as 2-fucosylactose (2'-FL); 2'-fucosyllactose/difucosyllactose (2'-FL/DFL); lacto-*N*-tetraose (LNT); 6'-sialyllactose (6'-SL) sodium salt; 3'-sialyllactose (3'-SL) sodium salt; 3-fucosyllactose (3-FL); and lacto-*N*-fucopentaose I/2'-fucosyllactose (LNFP-I/2'-FL). The characteristics of this parental host strain (K-12 DH1 MDO) has been described previously and is incorporated by reference to Sections II.B.1.1 through II.B.1.3 of GRN 659 (Glycom A/S, 2016a).

2.2.2 Description of the Production Process

Glycom's LNnT is manufactured in compliance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis Critical Control Point (HACCP). The manufacture of LNnT is largely comparable to the production processes previously evaluated for other HiMOs with GRAS status (see GRNs 650, 659, 815, 833, 880, and 881) (U.S. FDA, 2016a,b, 2019a,b, 2020b,c). All additives, processing aids, and food contact articles used during manufacturing are permitted by federal regulation, have been previously determined to be GRAS for their respective uses, or have been the subject of an effective Food Contact Notification.

The manufacture of LNnT includes upstream (fermentation) and downstream (purification) stages as described in GRN 659 (Glycom A/S, 2016a).

In Stage 1 [upstream processing (USP)], D-lactose is converted to LNnT by the adapted cellular metabolism of the production microorganism. The production microorganism is removed from the fermentation medium at the end of the fermentation process.

In Stage 2 [downstream processing (DSP)], a series of purification, isolation, and concentration steps are used to generate the final high-purity LNnT product. Production of LNnT includes a crystallization step (with methanol) to generate a higher-grade ingredient with further minimization of impurities.

A schematic overview of the manufacturing process for LNnT is presented in Table 2.2.2-1 below.

Table 2.2.2-1 Summary of the Overall Manufacturing Process for LNnT

Stage	Step No.	Process step	Purification
Upstream Processing (USP)	**01**	Media Preparation	
	02	Propagation	
	03	Seed Fermentation	
	04	Fermentation	Production of LNnT
	05	Ultrafiltration/Diafiltration (UF/DF)	Removal of cells and large biomolecules (*e.g.*, protein, nucleic acids and lipopolysaccharides)
Downstream Processing (DSP)	**06**	Nanofiltration or Nanofiltration-Diafiltration (NF/DF)	Concentration. Reduction water, minerals and very small biomolecules
	06a	*Optional Microfiltration*	*Removal of potential microbiological contamination*
	07	Ion Removal (*e.g.,* ion-exchange/adsorption resin)	Removal of small-charged molecules and salts (*e.g.,* trace metals)
	07a	*Optional Pre-concentration (e.g., evaporation or nanofiltration)*	
	08	Decolorization (*e.g.*, charcoal filtration)	Removal of color and impurities by adsorbent
	09	Microfiltration	Removal of potential microbiological contamination
	10	*Optional Pre-concentration (e.g., evaporation or nanofiltration)*	
	11	Chromatography	Removal of lactose, para-LNnH and lacto-*N*-triose II
	12	Pre-concentration (*e.g.,* evaporation and/or Nanofiltration)	
	13	Crystallization (from water with acetic acid)	Highly efficient removal of micro-impurities (traces of protein and DNA, amino acids, carbohydrate-type impurities, trace elements, *etc*.)
	14	Solid-Liquid-Separation (SLS)	
	15	Washing	
	16	Drying	Removal of water and acetic acid
	17	Milling	
	18	Sampling and Packaging	
	19	Quality Control	*Specifications are tested and CoA issued*
	20	Batch Release	

CoA = certificate of analysis; DSP = downstream processing; LNnH = lacto-*N*-neohexaose; LNnT = lacto-*N*-neotetraose; SLS = solid-liquid-separation; UF = ultrafiltration; USP = upstream processing.

2.2.3 Quality Control

The manufacture of LNnT by microbial fermentation is conducted in accordance with cGMP and HACCP principles. Considering the chemically well-characterized principal raw materials and final products, the whole production process can be followed in detail by a range of analytical techniques. These techniques are applied either as in-process controls or at batch release (by Certificate of Analysis) to allow full control of the production process (refer to Table 2.2.2-1).

Both manufacturing stages (USP and DSP) are controlled by a HACCP plan which includes specifications for equipment, raw materials, product, and packaging materials. Master operating instructions are followed, batch records are kept, a number of in-process controls are applied, and the isolated product is controlled by Certificates of Analysis and batch release routines. The HACCP plan for both manufacturing stages also includes in-process controls to reduce potential impurities to the lowest level technically possible. Glycom's production process (including all processing aids, raw materials, unit operations, and filter aids) and the food safety management system comply with the Food Safety Systems Certification (FSSC) 22000 and International Organization for Standardization (ISO) 9001.

Incorporation of sterile filtration units throughout the manufacturing process of the HiMOs, ensures high microbiological purity while the presence of the production microorganism is devoid in the final product. The product microorganism is efficiently removed in the ultrafiltration step, which is applied directly following fermentation. In addition, several additional purification steps are carried out in the DSP stage to help achieve a highly purified LNnT, which is free from bacterial cells and residual fermentation by-products. The absence of the microorganisms can be measured by analysis for Enterobacteriaceae in the final product according to an internationally recognized method (ISO 21528-2). This specification for Enterobacteriaceae is set at "≤ 10 colony-forming units per gram" of test article, which also ensures absence of enumerable production microorganism as *E. coli* belong to the Enterobacteriaceae family. As further assurance of the absence of viable production organism in the finished products, batches of LNnT have been tested for *E. coli*, specifically, in accordance with ISO 16649-2. The results have confirmed the absence of enumerable *E. coli* in all tested batches of LNnT (results available upon request).

As discussed in Section 2.2.2 above, LNnT that is the subject of this notice is the same ingredient that described in GRN 659 (Glycom A/S, 2016a). This production process has been determined to produce a high-purity crystallized ingredient that is free of allergenic milk protein. The effectiveness of the production process to produce LNnT that is free of allergic milk protein is described in Petition No. FDA-2021-FL-0655 exempting LNnT from allergy labeling requirements of the *Food Allergen Labeling and Consumer Protection Act of 2004* (FALCPA) (U.S. FDA, 2018). Although the production process for LNnT as described in GRN 659 does not result in the transfer of allergenic milk protein to LNnT, the following quality control checks are used as critical control points to ensure that residual milk proteins originating from the production media are not transferred to the LNnT ingredients that will be added to exempt infant formula:

1. To limit the introduction of allergenic milk protein to the production process, an internal specification of < 100 parts per million (ppm) total protein is applied to all lots of lactose used during fermentation. This specification is applied exclusively to lots of LNnT that are intended for use in exempt infant formula.

2. The specification of LNnT intended for use in exempt infant formula will also comply with a stricter level for residual lactose (1 w/w %)

3. The specification for total protein in the final lots of LNnT is reduced from 0.01% (100 ppm) to non-quantifiable (*i.e.*, < 17 ppm) using a validated modified Bradford method developed by Glycom.

4. Final lots of LNnT intended for use in exempt infant formula will be tested for allergenic milk protein using a sensitive enzyme-linked immunosorbent assay (ELISA) assay for β-lactoglobulin (Euroclone BLG). This assay has a limit of detection (LOD) of 1.5 ppb, a limit of quantitation (LOQ) of 10 ppb and has been validated for sensitivity for detecting β-lactoglobulin in milk protein within the LNnT matrix by third-part experts (Neotron, Italy).

LNnT ingredients passing the above quality control criteria are labeled "GlyCare™ LNnT 9000 HA" for differentiation of the ingredient from other lots of LNnT that have not been subjected to the extended quality control verification analyses.

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

Food-grade specifications for LNnT are presented in Table 2.3.1-1 below. All methods of analysis are either internationally recognized or developed internally and validated by Glycom. The LNnT ingredient is specified as a crystallized white to off-white powder with a purity of at least 92%. Upper limits have been established for the raw materials and processing aids used in the manufacturing (*e.g.*, D-lactose, methanol), the carbohydrates formed during the fermentation (*e.g.*, lacto-*N*-triose II, *para*-lacto-*N*-neohexaose, LNnT fructose isomer), chemical impurities, heavy metals, and microbiological parameters, to ensure the purity of the final product.

Table 2.3.1-1 Product Specifications for LNnT

Parameter	Specification	Method
Appearance	Powder or agglomerates	ISO 6658
Color	White to off white	ISO 6658
Identification	RT of standard ± 3%	Glycom method HPLC-106-1C6-002
Assay (water free) Human-identical Milk Saccharides[a]	≥ 95.0 w/w %	Glycom method HPLC-106-1C6-002, HPAEC-HMO-016
Assay (water free) Lacto-*N*-neotetraose	≥ 92.0 w/w %	Glycom method HPLC-106-1C6-002
D-Lactose	≤ 1.0 w/w %	Glycom method HPAEC-HMO-016
Lacto-*N*-triose II	≤ 3.0 w/w %	Glycom method HPAEC-HMO-016
para-Lacto-*N*-neohexaose	≤ 3.0 w/w %	Glycom method HPAEC-HMO-016
LNnT fructose isomer	≤ 1.0 w/w %	Glycom method HPLC-106-1C6-002
pH (20°C, 5 % solution)	4.0 to 7.0	Eur. Ph. 2.2.3
Water	≤ 9.0 w/w %	Glycom method KF-001
Ash, sulfated	≤ 1.5 w/w %	Eur. Ph. 2.4.14
Methanol	≤ 100 mg/kg	Glycom method GC-109-1C6-001
Residual proteins by Bradford assay	≤ 0.002 w/w %	Glycom method UV-001
β-Lactoglobulin	≤ 0.05 mg/kg	ELISA
Casein	≤ 0.5 mg/kg	ELISA
Heavy Metals		
Lead	≤ 0.1 mg/kg	EN 13805:2002; EPA-6020A:2007

Table 2.3.1-1 Product Specifications for LNnT

Parameter	Specification	Method
Microbiological Parameters		
Aerobic mesophilic total plate count	≤ 500 CFU/g	ISO 4833-1 or ISO-4833-2
Yeasts	≤ 10 CFU/g	ISO 21527-2
Molds	≤ 10 CFU/g	ISO 21527-2
Enterobacteriaceae	Absent in 10 g	ISO 21528-1 or NMKL 144
Salmonella	Absent in 25 g	ISO 6579 or AFNOR BRD 07/11-12/05
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	ISO 22964
Listeria monocytogenes	Absent in 25 g	ISO 11290-1
Bacillus cereus	≤ 50 CFU/g	ISO 7932
Residual endotoxins	≤ 10 E.U./mg	Eur. Ph. 2.6.14 (LAL kinetic chromogenic assay)

CFU = colony forming units; EPA = United States Environmental Protection Agency; Eur. Ph. = European Pharmacopeia; E.U. = endotoxin units; GC-HS = headspace gas chromatography; HPAEC = high-performance anion exchange chromatography; HPLC = high performance liquid chromatography; ISO = International Organization for Standardization; LAL = limulus amebocyte lysate; LNnT = lacto-*N*-neotetraose; NMKL = Nordisk Metodikkomite for Levnedsmidler; RT = retention time.
[a] Human-identical milk oligosaccharides is defined as the sum of LNnT, lactose, lacto-*N*-triose II, and *para*-lacto-*N*-hexaose.

2.4 Batch Analysis

The analytical results of three independent production batches of LNnT are summarized in Table 2.4.1-1. The stability of LNnT has been previously determined to be at least 5 years when protected from light and stored at room temperature and ambient humidity (see Section II.D of GRN 650 – Glycom A/S, 2016b).

