N-glycan profiling of bovine follicular fluid at key dominant follicle developmental stages

T Tharmalingam-Jaikaran^{1,2,†}, S W Walsh^{3,‡}, P A McGettigan³, O Potter¹, W B Struwe¹, A C O Evans³, P M Rudd^{1,*} and S D Carrington^{3,*}

¹Dublin-Oxford Glycobiology Laboratory, NIBRT – The National Institute for Bioprocessing Research and Training, Fosters Avenue, Mount Merrion, Blackrock, Co. Dublin, Ireland, ²School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland and ³School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland

Correspondence should be addressed to S D Carrington; Email: stephen.carrington@ucd.ie

*(P M Rudd and S D Carrington contributed equally to this work and share senior authorship)

[†]T Tharmalingam-Jaikaran is now at Amgen, Cell Sciences and Technology, One Amgen Centre Drive, Thousand Oaks, California 91320, USA

[‡]S W Walsh is now at Department of Chemical and Life Sciences, Waterford Institute of Technology, Waterford, Ireland

Abstract

Follicular fluid (FF), an important microenvironment for the development of oocytes, contains many proteins that are glycosylated with N-linked glycans. This study aimed i) to present an initial analysis of the N-linked glycan profile of bovine FF using hydrophilic interaction liquid chromatography, anion exchange chromatography, high performance liquid chromatography (HPLC)-based separations and subsequent liquid chromatography–mass spectrometry/mass spectrometry analysis; ii) to determine differences in the N-glycan profile between FF from dominant and subordinate follicles from dairy heifers and lactating dairy cows and iii) to identify alterations in the N-glycan profile of FF during preovulatory follicle development using newly selected, differentiated (preovulatory) and luteinised dominant follicles from dairy heifers and lactating cows. We found that the majority of glycans on bovine FF are based on biantennary hypersialylated structures, where the glycans are sialylated on both the galactose and N-acetylglucosamine terminal sugars. A comparison of FF N-glycans from cows and heifers indicated higher levels of nonsialylated glycans with a lower proportion of sialylated glycans in cows than in heifers. Overall, as the follicle develops from Selection, Differentiation and Luteinisation in both cows and heifers, there is an overall decrease in sialylated structures on FF N-glycans.

Reproduction (2014) **148** 569–580

Introduction

Low reproductive efficiency within the Holstein–Friesian breed is a significant issue faced by the major dairy producing countries worldwide. A marked decrease in reproductive performance, coincident with substantial increases in milk production, has been detected over the past five decades (López-Gatius 2003, Walsh *et al.* 2011). It is widely accepted that this has been caused by an aggregate of genetic, environmental and managerial factors which have influenced the metabolic and physiological status of dairy cows at the cellular, tissue and whole-animal level (Evans & Walsh 2011, Walsh *et al.* 2011). There is evidence to suggest that the mechanism by which some of these factors impair fertility is attributable to their effects on the intrafollicular environment (Leroy *et al.* 2005, 2011, Bender *et al.* 2010). Follicular fluid (FF) is the product of both the transfer of blood plasma constituents that cross the blood–follicular barrier and

of the secretory activity by the granulosa and thecal cells (Rodgers & Irving-Rodgers 2010). It provides a micro-environment in which the cumulus–oocyte complex can develop and mature. Negative energy balance (NEB) occurring in the early *postpartum* period in high-producing lactating cows can be detrimental, since it alters circulating metabolite concentrations with resulting changes in FF (Bender *et al.* 2010, Leroy *et al.* 2011). For example, increased circulating non-esterified fatty acids (NEFA), associated with NEB and reflected in the FF of the preovulatory ovarian follicle (Leroy *et al.* 2005), can directly affect granulosa cell function and steroidogenesis (Jorritsma *et al.* 2004, Vanholder *et al.* 2005) as well as oocyte competence, early embryo quality, viability and metabolism (Jorritsma *et al.* 2004, Leroy *et al.* 2005, Aardema *et al.* 2011, Van Hoeck *et al.* 2011).