Table 2.4-1 Batch Analysis of LNnT Produced by Fermentation

Specification Parameter	Specification Limit	Manufacturing Batch Number			
		[redacted]	[redacted]	[redacted]	[redacted]
Appearance	Powder or agglomerates	Agglomerates	Powder with agglomerates	Powder with agglomerates	Powder with agglomerates
Color	White to off white	White	White	White	White
Identification	RT of standard ± 3%	Conforms	Conforms	Conforms	Conforms
Assay (water free) Human-identical Milk Saccharides[a]	≥ 95.0	98.1	98.7	100.2	101.2
Assay (water free) Lacto-*N*-neotetraose (w/w %)	≥ 92.0	97.4	98.1	99.6	100.6
D-Lactose (w/w %)	≤ 1.0	0.30	0.09	0.18	0.16
Lacto-*N*-triose II (w/w %)	≤ 3.0	0.31	0.47	0.32	0.35
para-Lacto-*N*-neohexaose (w/w %)	≤ 3.0	0.10	< 0.03	< 0.03	< 0.03
LNnT fructose isomer (w/w %)	≤ 1.0	0.14	0.05	0.03	0.14
pH (20°C, 5 % solution)	4.0 to 7.0	4.9	5.0	5.0	4.6
Water (w/w %)	≤ 9.0	3.98	6.39	7.77	6.40
Ash, sulfated (w/w %)	≤ 1.5	< 0.10	0.20	< 0.1	0.32
Methanol (mg/kg)	≤ 100	< 10	< 20	42	< 20

Residual proteins by Bradford assay(w/w %)	≤ 0.002	< 0.0017	< 0.0017	< 0.0017	< 0.0017
β-Lactoglobulin (mg/kg)	≤ 0.05	< 0.01	< 0.01	< 0.01	< 0.01
Casein (mg/kg)	≤ 0.5	< 0.2	< 0.2	< 0.2	< 0.2
Lead (mg/kg)	≤ 0.1	< 0.05	< 0.05	< 0.05	0.002
Aerobic mesophilic total plate count (CFU/g)	≤ 500	< 10	< 10	< 10	< 10
Yeasts (CFU/g)	≤ 10	< 10	< 10	< 10	< 10
Molds (CFU/g)	≤ 10	< 10	< 10	< 10	< 10
Enterobacteriaceae (in 10 g)	Absent	Absent	Absent	Absent	Absent
Salmonella (in 25 g)	Absent	Absent	Absent	Absent	Absent
Cronobacter (Enterobacter) sakazakii (in 10 g)	Absent	Absent	Absent	Absent	Absent
Listeria monocytogenes (in 25 g)	Absent	Absent	Absent	Absent	Absent
Bacillus cereus (CFU/g)	≤ 50	< 10	< 10	< 10	< 10
Residual endotoxins (E.U./mg)	≤ 10	0.00020	< 0.00025	< 0.00025	< 0.0003

CFU = colony-forming units; E.U. = endotoxin units; HiMS = human-identical milk saccharides; LNnT = lacto-*N*-neotetraose; LOQ = Limit of Quantitation; RT = retention time.

[a] Human-identical milk oligosaccharides is defined as the sum of LNnT, lactose, lacto-*N*-triose II, and para-lacto-*N*-hexaose.

2.4.1 Manufacturing By-Products, Impurities, and Contaminants

Carbohydrate-type by-products (*e.g.*, lacto-*N*-triose II, *para*-lacto-*N*-neohexaose, LNnT fructose isomer) are the main manufacturing impurities present in LNnT. These compounds are detectable, and levels are limited by appropriate specifications. Glycom also has established internal quality control measures that include microbial endotoxins and residual proteins and precautionary analyses demonstrating the absence of deleterious levels of several other potential residual compounds and trace elements that may originate from fermentation. These include amino acids and biogenic amines, trace elements and the presence/absence of genes characteristic for the production microorganism. These by-products, impurities, and contaminants are confirmed to be absent at any relevant levels of safety concern, and as such are not proposed for addition to the product specifications (see Sections II.C.3 and II.C.4 of GRN 659 – Glycom A/S, 2016a).

2.4.1.1 Critical Control Point Analyses for Protein and Allergenic Milk Protein

As discussed in Section 2.2.3 above, the production process and downstream purification steps used for the manufacture of crystallized LNnT are sufficient to ensure that the transfer of allergenic milk protein—originating from the milk derived lactose used during fermentation—are not present in LNnT at levels that would cause a risk to human health among individuals with milk allergy. The absence of protein in crystallized LNnT has been verified by analyses of multiple lots of the ingredient using Glycom's modified Bradford assay at a detection limit of 17 ppm. The absence of allergenic milk protein against multiple milk antigens was demonstrated using four enzyme-linked immunosorbent assay (ELISA) assays against casein, milk protein, and β-lactoglobulin; the detection limits of these assays ranged from 1.5 parts per billion (ppb) to 1.0 ppm. Additional highly sensitive and indiscriminate analyses for milk protein were conducted using liquid chromatography with tandem mass spectrometry (LC-MS/MS) proteomic analyses with a limit of

quantitation of 20 ppb. All of the aforementioned assays have been validated by third-party experts for use on LNnT and an incorporated appropriate spiking methodology for verification of the assay sensitivity. This analytical data and validation work establishing the suitability of LNnT for use in hypoallergenic infant formula were reviewed by the U.S. FDA during the Agency's review of FALCPA petition No. FDA-2021-FL-0655 exempting LNnT from allergy labeling requirements of the FALCPA (U.S. FDA, 2018, 2020a). Based on findings from the Glycom's milk protein analysis it was concluded with a high degree of confidence that LNnT does not contain milk protein above a detection limit of 20 ppb as established from the proteomics analyses. This value is considered conservative as no milk proteins have been detected in Glycom's LNnT samples in any assay and include investigational proteomic analyses with an extrapolated detection limit of 5 ppm, and ELISA analyses with a detection limit sensitivity of 1 ppb for β-lactoglobulin.

Based on the above protein analyses work conducted on Glycom's LNnT ingredient, it was concluded that LNnT as described in GRN 659 is of suitable purity for consumption by infants with milk allergy (see Section 6.3 for the risk assessment analyses) (Glycom A/S, 2016a). Although it is Glycom's view that inclusion of further protein analyses in the ingredient specification is not necessary for ensuring safety for use in hypoallergenic exempt formula, for conservative reasons, LNnT samples used in exempt infant formula will be lot selected for samples with total protein levels below the detection limit of Glycom's modified Bradford method (*i.e.*, < 17 ppm total protein). These samples will be subjected to an additional quality control analyses to ensure the absence of detectable milk protein using an ELISA assay for β-lactoglobulin (Euroclone; LOD = 1 ppb; LOQ = 10 ppb). Analyses of five lots of LNnT demonstrating the absence of protein/milk protein are presented below in Table 2.4.1.1-1 below.

Table 2.4.1.1-1 Detection of Milk Residues in LNnT

Sub-Lot	Batch Number	Modified Bradford (LOQ = 17 ppm)	Euroclone ELISA against β-lactoglobulin (LOQ = 10 ppb)
[redacted]	[redacted]	< LOQ	< LOQ
[redacted]	[redacted]	< LOQ	< LOQ
[redacted]	[redacted]	< LOQ	< LOQ
[redacted]	[redacted]	< LOQ	< LOQ
[redacted]	[redacted]	< LOQ	< LOQ

ELISA = enzyme-linked immunosorbent assay; LNnT = lacto-*N*-neotetraose; LOQ = limit of quantitation; ppb = parts per billion; ppm = parts per million.

Part 3. Dietary Exposure

3.1 Background Consumption of LNnT in Human Breast Milk

The concentration of LNnT in human milk has been measured and reported by numerous investigators. A discussion on the background intakes of LNnT from breast milk are summarized in Section IV.B of GRN 659 (Glycom A/S, 2016a). Table 3.1-1 summarizes the levels of LNnT that have been reported in breast milk across various studies.

Table 3.1-1 LNnT Concentration in Human Milk after Full-Term Birth

Lactation time	Key findings	References
Pooled milk		
Days 1-4 ("colostrum")	**Reported Range: 0.21 to 1.42 g/L** Mean of means: 0.53 g/L	Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Asakuma *et al.*, 2008; Spevacek *et al.*, 2015; Ma *et al.*, 2018
Days 5-14 ("transitional milk")	**Reported Range: 0.12 to 1.03 g/L** Mean of means: 0.38 g/L Outlier: 1.83 g/L (Coppa)	Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Leo *et al.*, 2009; Spevacek *et al.*, 2015; Austin *et al.*, 2016; Ma *et al.*, 2018; Ferreira *et al.*, 2020
Days 10-60 ("mature milk")	**Reported Range: 0.04 to 1.01 g/L** Mean of means: 0.37 g/L	Chaturvedi *et al.*, 1997; Nakhla *et al.*, 1999; Erney *et al.*, 2000, 2001; Sumiyoshi *et al.*, 2003; Leo *et al.*, 2009, 2010; Asakuma *et al.*, 2011; Spevacek *et al.*, 2015; Austin *et al.*, 2016; Sprenger *et al.*, 2017; McGuire *et al.*, 2017; Ma *et al.*, 2018
After 2 months ("mature milk")	**Reported Range: 0.06 to 0.64 g/L** Mean of means: 0.34 g/L	Smilowitz *et al.*, 2013; Austin *et al.*, 2016; Sprenger *et al.*, 2017; Azad *et al.*, 2018a; Ma *et al.*, 2018
Secretor milk		
Days 1-30	**Reported Range: 0.047 to 4.10 g/L** Mean of means: 0.58 g/L	Thurl *et al.*, 1997, 2010; Coppa *et al.*, 1999; Galeotti *et al.*, 2012, 2014; Bao *et al.*, 2013; Hong *et al.*, 2014; Kunz *et al.*, 2017; Sprenger *et al.*, 2017; McGuire *et al.*, 2017; Aakko *et al.*, 2017; Azad *et al.*, 2018b; Austin *et al.*, 2019; Ferreira *et al.*, 2020; Lefebvre *et al.*, 2020
Non-secretor milk		
Days 1-30	**Reported Range: 0.01 to 3.53 g/L** Mean of means: 0.66 g/L	Thurl *et al.*, 2010; Galeotti *et al.*, 2012; Hong *et al.*, 2014; Kunz *et al.*, 2017; Sprenger *et al.*, 2017; McGuire *et al.*, 2017; Azad *et al.*, 2018b; Austin *et al.*, 2019; Ferreira *et al.*, 2020; Lefebvre *et al.*, 2020

It is important to recognize that LNnT is present in the milk of all mothers. The average[2] levels in pooled milk are highest in colostrum (0.53 g/L), followed by transitional milk (0.38 g/L) and continue to decline slowly in mature milk (0.37 g/L) and mature milk from a lactation stage later than 2 months (0.37 g/L). The reported ranges generally fall between 0.01 and 1.5 g/L. It is further noted that a group of investigators (Galeotti *et al.*, 2012, 2014; Coppa *et al.*, 1999) have reported LNnT concentrations in a small sample of European mothers of excess of 2.0 g/L.

[2] Mean of reported average values in literature are presented here.

3.2 Estimated Intake of LNnT from Proposed Uses

Dietary intake of LNnT from use in infant formula has been estimated previously during Glycom's GRAS evaluation of LNnT for use in non-exempt infant formula for term infants. The intake estimations were conducted using statistical modeling software and food consumption data from the U.S. National Center for Health Statistics' 2011-2012 National Health and Nutrition Examination Surveys (NHANES) (USDA, 2014; CDC, 2015). A detailed description of the methodology and results are reported in Section IV.A of GRN 659 (Glycom A/S, 2016a). As infant formula consumption is not expected to change over time, results of the dietary intake estimate for LNnT calculated using the 2011-2012 NHANES data were used for estimating dietary intake of LNnT among infant consumers of exempt infant formula. These dietary intake estimates would be considered conservative as infants with atopic gastrointestinal diseases/disorders are unlikely to consume greater quantities of infant formula than healthy term infants. A summary of the estimated dietary intake of LNnT from the proposed and existing GRAS uses (*i.e.*, use in exempt infant formula and conventional foods described in GRN 659 (Glycom A/S, 2016a) are shown below in Section 3.3.1. Dietary intake estimates from infant formula consumption alone are presented in Section 3.3.2.

3.2.1 Dietary Intake of LNnT by Infant Consumers from Exempt Formula and Background Diet

A summary of the estimated daily intake of LNnT by infant consumers from background food uses of LNnT as described in GRN 659 (Glycom A/S, 2016a) in conjunction with dietary intake of LNnT from exempt infant formula is provided in Table 3.2.1-1. On an absolute basis, the mean and 90th percentile consumer-only intakes of LNnT from all food uses were determined to be 0.8 and 1.45 g/person/day, respectively, among infants aged 0 to 6 months. The mean and 90th percentile consumer-only intakes of LNnT were estimated to be 1.18 and 2.35 g/person/day, respectively, among infants aged 7 to < 12 months.

Table 3.2.1-1 Summary of the Estimated Daily Intake of LNnT from Food Uses of LNnT in the U.S. by Population Group (2011-2012 NHANES Data)

Population Group	Age Group (Months)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6	0.65	1.35	81.5	173	0.8	1.45
Infants	7 to < 12	1.18	2.23	99.5	127	1.18	2.35

LNnT = lacto-*N*-neotetraose; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

On a body weight basis, the mean and 90th percentile consumer-only intakes of LNnT from all food uses among infants aged 0 to 6 months, were determined to be 116 and 199 mg/kg body weight/day, respectively. The mean and 90th percentile consumer-only intakes of LNnT were estimated to be 128 and 237 mg/kg body weight/day, respectively, among infants aged 7 to < 12 months (see Table 3.2.1-2).

Table 3.2.1-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of LNnT from All Proposed Food Uses in the U.S. by Population Group (2011-2012 NHANES Data)

Population Group	Age Group (Months)	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6	94	189	81.5	173	116	199
Infants	7 to < 12	127	237	99.5	127	128	237

bw = body weight; LNnT = lacto-*N*-neotetraose; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

3.2.2 Dietary Intake of LNnT by Infants from Exempt Infant Formula Only

A summary of the estimated daily intake of LNnT from infant formula use only is provided in Table 3.2.2-1 on an absolute basis (g/person/day). To understand dietary intakes among heavy consumers of infant formula 99th percentile intake data was included in the evaluation. On an absolute basis, the mean and 99th percentile consumer-only intakes of LNnT from infant formula only were determined to be 0.53 and 1.45 g/person/day, respectively, among infants aged 0 to 6 months. The mean and 99th percentile consumer-only intakes of LNnT were estimated to be 0.45 and 0.88 g/person/day, respectively, among infants aged 7 to < 12 months. Due to the overestimation of intakes that occurs when 99th percentile intake estimates are used across multiple food use categories it was not considered appropriate to evaluate dietary intakes to LNnT by 99th percentile consumers of infant formula and conventional foods to which LNnT may be added.

Table 3.2.2-1 Summary of the Estimated Daily Intake of LNnT from Infant Formula Only in the U.S. by Population Group (2011-2012 NHANES Data)

Population Group	Age Group (Months)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	99th Percentile	%	n	Mean	99th Percentile
Infants	0 to 6	0.4	1.45	76.0	161	0.53	1.45
Infants	7 to < 12	0.33	0.85	75.8	94	0.45	0.88

LNnT = lacto-*N*-neotetraose; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

Part 4. Self-Limiting Levels of Use

No known self-limiting levels of use are associated with LNnT.

Part 5. Experience Based on Common Use in Food Before 1958

Not applicable.

Part 6. Narrative and Safety Information

6.1 Introduction

Crystallized LNnT produced by Glycom is of high purity and has been demonstrated to be qualitatively identical to LNnT naturally present within human breast milk. Concentrations of LNnT in human milk are subject to significant interindividual variation. Concentrations of up to 1 g/L have an established safe history of consumption through breast milk by healthy infants. Accordingly, the use of LNnT in infant formula at concentrations within the upper mean percentiles of levels naturally present in human milk provides prima facie evidence of safety. To date, Glycom's LNnT ingredient has market access to over 160 countries for use in term infant formula.

Glycom intends to expand the use of LNnT to include exempt infant formula. The U.S. FDA defines exempt infant formula as:

> "*[…] an infant formula intended for commercial or charitable distribution that is represented and labeled for use by infants who have inborn errors of metabolism or low birth weight, or who otherwise have unusual medical or dietary problems*" (21 CFR §107.3 – U.S. FDA, 2021).

The dietary condition for which LNnT is intended for use includes infants with CMPA or multiple food allergies. The purpose of this notification is therefore to provide generally available data and information supporting Glycom's conclusion that the use of LNnT in hypoallergenic exempt infant formula would be concluded to be GRAS by qualified experts. Background information on CMPA and multiple food allergies is presented in Section 6.2.

As discussed, LNnT is manufactured using a modified strain of *E. coli* K-12 expressing biosynthetic enzymes that catalyze the conversion of lactose to LNnT. As the lactose used during fermentation is typically derived from cow's milk, small quantities of allergic milk protein are introduced during the manufacturing process. Although the potential introduction of milk allergens during LNnT manufacturing represents a potential hazard for use by infants with milk allergy, these levels are firstly significantly diluted during fermentation and secondly LNnT preparations produced by various manufacturers are subjected to significant DSP to further remove/reduce such impurities; however, the efficiency of the purification process will vary by production process and therefore are specific to each manufacturer. Glycom has therefore provided analytical data demonstrating that the company's manufacturing process controls are sufficient to ensure that allergic milk protein is not detected in the ingredient at levels that would cause an allergenic response that poses a risk to human health. This safety standard was established in a manner that is consistent with the petition requirements under 21 U.S.C. 343(w)(6) for exemption of LNnT from the allergen labeling requirements of FALCPA. Data and information supporting the safety of LNnT for use in hypoallergenic infant formula are therefore incorporated by reference to FALCPA Petition No. 006. The results of validated analytical data demonstrating the absence of milk protein in LNnT is discussed in brief in Section 2.4.1.1 along with confirmatory batch analyses obtained for multiple lots of LNnT subjected to Glycom's extended analytical allergen control processes that have been implemented for lots of LNnT used in infant formula.

Recognizing the challenges of demonstrating that an ingredient is wholly "absent" of milk protein, a risk-based approach (see Section 6.3) to the safety assessment of using LNnT in infant formula is presented that leverages a numerical detection limit for milk protein in LNnT obtained using multiple qualitatively distinct validated methods against generally recognized threshold levels for milk allergenicity reported by scientific experts of the Voluntary Incidental Trace Allergen Labeling (VITAL) program of the Allergen Bureau of

Australia & New Zealand (ABA) (Allen *et al.*, 2014; Taylor *et al.*, 2014; Remington *et al.*, 2020). Using a detection limit of 25 ppb for potential residues of milk protein in LNnT and a maximum use level of 0.6 g/L of LNnT in exempt infant formula, a possible dietary intake of up to 36 ng/day can be estimated for infant consumers 0 to 6 months of age from exempt infant formula. This value is 5,556-fold below the ED_{01} threshold of 0.2 mg.

In addition to the risk-based approach for assessing the safety of LNnT for use in exempt infant formula, additional supporting clinical data evaluating the safety and tolerance of Glycom's LNnT ingredient in hypoallergenic infant formula was evaluated in randomized controlled cross-over study in infants with confirmed CMPA (Nowak-Wegrzyn *et al*., 2019a). The study was conducted in accordance with the American Academy of Pediatrics (AAP) statement guidance on hypoallergenic infant formulas, and the base formula was validated for hypoallergenicity. The authors reported that LNnT was safe and well tolerated by infants with CMPA and no differences in incidences of adverse responses to the formula were observed between the groups. This study provides strong supporting information that LNnT manufactured by Glycom is safe for use in infants with CMPA and corroborates conclusions of the risk-based assessment.

For the purposes of identifying any new data relevant to the safety of LNnT published since the most recent LNnT GRAS determination notified to the U.S. FDA with a no questions response (*i.e.*, GRN 659; U.S. FDA, 2016a), a comprehensive search of the published scientific literature was conducted on 02 September 2021 spanning the period of March 2016 to September 2021. The search was conducted using the electronic search tool, ProQuest, with several databases, including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS reviews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. A discussion of all newly available published and unpublished studies, both favorable and unfavorable, is presented below.

Several clinical studies in infants administered LNnT alone or combination with 2'-FL in infant formula have been reviewed previously in GRN 547 and 659 (Prieto, 2005; Puccio *et al.*, 2017). Comprehensive discussions of these studies can be found in Section IV.B.6 of GRN 547 and Section IV.F.1 of GRN 659. The updated literature search identified 2 new interventional infant clinical trials in which endpoints related to the safety of LNnT were identified and these studies are reviewed in Section 6.4.2 below.

The results of published and unpublished toxicological studies in neonatal and mature rats further corroborate the safety of the ingredient. Comprehensive discussions of the published toxicity studies as they apply to the safety of LNnT for use in infant formula and foods are incorporated by reference to Section IV.B.5 of GRN 547 Section IV.E of GRN 659. These studies included a 90-day oral toxicity study in neonatal rats, as well as *in vivo* and *in vitro* genotoxicity assays. Findings from these studies demonstrated that LNnT is not genotoxic and is of low toxicity potential following gavage dosing in neonatal pups. The absence of adverse or toxicity effects reported in the literature on LNnT in animal models is consistent with its natural presence in human milk at appreciable levels. No new animal toxicology studies were identified from Glycom's updated literature.

Based on the findings from Glycom's risk-based assessment combined with supporting clinical data evaluating Glycom's LNnT ingredient in infants with CMPA it can be concluded that the ingredient is GRAS for use in hypoallergenic exempt infant formula.

6.2 Metabolic Fate

Reviews of published data and information characterizing the absorption, distribution, metabolism and excretion of LNnT have been the subject of previous comprehensive evaluations, and this information is incorporated herein by reference to Section IV.D of GRN 659 (U.S. FDA, 2016a). In brief, it is generally recognized that HMOs, including LNnT, are highly resistant to hydrolysis by digestive enzymes under conditions simulating the infant gastrointestinal tract (Engfer *et al*., 2000; Gnoth *et al*., 2000). Therefore, the intended uses of LNnT as described herein will not be a safety issue to infants with malabsorptive conditions.

6.3 Cow's Milk Protein Allergy (CMPA) and Multiple Food Allergies

Food allergy is defined as an adverse health effect arising from a specific immune mediated response that occurs reproducibly from the ingestion of specific foods. Food allergy can be segregated into one of two major categories: IgE-mediated and non-IgE-mediated, based upon the immunological response. A third category of mixed IgE- and non-IgE-mediated food allergy responses also has been characterized. IgE-mediated reactions are the most well characterized and are easily diagnosed by the presence of specific serum IgE or a positive skin prick test response to a food antigen challenge (Calvani *et al*., 2021). The prevalence of milk allergy in the developed world is 2 to 3% and is the most common type of food allergy in the pediatric population (Lifshitz and Szajewska, 2015). Milk allergy occurs most frequently in the first years of life and produce a range of symptoms from acute urticaria of the skin, gastrointestinal reactions (pain, discomfort, diarrhea, vomiting) to the most severe reactions of anaphylaxis affecting multiple organ systems and potentially leading to cardio-respiratory collapse and death (Høst and Halken, 2014). Non-IgE-mediated food allergy disorders are believed to represent up to 40% of milk protein allergy in infants and young children and include food protein-induced enterocolitis syndrome (FPIES), food protein-induced allergic proctocolitis (FPIAP), food protein-induced enteropathy, Heiner's syndrome (pulmonary hemosiderosis), and cow's milk protein-induced iron deficiency anemia. Mixed IgE- and non-IgE-mediated allergic disorders also have been characterized for milk and include eosinophilic esophagitis and eosinophilic gastroenteritis (Nowak-Węgrzyn, 2015).

The major allergens responsible for IgE-mediated milk allergy belong to the casein (αs1-, αs2-, β-, and κ-casein) and whey (β-lactoglobulin, and α-lactalbumin) fractions of milk; co-sensitization with soy is known to occur in some infants (Lifshitz and Szajewska, 2015). Although milk is one of the most frequent food triggers for non-IgE-mediated allergic reactions, non-IgE-mediated allergy to soy, egg, and cereals (wheat, rice, and oats) are also relatively common. Depending on the geographical region, non-IgE-mediated reactions to other dietary food proteins such as fish, pulses, poultry, and nuts have been identified. FPIES is typically caused by a single food in most children (65 to 80%), with milk and soy being the most common. Approximately 5 to 10% of infants with FPIES are allergic to more than three foods (Nowak-Węgrzyn, 2015).

Extensively hydrolyzed formula (EHF) is typically recommended as a first line intervention for infants with CMPA. When an EHF is provided for the first time to infants with CMPA, it should be provided under supervision of a physician experienced and equipped to treat anaphylaxis (Zeiger, 2003). Per definition by the AAP, to be labelled hypoallergenic, an infant formula needs to be tolerated by at least 90% of infants with CMPA (with 95% statistical confidence) (AAP, 2000). EHFs derived from bovine casein or whey are tolerated by approximately 95% of infants with CMPA (Bahna, 2008); however, some infants display very high sensitivities to low levels of intact/partially intact milk protein and cannot tolerate EHFs. Infants unable to ingest EHFs are then typically provided elemental formulas containing amino acids as a source of protein.

Consistent with the fact that most infants with CMPA respond favorably to EHF, the addition of LNnT to infant formula is expected to be well tolerated by these infants. The fact that a majority of infants with CMPA can tolerate lactose, which is derived from cow's milk tends to support this conclusion (Heine *et al*., 2017). Notwithstanding these conclusions, a small population of infants with CMPA will be expected to be highly sensitive to low levels of intact/partially intact milk proteins, and there remains a possibility that low-level milk protein residues in LNnT originating from the use of lactose during fermentation could induce an allergic response in these individuals should small quantities of milk protein be present at sufficiently high levels. The safety of LNnT for use in hypoallergenic exempt infant formula will therefore be determined on the basis of establishing the absence of detectable milk allergenic protein in the ingredient and through evidence demonstrating that the current detection limits for residual milk protein in the HMO ingredient added to infant formula is safely below the minimum level deemed necessary to produce an allergenic response that represents a safety risk to the infant based on available data.

6.4 Risk-Based Safety Assessment

The use of risk-based evaluation procedures can be applied to evaluate the safety of LNnT as an ingredient for use in hypoallergenic infant formula. Risk-based procedures incorporate statistical findings from a large dataset of clinical trials to derive threshold doses for allergenic responses to milk protein. The advantage of this approach is the quality and robustness of the data that is provided and the ability to pool findings on thresholds for the most sensitive sub-populations of milk-allergic infants. The advantages of using a risk-based safety approach have been discussed previously by the U.S. FDA (Buchanan *et al*., 2008).