Bovine follicular growth results from an intricately regulated sequential pattern of follicle development (Ginther *et al.* 2001, Mihm *et al.* 2008). It occurs in

waves with two or three waves per oestrous cycle (Rajakoski 1960, Savio *et al.* 1988, Ginther *et al.* 1989). The initiation of follicle waves involves the emergence and growth of a cohort of follicles, wherein one follicle is selected and becomes dominant. The remaining subordinate follicles in the cohort stop growing and undergo atresia. Differentiation of the newly selected dominant follicle to preovulatory size is characterised by enhanced growth and oestradiol synthesis. This ultimately initiates the preovulatory LH surge leading to the final maturation of the oocyte and cumulus expansion. Initial luteinisation of the follicle cells then culminates with ovulation (Eppig 2001). Following ovulation, the corpus luteum is formed and secretes progesterone. If fertilisation of the oocyte does not occur, progesterone declines following luteolysis and this allows the emergence of a new cohort of follicles.

Many glycosylated hormones and proteins isolated from human FF are reported to be good biochemical predictors of oocyte quality (Revelli *et al.* 2009). High concentrations of follicle-stimulating hormone (Suchanek *et al.* 1988), human chorionic gonadotrophin (Ellsworth *et al.* 1984) and luteinising hormone (Barnes *et al.* 1986) within the follicle are linked with oocyte maturation. High concentrations of bone-morphogenetic protein-15 (BMP-15) have also been positively correlated with oestradiol concentrations, and indicate a more advanced stage of oocyte maturation (Wu *et al.* 2007). Other glycosylated proteins that are positively correlated with follicle maturation and oocyte quality include endothelin-2 (Sudik *et al.* 1996) and lactoferrin (Yanaihara *et al.* 2007). A change in the glycosylation of specific proteins can be attributed to a change in the expression, intracellular localisation, stability of glycosyltransferases, the availability and transport of activated sugar nucleotides and/or the intracellular trafficking of glycoprotein acceptors (Gornik *et al.* 2007). These changes may have major implications in the functioning of proteins and ultimately the functioning of the other biomolecules with which they interact. Alterations in glycosylation in the reproductive system have been linked with infertility (Miller *et al.* 2010, Miller 2011).

Glycomics is the study of glycans (oligosaccharides) and glycoproteins. Glycosylation is a major post translational modification and involves the addition of various sugar structures to proteins within the endoplasmic reticulum and Golgi apparatus. The two most common forms of glycans that can be added to proteins are *N*- and mucin-type *O*-linked glycans, but other forms of *O*-linked glycosylation are also biologically important (Zachara & Hart 2006, Okajima *et al.* 2008). Glycans are known to mediate cell-to-cell communication, signal transduction, embryogenesis and are also involved in immune function and disease processes such as cancer, autoimmunity and neurological dysfunction (Tharmalingam *et al.* 2010, Adamczyk *et al.* 2012, Telford *et al.* 2012). In mammals, over 50% of membrane-associated and secreted proteins are *N*-glycosylated (Kornfeld &

Kornfeld 1985, Apweiler *et al.* 1999, Spiro 2002, Mariño *et al.* 2010). *N*-linked glycans are attached at their reducing terminus through *N*-acetylglucosamine (GlcNAc) to the amide group of asparagine residues within Asn-X-Ser/Thr motifs. *N*-linked glycans are characterised by their 'core' which is comprised of a chitobiose (a dimer of β -1,4-linked GlcNAc units) with a mannose linked to the GlcNAc in a β -1,4-linkage and two mannoses attached to the β 1,4 mannose by α 1,3 and α 1,6 linkages (for a comprehensive review refer to Mariño *et al.* (2010)). Protein glycosylation may be regulated by steroid hormones, in particular oestrogen, and thus is altered during the normal human menstrual cycle and during pregnancy (Carson *et al.* 1998).

Given that the proteins present in FF can be glycosylated, and that changes in their glycosylation are likely to affect their function, oocyte competence and ultimately fertility, we hypothesise that differences between the *N*-glycome of FF from heifers and lactating dairy cows are detectable. Therefore, the objectives of the current study were to i) characterise the *N*-glycan profile of bovine FF using hydrophilic interaction liquid chromatography (HILIC) and anion exchange chromatography (AEC)-based HPLC with orthogonal mass spectrometry; ii) determine baseline differences in the *N*-glycan profile between FF from dominant and subordinate follicles from heifers and lactating cows; and iii) compare temporal changes of the *N*-glycan profile of FF during preovulatory follicle development between heifers and lactating cows.

Materials and methods

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the cruelty to animals act (Ireland 1897) and European Community Directive 86/609/EC and the University's Animal Research Ethics Committee.