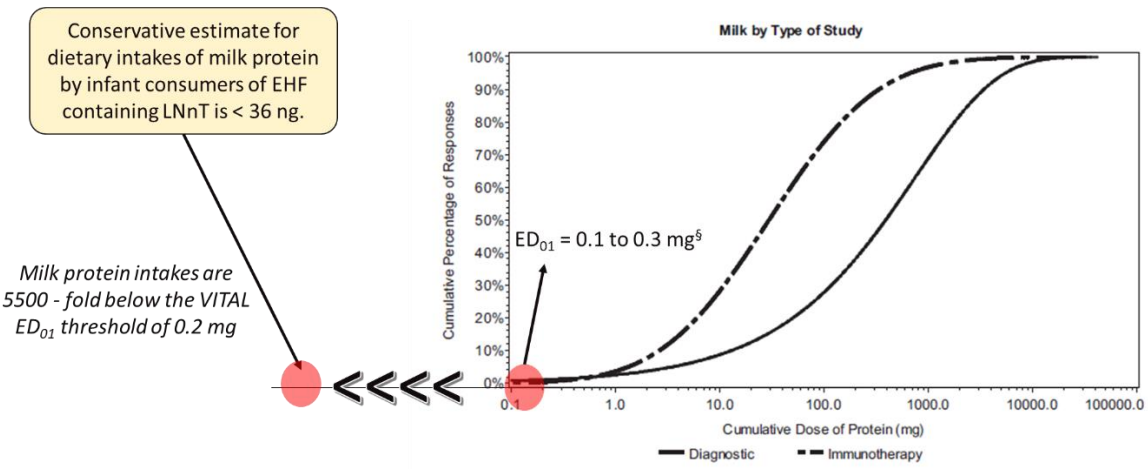
One of the most comprehensive risk-based evaluations of milk protein allergy thresholds was conducted in 2011 by an Expert Panel assembled as part of the VITAL program of the ABA (Taylor *et al*., 2014). The Panel applied statistical approaches described previously by Crevel *et al*. (2007) to model the dose distribution of allergy thresholds from oral clinical challenge studies. The authors incorporated the concept of a predicted population elicited dose (ED), where EDp, refers to the dose of allergen that is predicted to produce a response in (100-p)% of the allergic population. Food challenge studies used for dose-modeling were selected based upon criteria outlined previously (Taylor *et al*., 2009) and placed an emphasis on low-dose oral challenge studies with a preference for double-blind placebo-controlled studies (except for data from infants and young children where double-blinding was not considered necessary). Data were modeled using both discrete and cumulative dose-response effects, and the lowest-observed-effect level was selected based on the first reported objective symptoms of an allergic response; the NOAEL was then set at the previous dose.

For characterization of milk allergy hazard, the VITAL Expert Panel used a clinical data set that included 17 published studies and two unpublished studies, containing a total of 351 subjects reporting objective symptoms. Objective symptom reporting favored children with 323 objective symptoms reported for children, 25 from adults and 3 from subjects where the age was uncertain. Data for both discrete and cumulative dosing was modeled, with discrete dosing considered the most conservative approach; however, the authors reported that little difference existed between the ED values based on the discrete *vs*. cumulative doses for any of the parametric models. The data for milk allergy was sufficiently robust for calculation of an ED_{01}, a dose that would protect 99% of milk allergic individuals from developing any objective reaction. The Expert Panel recognized that an ED_{01} would imply that a small percentage of the population (*i.e*., 1%) of allergic individuals may elicit objective reactions at this dose; however, as adverse reaction experiences by individuals in the low-dose trials were characterized as mild to moderate, and never resulted in provocation of severe reactions, the risk of individuals developing severe reactions that would pose a risk to human health would be very low. Based on the dose-response modeling an ED_{01} of 0.1 mg was

established by the Panel. Additional qualitative analyses of the VITAL reference doses according to different statistical modeling techniques and analyses of the effects of age, geographic origin, nature of the challenge materials and dosing regimen were reported by Allen *et al*. (2014). The authors reported that the heterogeneity of the dataset for milk thresholds did not impact conclusions on the ED_{01} of 0.1 mg for milk. The reference doses were recently updated in 2020 and included new clinical data on milk allergy thresholds and re-analyses of the dose-threshold distributions using newly developed Stacked Model Averaging statistical modeling techniques (Remington *et al*., 2020). Using an updated dataset of 450 individuals, the authors reported a model averaged ED_{01} for discrete dosing[3] of 0.2 mg [95% confidence interval (CI) = 0.1, 0.5].

The ED_{01} threshold can be used to evaluate the risk of using LNnT in exempt infant formula by comparing theoretical dietary intakes of milk protein from the use of LNnT in infant formula to the ED_{01} value of 0.2 mg. LNnT is intended for use in non-exempt term infant formula at a use level of up to 0.6 g/L. The estimated 90th percentile intake of LNnT by infant consumers of term formula were reported to be 1.45 g/day (infants 0 to 6 months) and 0.88 g/day (infants 7 to 12 months). Using a detection limit for total milk protein of 25 ppb measured using validated LC-MS/MS proteomic analyses (see FALCPA No. 006), dietary intakes of milk protein would be 36 ng and 22 ng in infants aged 0 to 6 and 7 to 12 months, respectively. These intake levels are 5,556- and 9,090-fold below the ED_{01} threshold of 0.2 mg for infants 0 to 6 months and 7 to 12 months respectively (see Figure 6.3-1). This margin of safety is considered sufficiently high to protect the most highly sensitive population of infants with IgE-mediated food allergy.

Figure 6.3-1 Comparison of Estimated Dietary Intake of Milk Protein to Allergy Thresholds Using Risk-Based Assessment



Note: Dose-Distribution models for individual thresholds (expressed as milligrams of protein) based on allergic patients for diagnostic studies, threshold studies, and immunotherapy studies (Modified from Allen *et al*., 2014).
§ ED_{01} value reported as 0.1 mg (Allen *et al*., 2014; Taylor *et al*., 2014). ED_{01} increased to 0.2 and 0.3 mg for discrete and cumulative dosing based on updated analyses by Remington *et al*. (2020).

With respect to the use of LNnT as an ingredient in exempt amino acid-based infant formula for sensitive subpopulations of infants with non-IgE-mediated milk allergy, thresholds for severe reactions by sensitive subpopulations have not been established; however, as reported by Munblit *et al*. (2020), "[…] *available data suggests that thresholds of reactivity in infants with non-IgE mediated CMA* [cow's milk allergy] *are*

[3] Discrete dosing schemes are reported as the mg protein amount of each separate dose within a food challenge.

usually higher than thresholds of reactivity for IgE-mediated CMA". Therefore, the margin of safety between the ED_{01} values for IgE milk allergy and potential exposure to milk protein from the use of LNnT in infant formula strongly suggest that any risk of allergic reactions in sensitive infants with severe non-IgE food allergy would be very low. It also is noteworthy that children with non-IgE food allergy are not at risk for anaphylaxis (Calvani *et al*., 2021).

Glycom's LNnT is produced using fermentation technology that utilizes a modified strain of *E. coli* K-12 expressing genes required for the synthesis of LNnT from lactose. The HMO is then purified through a variety of downstream processes such as micro-filtration, chromatographic separation, and crystallization to produce high-purity ingredients that are free of fermentation contaminants and contain virtually no detectable protein. LNnT manufactured by Glycom have GRAS status for use in term infant formula, and therefore, data and information characterizing the identity, quality, manufacturing, and safety of LNnT for use as infant formula ingredients can be incorporated by reference to GRN 659 (U.S. FDA, 2016a). LNnT intended for use in hypoallergenic infant formula will meet specifications set forth as described in GRN 659 and will be manufactured using the same methods and purification techniques described in the Notice. Glycom has conducted extensive analytical testing for residues of protein in LNnT and has demonstrated the absence of detectible milk protein in the ingredients using four validated ELISA kits for casein, total milk, and lactoglobulin. Lactose is produced from whey and therefore the major milk protein that could be transferred from lactose into LNnT is expected to be lactoglobulin. Using the most sensitive ELISA assay available for lactoglobulin, Glycom has demonstrated the absence of lactoglobulin at a detection limit of 10 ppb. As lactoglobulin comprises approximately 50% of the total protein content of whey (Regester and Smithers, 1991), a detection limit of 10 ppb total lactoglobulin would correspond to a detection limit for total milk whey protein of 20 ppb. Proteomics analyses of LNnT using LC-MS/MS have demonstrated the absence of milk protein fragments at a detection limit of 25 ppb based on findings from validated spiking assays. It also is noteworthy that findings from additional proteomic analyses on samples of LNnT using LC-MS/MS were able to detect low-level quantities of *E. coli* protein from the fermentation organism at an extrapolated detection limit of 5 ppb corroborating the sensitivity of the assay; however, no milk protein fragments have ever been detected in any LNnT samples that have been analyzed to date. Due to technical challenges with spiking and sample preparation at such low concentrations, validation of the 5 ppb detection limit was deemed impractical; however, the totality of evidence from ELISA assays and proteomic analyses provide support that no milk protein is present in the Glycom's LNnT. Based on findings from the proteomics assay a detection limit of 25 ppb was used for risk assessment purposes (*i.e*., it was assumed that 25 ppb of milk protein will be present in LNnT). The 25 ppb detection limit from the proteomics assay was preferred over the ELISA, as the proteomic analyses is a more robust method that is not impacted by potential protein hydrolysis or denaturation of the milk protein. The totality of information characterizing the protein content of LNnT obtained using the most sensitive analytical methods available to date, have demonstrated that milk protein cannot be detected in LNnT under the conditions of manufacture as described in GRN 659 (U.S. FDA, 2016a). Although the current production controls have been deemed sufficient to ensure that milk protein will not be transferred to LNnT above a detection limit of 25 ppb, Glycom has applied additional quality control limits to production process for LNnT batches that will be used for hypoallergenic infant formula; these controls include limits for total protein on the incoming lactose used for production of LNnT and two ELISA-based assays for β-lactoglobulin and casein demonstrating the absence of detectable milk protein in the ingredients. The strong congruence between findings from the ELISA and LC-MS/MS data support a conclusion that the ELISA assay is appropriate for this purpose.

6.5 Infant Studies

6.5.1 Infants with Cow's Milk Protein Allergy (CMPA)

It is generally recognized that a majority of infants with IgE- and non-IgE-mediated allergy to cow's milk protein can tolerate EHF, analytical data demonstrating the absence of milk protein in LNnT strongly supports the view that the majority of infants with IgE- and non-IgE-mediated allergy will tolerate infant formula containing this HiMO. This conclusion is supported by findings reported by Nowak-Wegrzyn *et al*. (2019a) who evaluated the safety and tolerance of adding LNnT to an extensively hydrolyzed hypoallergenic infant formula. The study was conducted in accordance with the AAP statement guidance on hypoallergenic infant formulas, which requires that for a formula to be:

> "[…] *labeled hypoallergenic, these formulas, after appropriate preclinical testing, must demonstrate in clinical studies that they do not provoke reactions in 90% of infants or children with confirmed cow's milk allergy with 95% confidence when given in prospective randomized, double-blind, placebo-controlled trials*" (AAP, 2000).

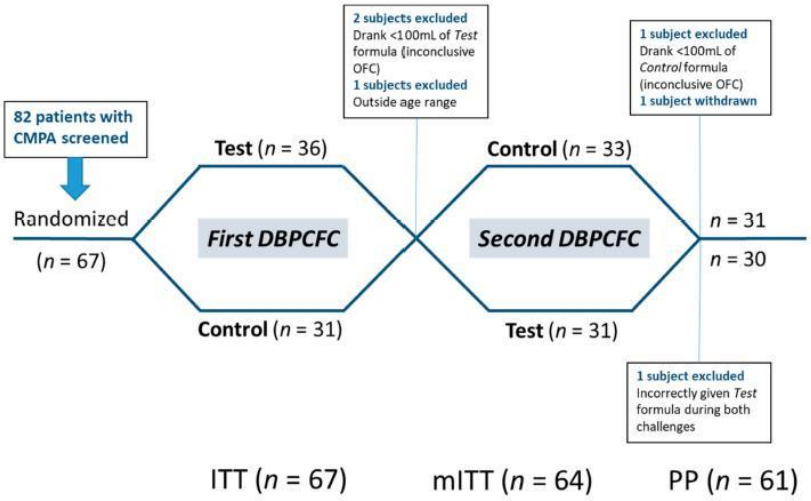
These criteria for hypoallergenic infant formula are also endorsed by other relevant scientific bodies such as the World Allergy Organization (Fiocchi *et al*., 2010), the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition (Koletzko *et al*., 2012), and the European Academy of Allergy and Clinical Immunology (Muraro *et al*., 2014).

The test formula used in the study was a 100% whey-based EHF supplemented with 2'-FL (produced by Glycom as described in GRN 650 – Glycom A/S, 2016b) and LNnT (produced by Glycom as described in GRN 659 – Glycom A/S, 2016a). Infants and children between 2 months and 4 years of age (modified intention-to-treat (mITT) cohort: mean age at enrollment of 24.1 ± 13.2 months) with clinically diagnosed CMPA were recruited for the study. The infants were randomized into one of two groups provided a commercially available control infant formula (Althéra®, Nestlé, Vevey, Switzerland) without HMOs, or a test infant formula [Althéra® supplemented with 2'FL (1.0 g/L) and LNnT (0.5 g/L)] in cross-over fashion resulting in two double-blind placebo-controlled food challenges (DBPCFCs) (see Figure 6.4.1-1). The control formula was previously qualified as hypoallergenic in accordance with AAP guidelines (Nowak-Wegrzyn *et al*., 2019b)[4]. The control and test formula were demonstrated to be free of residual milk proteins, as confirmed by gel electrophoresis (SDS-PAGE, Pharmacia PhastSystem™ with silver staining) and high-sensitivity ELISA testing (Euroclone Spa, Pero, Italy; limits of quantification 10 ppb for β-lactoglobulin and 20 ppb for casein). The first DBPCFC occurred within 3 to 28 days after enrollment, and the second DBPCFC within 2 to 7 days of the first DBPCFC. For subjects < 1 year of age, the initial dose was a lip smear with the assigned infant formula, followed by oral doses of 5, 10, 20, 30, 30, 35, and 50 mL at 10- to 15-minute intervals (total volume of 180 mL). For subjects > 1 year of age, the initial dose was a lip smear, followed by oral doses of 5, 10, 25, 45, 45, 45, and 65 mL at 10- to 15-minute intervals (total volume of 240 mL). A DBPCFC was considered evaluable if subjects had consumed a minimum of 100 mL of formula. The subjects were observed for a minimum period of 1 hour after the second DBPCFC for any allergic signs or symptoms (cutaneous, gastrointestinal, respiratory, or cardiovascular) attributable to the challenge formula. If both DBPCFCs were negative, subjects participated in a one-week (7 to 9 days), open food challenge (OFC) with the test infant formula (instructed to drink a minimum of 240 mL daily). During this time, daily formula intake as well as several clinical parameters, including allergenic or adverse events were

[4] The production of hypoallergenic formula involves enzymatic hydrolysis, heat-treatment, and ultrafiltration steps that are specific to each manufacturer. Accordingly, both the control formula and the test formula must be qualified as hypoallergenic in accordance with AAP guidelines. Extrapolation of findings between studies is therefore not possible.

recorded: (1) Daily stool frequency, color, consistency, and odor; (2) frequency of flatulence; (3) frequency of spitting-up and/or vomiting; (4) any potential allergic symptoms; and (5) any other adverse or serious adverse events.

Figure 6.4.1-1 Double-Blind Placebo-Controlled Food Challenge Study Flow Chart (Nowak-Wegrzyn *et al.*, 2019a)



ITT = Intention-to-treat; mITT = modified intention-to-treat; OFC = open food challenge; PP = per protocol analysis cohorts.
Patients were allocated to perform two double-blind, placebo-controlled food challenges (DBPCFC) with the Test and Control formula in randomized order.

The results of the DBPCFCs reported one positive allergic reaction to the test infant formula during the first DBPCFC, and one positive allergic reaction to the control infant formula in the second food DBPCFC (same 12-month-old female). Based on the mITT analysis, 63 out of 64 (98.4%; 95% CI lower bound of 92.8%) participants tolerated the test infant formula. Based on the per protocol (PP) analysis, 60 out of 61 (98.4%; 95% CI lower bound of 92.5%) participants tolerated the test infant formula (see Table 6.4.1-1). Therefore, under both analyses, the test infant formula with two HMO (2'-FL and LNnT) met the defined criteria for hypoallergenic formulas (AAP, 2000). Additionally, no serious adverse events occurred during the OFC.

Table 6.4.1-1 Outcome of Double-Blind Placebo-Controlled Food Challenge to the Test and Control Formula by Group Allocation in the Modified Intention-to-Treat Cohort (Nowak-Wegrzyn *et al*., 2019a)

	Challenge Outcome	DBPCFC 1 n (%)	DBPCFC 2 n (%)	Total n (%)	Exact 96.5% Lower Bound*
Test Formula (EHF + 2'-FL/LNnT)	Positive	1 (3.0%)	0 (0.0%)	**1 (1.6%)**	92.8%
	Negative	32 (97.0%)	31 (100%)	63 (98.4%)	
Control Formula	Positive	0 (0.0%)	1 (6.1%)	**1 (2.9%)**	92.6%
	Negative	31 (100%)	30 (93.9%)	61 (97.1%)	

2'-FL = 2'-fucosyllactose; DBPCFC = double-blind placebo-controlled food challenge; EHF = extensively hydrolyzed formula; LNnT = lacto-*N*-neotetraose.
*The 95% confidence interval lower bound was greater than 90% for both formulas.

The authors concluded that "*the hypoallergenicity of this novel EHF supplemented with two HMOs* [2'-FL and LNnT] *was confirmed by DBPCFC in children with CMPA, in line with the established guidelines for hypoallergenic formulas*" (Nowak-Wegrzyn *et al*., 2019a); therefore, this conclusion supports the safety and tolerance of 2'-FL and LNnT as ingredients for use in exempt hypoallergenic infant formula.

6.5.2 Other Infant Studies

The results of the updated literature search identified two new interventional infant clinical trials in which endpoints related to the safety of 2'-FL were identified. These studies are summarized in the subsections below and tabulated in Section 6.5.2.6. Studies exclusively examining benefits of LNnT supplementation were not included herein.

6.5.2.1 Randomized, Double-Blind, Controlled Clinical Study Examining Safety of 2'-FL and LNnT in a Liquid Supplement for Premature Infants (Hascoët et al., 2021 [abstract])

The effect of a supplement containing 2'-FL and LNnT on growth, safety, and feeding tolerance was examined in a multi-center, randomized, double-blind, controlled clinical study conducted in France (Hascoët *et al.*, 2021 [abstract]; NCT03607942). In this study, preterm infants (27 to 33 weeks gestation, birth weight < 1,700 g) were randomly allocated to receive either a supplement containing 2'-FL and LNnT in a 10:1 ratio (administered as a total of 0.374 g/kg body weight/day, dissolved in water buffered with a pH adjusting agent) or an isocaloric placebo supplement consisting of only glucose (0.140 g/kg body weight/day) from randomization (as early as possible) to discharge from the neonatal unit. The primary outcome was feeding tolerance, measured by non-inferiority in days to reach full enteral feeding from birth in the 2'-FL + LNnT group compared to the placebo group (non-inferiority margin of +4 days). Anthropometric z-scores were calculated using Fenton growth standards.

A total of 43 infants were allocated to the 2'-FL + LNnT supplement group and 43 to the placebo control group. The mean chronological age at the initiation of supplementation were 6.3 days in the 2'-FL + LNnT group and 6.2 days in the placebo group. The mean total duration of intervention was 41 (range: 2 to 80) days in the 2'-FL + LNnT group and 34.5 (range: 2 to 125) days in placebo group. Non-inferiority in time to reach full enteral feeding in the 2'-FL + LNnT group versus the placebo was achieved in the full analysis set (least squares mean difference = 2.16 days; 95% confidence level -5.33, 1.00; upper bound of 95% confidence interval < non-inferiority margin). Similar results were observed in the per protocol set. The adjusted mean time to reach full enteral feeding from birth was two days shorter in the 2'-FL + LNnT group compared to placebo (12.2 days *vs.* 14.3 days) but this finding did not reach statistical significance (p=

0.177). There was no difference in weight-for-age z-scores between the groups. Length-for-age z-scores were statistically significantly higher in the 2'-FL + LNnT supplement group versus the control group at full enteral feeding days 14 (least squares mean difference of 0.29; p= 0.037) and 21 (least squares mean difference of 0.31; p= 0.037). Head circumference-for-age z-score was significantly higher in the group receiving 2'-FL + LNnT versus the control at discharge (least squares mean difference of 0.42; p= 0.007). Gastrointestinal tolerance measures, incidence of gastrointestinal adverse events, incidence of necrotising colitis, and incidence of other illnesses and infections were similar between groups. No cases of illnesses and infections were deemed related to the intervention.

6.5.2.2 Real-World Study in Infants Fed 2'-FL and LNnT (Román Riechmann et al., 2020)

A non-randomized, open-label, prospective study was conducted in healthy, term infants (Román Riechmann *et al.*, 2020; clinical trial registry number NCT04055363). In this real-world study, infants were enrolled at age 7 days to 2 months and fell into one of three groups: an exclusively formula-fed group, a mixture of formula and human milk fed, or exclusively breastfed infants (serving as a reference population). Formula fed infants received a partially hydrolyzed, 100% whey, term infant formula (67 kcal/100 mL, 1.9 g protein/199 kcal, 11.5 g carbohydrates/100 kcal, 5.1 g lipids/100 kcal, 1.0 g 2'-FL/L, and 0.5 g LNnT/L) that contained *Lactobacillus reuteri*[5] (dose not reported), vitamins, and minerals, *ad libitum* for 8 weeks.

Anthropometry measures (weight, length, head circumference) were measured at baseline and at Week 8. Z-scores for weight-for-age, length-for-age, head circumference-for-age, and body mass index-for-age were calculated. Gastrointestinal symptoms were evaluated *via* the Infant Gastrointestinal Symptom Questionnaire (IGSQ). Adverse events were recorded from the time of enrolment through the end of study.

A total of 66 exclusively formula fed, 48 mixed fed, and 45 exclusively breastfed infants were included in the analyses. When comparing baseline characteristics of the enrolled infants, the exclusively formula fed group was slightly younger at enrollment ($p < 0.01$) and had a higher proportion of male infants ($p > 0.05$) compared to the mixed-fed and breastfed group. Consistent with the slightly younger age group, baseline weight and length were slightly lower in the exclusively formula-fed group. Other baseline anthropometric characteristics were comparable across groups.

Through the study, age-appropriate growth was reported in all groups. Differences in baseline weight and length did not persist by Week 8; there were no significant differences between any groups for any of the anthropometric measures. The composite IGSQ scores showed low gastrointestinal distress in all groups at all time points. No significant differences were reported in four of the subdomains of gassiness, fussiness, crying, and spitting-up/vomiting. In the last subdomain of stooling, the formula-fed and mixed feeding group exhibited a statistically significant different score at baseline compared to exclusively breastfed infants. This was significantly improved at Week 8 in exclusively formula-fed infants, with scores moving closer to the stooling profile of the exclusively breastfed group. Stooling scores in mixed fed infants remained significantly different at Week 8.

Three patients experienced potentially product-related adverse events, including two instances of cow's milk intolerance (one in exclusively formula fed group, one in the mixed-feeding group, and one instance of irritability in the exclusively formula fed group. No serious adverse events were attributed to the study

[5] Published as *Lactobaccllus reuteri* but current day is referred to as *Limosilactobacillus reuteri.*

feeding. The authors noted that the incidence of adverse events was low overall and was not significantly different between the groups.

6.5.2.3 Summary of Interventional Clinical Infant Studies Identified

Glycom performed a search of the scientific literature for new interventional infant studies relevant to the safety of LNnT. Overall, the new clinical studies examining the effect of the administration of LNnT to infants have not identified any safety concerns (see Table 6.5.2.6-1).

Table 6.5.2.6-1 Summary of the Interventional Clinical Infant Studies Conducted on LNnT

Study Population	Duration of Intervention	Study Groups and Test Articles	References
86 preterm infants (27 to 33 weeks gestation, birth weight < 1,700 g) 43 per group Average 6 days of age at intervention initiation	Enrolment to discharge from neonatal unit	***Control Supplement:*** Glucose (0.140 g/kg bw/day) ***Test Supplement:*** 2'-FL and LNnT in 10:1 ratio (0.374 g/kg bw/day)	Hascoët *et al.*, 2021 ***Clinical trial number*** NCT03607942
159 healthy full-term infants 45 to 66 per group 7 days to 2 months old at enrolment	8 weeks	***Exclusively Formula Fed Group:*** *Ad libitum* formula containing 1.0 g 2'-FL/L and 0.5 g **LNnT**/L ***Mixed Formula Fed and Breastfed Group***: *Ad libitum* formula containing 1.0 g 2'-FL/L and 0.5 g **LNnT**/L ***Exclusively Breastfed Group (Reference Group):*** Breastfed enrolled at the same time as formula fed infants	Román Riechmann *et al.* (2020) ***Clinical trial number*** NCT04055363

2'-FL = 2'-fucosyllactose; LNnT = lacto-*N*-neotetraose.

6.6 Other Considerations – Additive Dietary Intakes of LNnT with Other HiMOs

While Glycom is not a manufacturer of infant formula, the company anticipates that their portfolio of HiMOs will be used in combination to produce infant formula products that are as compositionally representative of human breast milk as possible, taking into account their natural variation. Glycom recognizes that there are known gastrointestinal tolerance issues that can develop if consumed levels of indigestible carbohydrates, such as HiMOs, are too high in sensitive populations including infants. As discussed in detail previously, in Glycom's view, GRAS uses of individual HiMOs in infant formula should be representative of levels that have been reported for human milk samples obtained from lactating women across all lactational stages considering natural variation. Consequently, the maximum level of HiMOs used in combination (*i.e.*, an additive manner) in infant formula should not exceed mean quantities of total HMOs that have been measured in pooled samples of human breast milk (Kunz *et al*., 1999, 2000).