Animals and tissue preparation

A description of the experimental design, animal treatments and tissue recovery has been presented previously (Bender *et al.* 2010, Walsh *et al.* 2012a). Briefly, the oestrus cycles of Holstein-Friesian lactating cows ($n=16$; lactation number 3.63 ± 0.48 , days in milk *postpartum* 81 ± 2.4 ; mean $\pm$ S.E.M.) and nulliparous heifers ($n=17$; 1.6 years ± 0.06) were synchronised to manage the development of the second-dominant follicle. Heifers and lactating cows were randomly assigned to one of three stages of follicular development: i) newly selected dominant follicle in the luteal phase (Selection, cows $n=5$; heifers $n=6$); ii) follicular phase before the LH surge (Differentiation, cows $n=7$; heifers $n=5$); and iii) pre-ovulatory phase after the LH surge (Luteinisation, cows $n=4$; heifers $n=6$).

Subsequent to the detection of oestrus, the animals were treated using a controlled intravaginal drug-releasing device

(CIDR; containing 1.38 g of progesterone, CIDR, Pfizer Pharma GmbH) on Day 8 (Day 0=observed oestrus), and the development of the second-dominant follicle in the cycle was monitored daily by transrectal ultrasonography (Aloka SSD-900 linear array trans-rectal probe, 7.5-MHz transducer, BCF Ireland Ltd, Fermoy, Ireland) until the day of slaughter for the recovery of ovaries. The animals randomly assigned to the Selection group had their used CIDRs removed on the morning of Day 14 and were slaughtered within 6 h of CIDR removal. On the last ultrasound scan before slaughter, all animals had a newly selected dominant follicle, subordinate follicle and a corpus luteum present. The animals randomly assigned to the Differentiation and Luteinisation groups received a luteolytic dose of prostaglandin $F_{2\alpha}$ analogue (PG, Estrumate, Chanelle, Loughrea, Co. Galway, Ireland, equivalent to 0.5 mg cloprostenol) on Day 13, and the CIDR was removed on the morning of Day 15 to accurately synchronise the follicular phase differentiation of selected dominant follicles. The animals assigned to the Differentiation group were then slaughtered on Day 16 between 24 and 30 h after CIDR removal and before any animals displayed oestrus indicating the onset of the gonadotrophin surge. The animals assigned to the Luteinisation group received an i.m. injection of 5 ml GNRH (Receptal, Intervet/Schering-Plough Animal Health, Bray, Co. Wicklow, Ireland) 30 h post CIDR removal, which induces a synchronised gonadotrophin surge, and were slaughtered

18–22 h after the administration of GNRH (Day 17) timed to occur before ovulation but after the onset of luteinisation (Komar *et al.* 2001).

Following slaughter at a local abattoir, pairs of ovaries from each individual animal were recovered coinciding with the timings of follicular development described above and transported to the laboratory in ice-cold PBS (Sigma). Identification of dominant and subordinate follicles was aided by ovarian diagrams as recorded between Day 8 and the day of slaughter of each animal. The external diameter of each follicle was measured using callipers and FF was aspirated from both the dominant and the largest subordinate follicles, frozen in liquid nitrogen and stored at -80°C . Progesterone and oestradiol assays were carried out on the FF to ensure the correct classification of the subordinate and dominant follicle for each animal (data not shown). The dominant follicle was identified as being larger and containing more oestradiol than other follicles (Sunderland *et al.* 1994). The largest atretic subordinate follicle, ranked based on oestradiol concentration and diameter (Subordinate, cows $n=5$; heifers $n=5$), was used from animals in the selection group.

Release and labelling of N-glycans from whole FF

The workflow for the structural determination of the FF glycans is shown in Fig. 1. In summary, FF samples (5 μl) were reduced

Figure 1 The overall strategy for the analysis of N-glycans isolated from follicular fluid. The N-glycans were isolated from 5 μl of follicular fluid. The steps to release and label glycans are illustrated. The 2-AB-labelled glycans were then separated by HILIC-HPLC. Structural assignment was carried out by a combination of fractionation by AEC-HPLC, exoglycosidase digestions and mass spectrometry with subsequent fragmentation.

and alkylated in a polypropylene 96-well flat-bottomed microplate. Reduced and alkylated samples were set into gel blocks of SDS–polyacrylamide gel, and *N*-glycans were released using PNGase F (Prozyme, Hayward, CA, USA) as described previously (Royle *et al.* 2008). The released glycans were labelled using 2-aminobenzamide (AB) (Ludger 2-AB labelling Kit, Abingdon, UK). *N*-glycan nomenclature and symbolic representations used throughout are as previously described in Fig. 2.

Analysis by HPLC

The glycans were separated by HILIC–HPLC and AEC–HPLC. Additional exoglycosidase digestions (below) were undertaken to aid with structural assignment of glycan sequence and linkage. The charged fractions were collected during AEC separation and then digested with exoglycosidase enzymes. The AEC-separated exoglycosidase-digested fractions were further separated by HILIC to aid with structural determination. Preliminary structural assignments were made using database matching (GlycoBase; http://glycoBase.nibr.ie/glycoBase/show_nibr.action). The structures were confirmed by nano liquid chromatography–mass spectrometry (LC–MS) and MS² isolation and fragmentation. A sample of glycans for baseline analysis was constructed by pooling 10% of glycans derived from all FF samples.

Figure 2 Explanation of the alpha numeric code used to describe *N*-glycans. The glycan structure is illustrated. The alpha numeric code refers to structural subfeatures of these glycans. The trimannosyl chitobiose core (core *N*-glycan) is designated by 'M3'. An *N*-glycan core with the addition of a GlcNAc on each arm forms A2. Further extensions are designated by the addition of single letters denoting monosaccharide residues and numbers denoting the number of these residues. For example, G2 represents the addition of a single galactose residue to each arm of a biantennary glycan and S2 represents the further addition of a single sialic acid residue to each galactose. Sialic acids linked to the GlcNAc of a hypersialylated structure are denoted by an S preceding the G (as in the case of A2S1G2S2*). Core fucosylated variants are denoted by an initial F as in FM3. The structures in which a GlcNAc residue lies between the two antennae of the trimannosyl core are denoted by a B (bisect), such as in M3B. Further GlcNAc residues added to the terminal mannose residues of the M3 structure are the initiation point for further antennae (A3 and A4). *The sialic acids represented in the figure are Neu5Ac. However, Neu5Gc residues are also present in our data. The Oxford glycan notation was used to represent *N*-linked glycan composition and structure (Harvey *et al.* 2009).

Hydrophilic interaction liquid chromatography

HILIC–HPLC was carried out using a TSK-Gel Amide-80 5 μ m (250 $\times$ 4.6 mm) column (Tosoh Bioscience, Stuttgart, Germany) for 180- and 60-min runs, or on a 3- μ m (150 $\times$ 4.6 mm) column for 60-min runs (Tosoh Bioscience, Stuttgart, Germany), at 30 °C, with 50 mM formic acid adjusted to pH 4.4 with ammonia solution as solvent A and acetonitrile as solvent B. The 180- and 60-min runs were on a 2695 Waters Alliance separations module with a 2475 fluorescence detector (Waters) with excitation and emission wavelengths of 330 and 420 nm respectively. For the initial characterisation of the overall baseline glycan profile, an 180-min separation method utilised the following conditions: a linear gradient of 20–58% solvent A at a flow rate of 0.4 ml/min over 152 min followed by a series of equilibration steps to 180 min (Royle *et al.* 2008). This method was used to obtain the maximum peak separation and resolution of individual glycan structures. For the comparison of unpooled individual samples, a 60-min method was used as follows: a linear gradient of 35–47% solvent A over 48 min at a flow rate of 0.8 ml/min, followed by a series of equilibration steps to 60 min (Royle *et al.* 2008). The systems were calibrated by running an external standard of 2-AB–dextran ladder (2-AB–glucose homopolymer, Ludger) alongside the sample runs. A fifth-order polynomial distribution curve was fitted to the dextran ladder and used to allocate glucose unit (GU) values from retention times, as described previously (Royle *et al.* 2008).

Anion exchange chromatography–HPLC

Separation of neutral and acidic oligosaccharides by AEC–HPLC was carried out using a Waters BioSuite DEAE Anion-exchange column (1000 Å 10 μ m, 7.5 mm $\times$ 50 mm) on a 2695 Alliance Separation module with a 474 fluorescence detector set with excitation and emission wavelengths of 330 and 420 nm respectively (Waters, Milford, MA, USA). The solvent A consisted of 0.1 M ammonium acetate buffer of pH 7.0 in 20% v/v acetonitrile, and solvent B was 20% acetonitrile. The gradient conditions were as follows: a linear gradient of 0–5% solvent A over 12 min at a flow rate of 1 ml/min, followed by 5–21% solvent A over 13 min and then 21–50% solvent A over 25 min, 80–100% solvent A over 5 min and then 5 min at 100% solvent A. The samples were injected in water, and a fetuin *N*-glycan standard was used for calibration (Royle *et al.* 2006).