Glycom also recognized the possibility that the company's HiMOs may be used in combination with other non-digestible carbohydrate sources such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), which have GRAS status for use in infant formula. Although Glycom is not a manufacturer of infant formula, and is therefore not in a position to comment on the levels of resistant oligosaccharides such as

GOS or FOS that could be used with a HiMO, or even the likelihood that such combinations would be introduced to the market, Glycom notes that any new infant formula containing a new HiMO or new HiMO combination will be subject to the laws and implementing regulations governing infant formula under Section 412 of the *Federal Food, Drug, and Cosmetic Act* [21 USC §350(a)]. Specifically, under Section 412(d)(1) of the *Federal Food, Drug, and Cosmetic Act*, a manufacture of a new infant formula must notify the U.S. FDA at least 90 days before marketing their infant formula, and this must include, among other things, a description of any reformulation of the formula or change in processing of the infant formula. Accordingly, the manufacturer will need to provide the Agency with information supporting that a particular oligosaccharide combination (*e.g.*, use of LNnT with an indigestible oligosaccharide such as GOS in exempt infant formula) would be well tolerated as part of the Agency's 90-day notification procedure. Under 21 CFR §107.50, a manufacturer of a new exempt infant formula must notify the U.S. FDA at least 90 days before the first processing of the infant formula for commercial or charitable distribution, and include the infant formula label, a complete quantitative formulation, and a detailed description of targeted medical conditions (U.S. FDA, 2021).

6.7 General Recognition

Glycom has concluded that crystallized LNnT is GRAS for use in non-exempt term infant formula, as described in Section 1.3, on the basis of scientific procedures. This GRAS conclusion is based on general principles of risk-assessment of food allergens proposed by the Threshold Working Group of the U.S. Food and Drug Administration in 2008 (Buchanan *et al*., 2008). Clinical thresholds for milk allergenicity have now been validated by qualified scientific experts as a part of the Voluntary Incidental Trace Allergen Labeling (VITAL) program of the Allergen Bureau of Australia & New Zealand (ABA) using publicly available data and approaches that are generally accepted in the scientific community (Allen *et al*., 2014; Taylor *et al*., 2014; Remington *et al*., 2020). Using this risk-based approach Glycom has concluded that accepted milk allergy thresholds relative to the validated detection limits for milk protein in LNnT manufactured by Glycom are sufficient to protect the U.S. population of infants with CMPA. The safety of adding LNnT to infant formula is being evaluated by the U.S. FDA under the agency's petition procedure for exemption of 2'-FL from the allergen labeling requirements of FALCPA further supporting the general recognition standard (U.S. FDA, 2020a). The risk-based approach was further supported using published findings from a clinical safety study of LNnT in infants with CMPA conducted in accordance with AAP guidelines for the evaluation of hypoallergenic infant formula (AAP, 2000).

6.8 Conclusion

Based on the above data and information presented herein, Glycom has concluded that the intended uses of LNnT in exempt hypoallergenic infant formula, as described in Section 1.3, is GRAS based on scientific procedures. General recognition of Glycom's GRAS conclusion is supported by previously established and now widely accepted risk-based procedures for assessment of milk allergy thresholds ensuring the protection of 99% of the population of infants with CMPA.

LNnT therefore may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the *Code of Federal Regulations*.

Part 7. List of Supporting Data and Information

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Part	Section §	Section Title
101—Food labeling	101.12	Reference amounts customarily consumed per eating occasion
107—Infant formula	107.3	Definitions
	107.50	Terms and conditions
170—Food additives	170.3	Definitions
	170.30	Eligibility for classification as generally recognized as safe (GRAS)
	Subpart E (170.203 through 170.285)	Generally Recognized as Safe (GRAS) Notice

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From: Darch, Maryse
To: Morissette, Rachel
Cc: Roehrig, Christoph
Subject: RE: [EXTERNAL] RE: questions for GRN 001059
Date: Friday, September 30, 2022 8:10:42 AM
Attachments: image001.png
image002.png
image003.png
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image006.png
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GRN 001059 - Response to FDA Questions - 30Sept"22.pdf

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Dear Rachel,

Please find attached our responses to questions for GRN 001059. Please do not hesitate to contact us if any further clarification is necessary.

Kind regards,
Maryse

Maryse Darch | Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T 1 519 803 4002 | Maryse.darch@dsm.com | Stay connected:

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Friday, September 9, 2022 4:00 PM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: questions for GRN 001059

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Dear Maryse,

That's fine. We will expect to see responses on or before September 30. Have a good weekend.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

I GOT MY COVID-19 VACCINATION

From: Darch, Maryse <Maryse.Darch@dsm.com>
Sent: Friday, September 9, 2022 9:57 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: questions for GRN 001059

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For Internal Use Only

Dear Rachel,

Due to holidays and business travel, we would like to request an extension of the response to questions for GRN1059 to the end of September.

Kind regards,
Maryse

Maryse Darch | Regulatory & Scientific Affairs Manager | DSM Glycom A/S | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T 1 519 803 4002 | Maryse.darch@dsm.com | Stay connected:

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From: Darch, Maryse
Sent: Monday, August 29, 2022 2:54 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: questions for GRN 001059

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Dear Rachel,

Thank you for confirming the extension.

Kind regards,
Maryse

Maryse Darch| HMO Regulatory – Sr. Regulatory Affairs Specialist | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T +1 519 803 4002 | maryse.darch@dsm.com |
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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Monday, August 29, 2022 2:32 PM
To: Darch, Maryse <Maryse.Darch@dsm.com>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: RE: [EXTERNAL] RE: questions for GRN 001059

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Dear Maryse,

September 9 will be fine.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

I GOT MY COVID-19 VACCINATION

From: Darch, Maryse <Maryse.Darch@dsm.com>
Sent: Monday, August 29, 2022 4:17 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: [EXTERNAL] RE: questions for GRN 001059

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For Internal Use Only

Dear Rachel,

Apologies for the delay in response. Christoph was on holidays in August. We kindly request an extension to provide a response by September 9th. Please let us know if this time frame is suitable.

Kind regards,
Maryse

Maryse Darch| HMO Regulatory – Sr. Regulatory Affairs Specialist | Kogle Alle 4 | 2970 Hørsholm | Denmark | Reporting from ON, Canada | T +1 519 803 4002 | maryse.darch@dsm.com |
Glycom, the leading HMO expert is part of DSM

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Thursday, 11 August 2022 15:43
To: Roehrig, Christoph <Christoph.Roehrig@dsm.com>
Subject: questions for GRN 001059

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Dear Christoph,

Please see attached our questions for GRN 001059.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
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rachel.morissette@fda.hhs.gov

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Glycom A/S, Kogle Allé 4, 2970 Hørsholm, Denmark

30 September 2022

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist
Division of Food Ingredients
Center for Food Safety & Applied Nutrition
U.S. Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Re: GRAS Notice No. GRN 001059

Dear Dr. Morissette,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s letter dated 11 August 2022 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for the intended use of lacto-N-neotetraose (LNnT) in exempt hypoallergenic infant formula for term infants filed by the Agency under GRN 001059.

FDA.1. *We note that on the top of p.8 of the notice the crystallization step of the manufacturing process is described as using methanol; however, Table 2.2.2-1 shows water and acetic acid used in the crystallization. Please clarify the identity of the solvent used in crystallization step 13.*

We thank the FDA for catching this error. The only solvent used in crystallization step 13 of LNnT is methanol. Acetic acid is not used in the production of LNnT; rather, it was a "copy-and-paste" error from 2'-FL.

FDA.2. *On p.15 of the notice, Glycom states that infants with atopic gastrointestinal diseases/disorders are unlikely to consume greater quantities of infant formula than healthy term infants. Is Glycom aware of any data and information on the consumption rates of the intended type of formula or by the intended infant population? Healthy infants typically begin a transition to complementary foods after 6 months of age. Is the same rate of transition and consumption of formula expected for the intended infant population? Please provide justification for the above-mentioned statement.*

While Glycom is not aware of any data on the consumption rates of infant formula in term infants with atopic gastrointestinal diseases or disorders, the intended population is for infants with cow's milk protein allergy (CMPA) (see response to Question 6). Guidelines set forth by the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN), the World Allergy Organization's (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA), and the American Academy of Pediatrics (AAP) suggest management of care for formula-fed infants with moderate to severe CMPA include a reduction in cow's milk formula and substitution of extensively hydrolyzed formula for up to 6 months of age and beyond. However, solid foods without cow's milk protein can be

introduced around 6 months as long as the nutritional needs of the child are being met (Vandenplas *et al.,* 2007; Koletzko *et al.,* 2012; Barrera *et al.,* 2021). As term infants with CMPA have the same nutritional needs as healthy term infants, it is expected that the dietary intake estimates of LNnT for healthy infants would be representative of term infants with CMPA as well.

FDA.3. *Please state whether the batch analysis data provided is from three non-consecutive batches of LNnT.*

Glycom confirms that the batch analysis data provided in GRN 001059 is from four non-consecutive batches of LNnT (manufacturing batch numbers 18022002, 18154001, 18264001 and 18325001).

FDA.4. *Please provide additional description of the production microorganism, including the following: the process for producing the production strain from the parental strain, including any genetic modifications; deposition information for the production strain, if available; and whether the production strain is expected to produce any secondary metabolites.*

Glycom's LNnT for use in exempt hypoallergenic infant formula is produced by the original plasmid-based strain *Escherichia coli* K-12 DH1 MDO MP572 or by the alternative fully chromosomal strain *E. coli* K-12 DH1 MDO MP572b. Both strains have previously been notified to the U.S. FDA under GRAS filing number 659 (Glycom A/S, 2016), to which the Agency responded with no questions letters (U.S. FDA 2016, 2021).

The well-characterized host strain *E. coli* K-12 DH1 was optimized for general oligosaccharide expression features *via* seven genetic modification events related to the metabolism of various sugars, leading to the creation of the platform strain *E. coli* K-12 DH1 MDO. These genetic modifications have previously been described in GRN 659 and are incorporated by reference to Section II.B.1.2, pgs. 8-9 (Glycom A/S, 2016). Briefly, they include the deletion of the *lacZ, lacA, melA, wcaJ*, and *mdoH* genes, as well as deletion of the *nanKETA* gene cluster*,* and the insertion a Plac promoter.

The *E. coli* K-12 DH1 MDO strain is Glycom's platform strain for the manufacture of a number of HiMOs. Hence, both the *E. coli* K-12 DH1 MDO MP572 and MP572b production strains originate from this platform strain. Importantly, both production strains harbor two heterologous genes expressing enzymes necessary for LNnT biosynthesis, namely β-1,3-*N*-acetylglucosaminyltransferase (from *Neisseria meningitidis*) for the conversion of lactose into lacto-N-triose II, and β-1,4-galactosyltransferase (from *Helicobacter pylori*) for the conversion of lacto-*N*-triose II into LNnT.

Genetic modifications enabling the biosynthesis of LNnT and resulting in the original production strain *E. coli* K-12 DH1 MDO MP572 have previously been characterized in GRN 659 and are incorporated by reference to Section II.B.1.2 (pgs. 9-11) of the original GRAS notification (Glycom A/S, 2016). Briefly, the original MP572 production strain expresses β-1,4-galactosyltransferase from a multi-copy plasmid[1], while β-1,3-N-acetylglucosaminyltransferase is genomically expressed. The *nadC* gene was deleted from the genome of the platform strain to support an antibiotic resistance marker-free plasmid system.

[1] Glycom identified a typo in GRN 659. The **β-1,3-N-acetylglucosaminyltransferase** gene is introduced into the chromosomal DNA of the MDO platform strain; the **β-1,4- galactosyltransferase** gene is introduced to the plasmid carried by strain MP572.

Additionally, the *lacI* gene encoding the repressor of the Lac operon was deleted to enable gene expression from *Plac*-regulated genes.

Genetic modifications of MP572 resulting in the alternative production strain MP572b have also previously been described in GRN 659 and are incorporated by reference to Section 2.1 (pgs. 3-6) of the GRAS supplement (Glycom A/S, 2016). Briefly, the alternative and improved production strain MP572b does not employ a plasmid-based system. As such, both the β-1,3-N-acetylglucosaminyltransferase and β-1,4-galactosyltransferase encoding genes were introduced into the strain chromosomal DNA, and the native *nadC* gene was preserved. To improve fermentation performance, several gene deletions (*ldhA*, *focA-pflB,* and *iclR*) and gene insertions (*scrYA*, *scrBR*, *lacY*, *vag*, and *vgb*) were applied to the strain. Finally, the *hlyE* gene was deleted on a precautionary basis to eliminate any hypothetical risk of unintentional expression of the dormant hemolytic toxin cytolysin A.

The original and alternative LNnT production strains are both deposited at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, Germany. The original LNnT production strain, *E. coli* K-12 DH1 MDO MP572, is deposited under deposition number DSMZ 32272, and the alternative LNnT production strain, *E. coli* K-12 DH1 MDO MP572b, is deposited under deposition number DSMZ 33638.

Batch analysis results submitted in the GRAS notice have been updated to include results for at least three non-consecutive batches of LNnT produced by strain *E. coli* K-12 DH1 MDO MP572 or MP572b intended for use as an ingredient in exempt infant formula for term infants with CMPA (see revised Table 2.4-1 below).

Glycom interprets the term secondary metabolites as substances other than LNnT that may be produced by the production strain during fermentation. Glycom has established internal quality control measures to monitor for secondary metabolites, and only those that have been confirmed to occur at detectable levels in the final LNnT product are included in the specification for LNnT. Most notably, these consist of carbohydrate-type products resulting from the biosynthesis of LNnT by the production strain, including Lacto-*N*-triose II, *para*-Lacto-*N*-neohexaose, and LNnT fructose isomer. No significant detectable levels of other secondary metabolites potentially produced during fermentation (including biogenic amines and amino acids) have been identified in the finished LNnT ingredient.

Moreover, Glycom accounts for Cytolysin A (HlyE), a protein-type cytotoxin, and lipopolysaccharide (LPS, endotoxin), an immunogenic pyrogen. Although these are not metabolites resulting from the biosynthesis of LNnT, these compounds have been identified as bioactive secondary metabolites of *E. coli* K-12 by the European Food Safety Authority (EFSA). The concern for these secondary metabolites was highlighted in the publication: “Database on the taxonomical characterization and potential toxigenic capacities of microorganisms used for the industrial production of food enzymes and feed additives, which do not have a recommendation for Qualified Presumption of Safety” (EFSA, 2017). Out of the 474 bioactive secondary metabolites, 59 compounds were selected and examined for toxicology. Two of the 59 compounds are produced by *E. coli* K-12, namely Cytolysin A (HlyE), and lipopolysaccharide (LPS, endotoxin). Cytolysin A is a pore-forming toxin known to cause lysis of mammalian cells. Under laboratory conditions, the Cytolysin A encoding gene, *hlyE*, appears to be silent (del Castillo *et al.,* 1997). Following publication of the EFSA (2017) external scientific report, Glycom has deleted the *hlyE* gene in the alternative production strain *E. coli* K-12 DH1 MDO MP572b as a precautionary measure to eliminate the risk of the activation of Cytolysin A production.

Lipopolysaccharides are a major component of the outer membrane of gram-negative bacteria. Consequently, Glycom allows no more than 10 E.U./mg residual endotoxin in the final LNnT product.

Table 2.4-1 Batch Analysis of LNnT Produced by Fermentation [REVISED]

Specification Parameter	Specification Limit	LNnT from MP572				LNnT from MP572b		
		18022002	18154001	18264001	18325001	21153002[a]	21167001	21337001
Appearance	Powder or agglomerates	Agglomerates	Powder with agglomerates	Powder with agglomerates	Powder with agglomerates	Powder with agglomerates	Powder with agglomerates	Powder with agglomerates
Color	White to off white	White	White	White	White	White	White	White
Identification	RT of standard ± 3%	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Assay (water free) Human-identical Milk Saccharides[b]	≥ 95.0	98.1	98.7	100.2	101.2	101.2	101.3	100.0
Assay (water free) Lacto-*N*-neotetraose (w/w %)	≥ 92.0	97.4	98.1	99.6	100.6	99.9	98.8	98.1
D-Lactose (w/w %)	≤ 1.0	0.30	0.09	0.18	0.16	0.06	0.05	0.05
Lacto-*N*-triose II (w/w %)	≤ 3.0	0.31	0.47	0.32	0.35	1.94	2.28	1.53
para-Lacto-*N*-neohexaose (w/w %)	≤ 3.0	0.10	< 0.03[c]	< 0.03[c]	< 0.03[c]	< 0.03[c]	< 0.03[c]	0.03
LNnT fructose isomer (w/w %)	≤ 1.0	0.14	0.05	0.03	0.14	< 0.03[c]	0.04	0.11
pH (20°C, 5 % solution)	4.0 to 7.0	4.9	5.0	5.0	4.6	5.0	5.0	5.0
Water (w/w %)	≤ 9.0	3.98	6.39	7.77	6.40	5.9	6.5	7.2
Ash, sulfated (w/w %)	≤ 1.5	< 0.10[c]	0.20	< 0.1[c]	0.32	<0.05[c]	<0.05[c]	<0.05[c]
Methanol (mg/kg)	≤ 100	< 10	< 20	42	< 20	<20	<20	<20
Residual proteins by Bradford assay (w/w %)	≤ 0.002	< 0.0017[c]	< 0.0017[c]	< 0.0017[c]	< 0.0017[c]	< 0.0017[c]	< 0.0017[c]	< 0.0017[c]
β-Lactoglobulin (mg/kg)	≤ 0.05	< 0.01[c]	< 0.01[c]	< 0.01[c]	< 0.01[c]	< 0.01[c]	< 0.01[c]	< 0.01[c]
Casein (mg/kg)	≤ 0.5	< 0.2[c]	< 0.2[c]	< 0.2[c]	< 0.2[c]	< 0.2[c]	< 0.2[c]	< 0.2[c]
Lead (mg/kg)	≤ 0.1	< 0.05[c]	< 0.05[c]	< 0.05[c]	0.002	<0.005[c]	<0.005[c]	<0.005[c]
Aerobic mesophilic total plate count (CFU/g)	≤ 500	< 10[c]	< 10[c]	< 10[c]	< 10[c]	< 10[c]	< 10[c]	< 10[c]
Yeasts (CFU/g)	≤ 10	< 10[c]	< 10[c]	< 10[c]	< 10[c]	< 10[c]	< 10[c]	< 10[c]
Molds (CFU/g)	≤ 10	< 10[c]	< 10[c]	< 10[c]	< 10[c]	< 10[c]	< 10[c]	< 10[c]

Enterobacteriaceae (in 10 g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Salmonella (in 25 g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Cronobacter (Enterobacter) sakazakii (in 10 g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Listeria monocytogenes (in 25 g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Bacillus cereus (CFU/g)	≤ 50	< 10[c]	< 10[c]	< 10[c]	< 10[c]	< 10[c]	< 10[c]	< 10[c]
Residual endotoxins (E.U./mg)	≤ 10	0.00020[c]	< 0.00025[c]	< 0.00025[c]	< 0.0003[c]	< 0.00025[c]	< 0.00025[c]	< 0.00025[c]

CFU = colony-forming units; E.U. = endotoxin units; HiMS = human-identical milk saccharides; LNnT = lacto-*N*-neotetraose; LOQ = Limit of Quantitation; RT = retention time.

[a] Batch analysis results have previously been submitted in the supplement to GRN 659 for this batch of LNnT. Nevertheless, this batch also meets the stricter specifications for LNnT intended for use in exempt hypoallergenic infant formula.

[b] Human-identical milk oligosaccharides is defined as the sum of LNnT, lactose, lacto-*N*-triose II, and para-lacto-*N*-hexaose.

[c] Result is below the LOQ: 0.03 w/w % for *para*-Lacto-*N*-neohexaose or LNnT fructose isomer; 0.10 or 0.05 w/w % for ash depending on the testing laboratory; 10 or 20 mg/kg for methanol depending on the testing laboratory; 0.0017 w/w % for residual proteins; 0.01 mg/kg for β-Lactoglobulin; 0.2 mg/kg for casein; 0.05, 0.005, or 0.001 mg/kg for lead depending on the testing laboratory; 10 CFU/g for total plate count, yeasts, molds, and *Bacillus cereus*; and 0.050 E.U./mL for endotoxins (converted to approximately 0.0002 to 0.0003 E.U./mg depending on the sample weight).

FDA.5. *Please confirm that the production microorganism is non-pathogenic and non-toxigenic.*

Glycom confirms that the production microorganism is non-pathogenic and non-toxigenic. *E. coli* K-12-derived strains cannot colonize the human gastrointestinal system, and do not produce protein-type toxins (U.S. EPA, 1997). All introduced genes are well characterized with respect to their function, do not have homology to known protein toxins, and as enzymes involved in LNnT biosynthesis, are not reasonably expected to introduce toxicogenic/pathogenic attributes to the host.

FDA.6. *On pg. 5 of the notice, Glycom states:*

"Addition of LNnT to amino acid-based formula such as Alfamino (Nestle) would represent products targeted to infants not responding to EHF or for infants with moderate to severe CMPA, including those with anaphylaxis, food protein-induced enterocolitis syndrome (FPIES), multiple food protein allergy of infancy (non-IgE-mediated), or eosinophilic esophagitis."

In Table 1.3-1, Glycom further states that the target population includes infants suffering from fat malabsorption and short-bowel syndrome.

We find that the narrative in GRN 001059 does not provide sufficient publicly available safety data and information to support the intended use of LNnT in exempt hypoallergenic infant formula in infant populations other than those with CMPA. Thus, we recommend that Glycom narrows the intended targeted infant population in GRN 001059 to only term infants with CMPA. If Glycom has any questions about this recommendation, please request a teleconference to discuss further.

Glycom agrees to narrow the targeted infant population specified in GRN 1059 to only term infants with CMPA.

FDA.7. *Glycom discusses a clinical study conducted in infants and young children with cow milk protein allergy, which assessed the hypoallergenicity and safety of an extensively hydrolyzed formula supplemented with 1.0 g/L 2'-FL and 0.5 g/L LNnT against a control (Nowak-Wegrzyn et al. (2019). This study used a lower level of LNnT (0.5 g/L) compared to the proposed 0.6 g/L and was used in combination with 2'-FL. Please provide a brief discussion on why LNnT alone at the proposed use level would not have an impact on the health of infants requiring hypoallergenic infant formulas.*

The proposed use level of LNnT in the GRAS notice of 0.6 g/L was chosen based on the range of observed concentrations of LNnT naturally occuring in human milk that have an established safe history of consumption by breastfed infants. As described in Section IV.B.I of GRN 547 (pg. 23), the level of LNnT present in mature human breast milk typically ranges between 0.1 and 0.6 g/L, but may potentially be up to approximately 2.5 g/L (Glycom A/S, 2014).

The proposed use level of 0.6 g/L for the use of LNnT in exempt term infant formula from the current GRAS notice is the same as that notified as GRAS for Glycom's LNnT ingredient manufactured using the same production strain intended for use in non-exempt term infant formula (GRN 659). In the current GRAS notice, Glycom has demonstrated that the production process for LNnT as described in GRN 659 does not result in the transfer of allergenic milk protein to the final LNnT ingredient. Furthermore,

Glycom has extended quality control criteria for LNnT intended for use in exempt term infant formula for infants with CMPA.

Infants with CMPA have an allergy against cow's milk protein. Numerous published guidelines for the management of CMPA in breastfed infants recommend that breastfeeding should continue while mothers avoid the consumption of cow's milk and milk products from cow's milk in their own diet (Vandenplas *et al*., 2007; Koletzko *et al*., 2012; Caffarelli *et al*., 2010). Thus, breastfed infants with CMPA would continue to be exposed to carbohydrates, such as HMOs (including LNnT) produced in the mammary gland, from human milk. On a body weight basis, the intake of LNnT from the proposed conditions of use in exempt formula and from the background diet at the mean (116 to 128 mg/kg body weight/day, respectively – see Table 3.2.1-2 of the GRAS notice) and high-level (199 to 237 mg/kg body weight/day, respectively – see Table 3.2.1-2 of the GRAS notice) is within the intake of LNnT from mature human milk (up to 385 mg/kg body weight/day – see Section IV.B.1 of GRN 547, pg. 23).

FDA.8. *Glycom states that the updated literature search was performed though September 2021. Please confirm that no new information that may appear counter to your GRAS conclusion has been published since September 2021.*

Two new interventional infant clinical trials published since September 2021 in which endpoints related to the safety of LNnT were identified (Gold *et al*., 2022; Vandenplas *et al*., 2022). The study population in both studies consisted of infants with CMPA. Infants were administered either an amino acid-based infant formula (Gold *et al*., 2022) or an extensively hydrolyzed infant formula (Vandenplas *et al*., 2022) supplemented with 1.0 g/L of 2'-FL and 0.5 g/L of LNnT. Details of the newly identified studies are summarized below, and Table 6.5.2.6-1 of the GRAS notice has also been updated below to include these two new studies. Overall, the two recent clinical studies examining the effect of the administration of LNnT to infants with CMPA have not identified any safety concerns.

Furthermore, the European Food Safety Authority (EFSA) Panel on Nutrition, Novel Foods and Food Allergens (NDA) recently evaluated the safety of the extension of use of LNnT as a novel food in food supplements for infants at a maximum use level of 0.6 g/day (EFSA, 2022). The intake of LNnT per kg body weight from the proposed use in dietary supplements for a 5-kg infant (estimated at 120 mg/kg body weight/day) was determined to be lower than the highest estimated mean intake of naturally occurring LNnT from human milk by breastfed infants (estimated at 134 mg/kg body weight/day[2]). The Panel concluded that the use of LNnT in food supplements for infants is safe under the proposed conditions of use.

Therefore, Glycom's GRAS conclusion remains the same.

A) Multicenter, Double-Blind, Randomized, Controlled, Parallel-Designed Growth, Tolerability, Safety, and Infection Risk of an Extensively Hydrolyzed Formula Supplemented with 2'-FL and LNnT for Infants with Cow's Milk Protein Allergy (Vandenplas et al., 2022)

The ability of an extensively hydrolyzed formula (EHF) supplemented with 2'-FL and LNnT to support the growth, tolerability, safety, and infection risk in infants with cow's milk protein allergy (CMPA) was

[2] Considering an average daily intake of human milk intake of 800 mL and the maximum mean concentration of LNnT in human milk reported by in the systematic review by Thurl *et al*. (2017) of 1.12 g/L.

evaluated in a controlled, double-blind, randomized, multicenter study[3] (Vandenplas *et al*., 2022; Clinical Trial Registry NCT03085134). Full-term, formula-fed infants from 0 to 6 months of age with physician-diagnosed CMPA were enrolled in the study and randomized to receive either the control formula or the test formula for a minimum duration of 4 months and up to 12 months of age on a voluntary basis. The test formula was a whey-based EHF supplemented with 1.0 g/L of 2'-FL and 0.5 g/L of LNnT that was previously demonstrated to be hypoallergenic (Nowak-Wegrzyn *et al*., 2019), while the control formula was a commercially available whey-based EHF without HMO. The macronutrient and micronutrient profile of the study formulas were almost identical, with the exception of the test formula having a reduced protein/peptide content compared to the control formula (2.20 *vs.* 2.47 g/100 kcal, respectively).

The primary objective of the study was to demonstrate non-inferiority for weight gain in infants receiving the test formula compared to those receiving the control formula throughout 4 months of intervention. Secondary outcomes included the comparison of other growth parameters (body weight, body length, head circumference) to WHO growth standards (weight-for-age, length-for-age, head circumference-for-age, or BMI-for-age Z scores), symptom resolution (crying, regurgitation, stools, respiratory symptoms, and skin signs), the incidence of infections (respiratory, gastrointestinal, and other), medication use (antibiotics and antipyretics), and adverse events from enrolment through to 12 months of age. All analyses were conducted for the full-analysis set (FAS)[4] and the per-protocol (PP)[5] cohorts, with the exception of adverse events (including adverse events of interest) which were analyzed in the FAS only.

Overall, 200 infants were screened against study eligibility criteria and 194 infants were randomized in the study (97 infants per formula group). The majority of infants started taking the allocated formula (FAS cohort: n = 94 in the test formula group; n = 96 in the control formula group), while 151 infants completed the 4-month intervention without any major protocol deviations (PP cohort: n = 73 in the test formula group; n = 64 in the control formula group). Mean formula intake volumes were similar between groups during the first 2 months of intervention, significantly increasing in the test formula group compared to the control formula group from 3 to 5 months of intervention (10 to 13% higher in the FAS cohort), before reaching similar intake volumes again by the end-of-intervention.

Following 4 months intervention, daily weight gain for the test formula group was noninferior to the control formula group in both the FAS ($p< 0.0001$) and PP ($p<0.005$) cohorts. Furthermore, there were no significant group differences in any other anthropometric measures evaluated up to 12 months of age. Similarly, there were no significant group differences in the resolution of CMPA symptoms or adverse events. The number of adverse events that were considered to be "related" or "probably related" to the study product were low and similar between the study formula groups, plus none of the serious adverse events were considered to be "related" to the study formulas and there were no reports of anaphylaxis.

Although infants receiving the test formula supplemented with 2'-FL and LNnT had numerically lower rates of respiratory and gastrointestinal infections compared to infants receiving the control formula from enrollment to 12 months of age, these group differences were not statically significant in the FAS or PP cohorts. Still, on a monthly basis, there was a significant reduction in the frequency of upper respiratory tract infections in the test formula group. Furthermore, compared to infants receiving the

[3] Conducted in 41 clinical sites in Europe (Poland, Italy, United Kingdom, Spain, Hungary, and Belgium) and 3 sites in Singapore.
[4] All randomized infants who also commenced the allocated treatment formula.
[5] All subjects from the FAS without any major protocol deviations.

control formula, infants in the PP cohort receiving the test formula had significantly lower odds of otitis media during the first 4 months of intervention (p=0.045; exploratory analysis) and from enrollment to 12 months (p<0.05). Although 4 infants from the test formula group were diagnosed with a urinary tract infection while none were reported in the control formula group, these infections were considered by the study authors to be unrelated to the test formula or presence of HMOs. There was no significant difference between groups in antibiotic or antipyretic use from enrollment to 12 months of age; however, infants receiving the test formula from 4 months of intervention (*i.e.,* completed 4 months follow up) to 12 months of age had significantly lower antipyretic use compared to control.

Taken together, the study authors concluded that both formulas were safe and well-tolerated, and that the results suggest a protective effect of the 2'-FL and LNnT-supplemented EHF against respiratory and ear infections in the first year of life of infants with CMPA.

B) Open-Label, Non-Randomized, Multicenter Growth, Tolerability, Safety and Gut Microbiome Study of an Amino Acid-Based Formula Supplemented with 2'-FL and LNnT for Infants with Cow's Milk Protein Allergy (Gold et al., 2022)

The effect of an amino acid-based formula supplemented with 2'-FL and LNnT on growth, tolerability, safety, and the gut microbiome of infants with CMPA was evaluated in an open-label, non-randomized, multicenter study[6] (Gold *et al*., 2022; Clinical Trial Registry NCT03661736). Full-term, formula-fed infants from 1 to 8 months of age with physician-diagnosed moderate-to-severe CMPA were enrolled in the study for a minimum duration of 4 months and up to 12 months of age on a voluntary basis. The study formula was a lactose-free, nutritionally complete, amino acid-based formula for the management of infants with CMPA supplemented with 1.0 g/L of 2'-FL and 0.5 g/L of LNnT, suitable as a sole source of nutrition until 6 months of age.

The primary objective of the clinical study was to assess the weight gain of infants with CMPA fed the amino acid-based formula supplemented with the two HiMOs from enrolment to 4-month follow-up and compare to WHO child growth standard. Secondary outcomes included the assessment of other growth parameters (body length, head circumference, and BMI) to WHO growth standards over the 4-month period, anthropometric parameters to 12-months of age, as well as symptom resolution (crying, fussing, spitting up, vomiting, feeding problems, skin symptoms, respiratory symptoms), stool characteristics and frequency, changes in the composition of the gut microbiome and SCFA production, and adverse events from enrolment through to 12 months of age.

Overall, 34 infants were screened against study eligibility criteria, 32 infants were enrolled in the study (mean age of 18.6 weeks, ranging from 4 to 37 weeks), and 29 infants completed the trial to the primary endpoint (from enrolment to 4-mont follow-up). The mean duration of formula intake was 122.2 ± 6.14 days from enrolment to 4-month follow-up and 110.7 ± 47.01 from 4-month follow-up to 12 months of age, and formula intake progressively decreased with infant age.

Mean weight gain from enrolment to 4-month follow-up was reported to be 18.0 ± 6.13 g per day of formula intake (range 7.8–29.2 g/day). Similarly, the mean weight-for-age z-score (WAZ) increased from -0.31 at baseline to +0.28 at 4-month follow-up and tracked closely to WHO child growth standards. Z-scores for other growth parameters also increased from enrolment. Symptoms improved significantly from enrolment to 1-month follow-up, as demonstrated by a significant decrease in the proportion of

[6] Conducted in 6 clinical sites in Australia.

infants with frequent or persistent crying, fussing, regurgitation and vomiting, a significant reduction in the prevalence of feeding difficulties, and a nearly significant reduction in the prevalence of frequent or persistent skin problems Furthermore, persistent respiratory symptoms were resolved following intervention in one of two infants presenting with these symptoms at baseline. There was a trend towards more formed and less frequent stools with increasing age. The majority of infants experienced adverse events affecting the gastrointestinal system or due to infection. Overall, 2 infants experienced 4 adverse events determined to be 'related' or 'probably related' to the study formula, resulting in both infants discontinuing the intervention (one infant had milk gastroesophageal reflux, while the other infant had loose stools, flatulence, and a mild decrease in feeding). A total of 6 infants experienced 8 serious adverse events, all of which were determined to be unrelated to the study formula.

A total of 105 stool samples from 29 infants providing baseline and at least one other sample were included in microbiological analyses. Alpha diversity (assessed by Faith's phylogenetic diversity) increased significantly with age and from enrolment to 4-months follow-up and to 12 months of age. Similarly, beta-diversity (evaluated using weighted UniFrac distances) was significantly different when comparing by age or study visit. There was significant enrichment of Actinobacteria and bifidobacteria from enrolment to 1- and 4-months follow-up, whereas Proteobacteria, *Enterobacteriaceae*, *Escherichia* spp., *E. coli* decreased significantly from enrolment to 1- and 4-months follow-up and to 12 months of age. The abundance of several other genera increased (*e.g.*, *Bacteroides* spp.) and decreased (*e.g.*, *Enterococcus* spp.) from enrollment to 12 months of age. The relative abundance of the 11 metagenomic species annotated to the genus *Bifidobacterium* (as a set) increased significantly from enrollment to 4-months follow-up and to 12 months of age. Similar findings were obtained from taxon set enrichment analyses. Fecal concentrations of the SCFAs acetate, propionate and butyrate increased significantly from enrolment to 12 months of age.

Taken together, the study authors concluded infants with CMPA receiving the study formula supplemented with the 2'-FL and LNnT achieved adequate growth, and that the formula was well-tolerated and had an excellent safety profile. Furthermore, the study authors concluded that supplementation with 2'-FL and LNnT was associated with a significant enrichment of bifidobacteria and partial correction of gut microbial dysbiosis in infants with CMPA.

Table 6.5.2.6-1 Summary of Interventional Infant Clinical Studies Conducted on LNnT[a] [UPDATED]

Study Population	Duration of Intervention	Study Groups and Test Articles	References
86 preterm infants (27 to 33 weeks gestation, birth weight < 1,700 g) 43 per group Average 6 days of age at intervention initiation	Enrolment to discharge from neonatal unit	***Control Supplement:*** Glucose (0.140 g/kg bw/day) ***Test Supplement:*** 2'-FL and **LNnT** in 10:1 ratio (0.374 g/kg bw/day)	Hascoët *et al.*, 2021 [abstract]; Hascoët *et al.* (2022)[b] ***Clinical trial number*** NCT03607942
159 healthy full-term infants 45 to 66 per group 7 days to 2 months old at enrolment	8 weeks	***Exclusively Formula Fed Group:*** *Ad libitum* formula containing 1.0 g 2'-FL/L and 0.5 g **LNnT**/L ***Mixed Formula Fed and Breastfed Group***: *Ad libitum* formula containing 1.0 g 2'-FL/L and 0.5 g **LNnT**/L ***Exclusively Breastfed Group (Reference Group):*** Breastfed enrolled at the same time as formula fed infants	Román Riechmann *et al.* (2020) ***Clinical trial number*** NCT04055363
32 term **infants** with CMPA 1-8 months of age Single-arm design	4 moths, followed by voluntary continuation up to 12 months of age	***Test Formula:*** Amino acid-based formula supplemented with 1.0 g/L of 2'-FL and 0.5 g/L of **LNnT**	Gold *et al*., 2022 ***Clinical trial number*** NCT03661736
194 term **infants** with CMPA 94-96 per group (Full Analysis Set) 0 to 6 months of age at enrolment	4 months, followed by voluntary continuation up to 12 months of age	***Control Formula:*** Commercially available EHF without HiMO ***Test Formula:*** Whey-based EHF supplemented with 1.0 g/L of 2'-FL and 0.5 g/L of **LNnT**[c]	Vandenplas *et al*., 2022 ***Clinical trial number*** NCT03085134

2'-FL = 2'-fucosyllactose; bw = body weight; CMPA = cow's milk protein allergy; EHF = extensively hydrolysed formula; HiMO = human-identical milk oligosaccharides; LNnT = lacto-*N*-neotetraose.

[a] Studies shaded in grey were previously submitted in the GRAS notice. New studies published since September 2021 are presented in rows with green font.

[b] The Hascoët study conducted in preterm infants was recently published. Study details remain the same.

[c] The test formula was previously demonstrated to be hypoallergenic (Nowak-Wegrzyn *et al*., 2019).

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We hope this information adequately addresses the Agency's questions on GRN 001059, and if there is any additional information or further clarification that is required, Glycom will be happy to provide such information upon request.

Sincerely,

Maryse.Darch Digitally signed by Maryse.Darch
DN: cn=Maryse.Darch, email=Maryse.Darch@dsm.com
Date: 2022.09.30 14:07:52 +02'00'

Maryse Darch
Regulatory & Scientific Affairs Manager
Glycom A/S

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