Exoglycosidase digestions

To determine the sequence, monosaccharide type and linkage of sugar residues as described previously (Royle *et al.* 2006), whole pools and AEC-fractionated 2-AB-labelled *N*-glycans were digested with the combinations of linkage-specific exoglycosidase enzymes before separation on HPLC. The protocol used the following enzymes (with the specificities and activities in brackets) obtained from Prozyme, San Leandro, CA, USA: NAN1 sialidase (EC 3.2.1.18, releases α 2–3 sialic acid, 1 U/ml); ABS, *Arthrobacter ureafaciens* sialidase (EC 3.2.1.18, releases α 2–3,6,8, 9 sialic acid, 1 U/ml);

AMF, almond meal α -fucosidase (EC3.2.1.51, releases α 1–3,4, 3 mU/ml); BKF, bovine kidney α -fucosidase (EC3.2.1.51, releases α 1–6, >2,3,4, 1 U/ml); BTG, bovine testes β -galactosidase (EC 3.2.1.23, releases β 1–3,4,6, 1 U/ml); SPG, *Streptococcus pneumoniae* β -galactosidase (EC 3.2.1.23, releases β 1–4, 0.1 U/ml); GUH, *S. pneumoniae* β -N-acetylhexosaminidase (GlcNAc) (EC 3.2.1.30, releases β 1–2,4,6, bisect, 4 U/ml); JBH, jack bean α -N-acetylhexosaminidase (EC 3.2.1.30, releases α 1–2,3,4,6 >bisect, 50 U/ml); JBM, jack bean α -mannosidase (EC 3.2.1.24, releases α 1–2,6,3, 50 U/ml). The samples were incubated overnight at 37 °C in 50 mM sodium acetate buffer of pH 5.5. They were then passed through a Micropure-EZ enzyme remover (Millipore) before applying to the HPLC.

Liquid chromatography–mass spectrometry

Where required to confirm the structural assignments, LC–MS² analysis of 2-AB-labelled glycans from tri-sialylated and tetra-sialylated AEC fractionated samples was carried out. This was undertaken using an Agilent Technologies 1200 series capillary/nano liquid chromatography system in conjunction with an Agilent ChipCube interface and a 6340 ion trap mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The instrument was operated in a positive ion mode with a spray voltage of –1.8 kV. The samples were run on a custom HPLC-chip which consisted of a 500 nl porous graphitised carbon enrichment column and a 43 mm separation column packed with Tosoh TSK Gel Amide-80 3 μ m. (M+2H)²⁺ or (M+H+NH₄)²⁺ ions of the labelled glycans were isolated in the ion trap and fragmented therein by collision-induced dissociation to yield MS² spectra. NAN1 and BTG-combined exoglycosidase digestions were used to identify the sialic acid to GlcNAc linkage.

Statistical analysis

The N-glycomes from FF samples were analysed by HILIC–HPLC. The profiles were separated into 17 peaks. The area under each peak was expressed as a percentage of the total profile and logit transformed. Using MANOVA analysis of transformed data (SAS 9.1 SAS Institute, Inc., Cary, NC, USA), comparisons were made in order to identify temporal changes in glycosylation associated with dominant follicle selection (Subordinate vs Selection), dominant follicle differentiation (Selection vs Differentiation) and dominant follicle luteinisation (Differentiation vs Luteinisation). In addition, overall N-glycan profiles of FF associated with temporal changes in dominant follicle development were compared between heifers and lactating cows. The comparisons were considered significant when $P < 0.05$.

Results

Characterisation of the baseline N-glycan profile of bovine FF

Combined data from HILIC–HPLC, AEC–HPLC, mass spectrometry with combined fragmentation and LC–MS

combined with exoglycosidase digestions were used to determine structures in a pooled FF sample.

The HILIC–HPLC profile separated glycans that ranged from 5.5 to 11 glucose units (GU). The glycan profile was divided into 17 peak areas (GLY1–17) (Fig. 3). The major structures present in each of the peaks are given in Fig. 4; however, due to the coelution of multiple glycans in GLY peaks 6–11, glycans of low abundance could not be identified.

The major structures determined from the analysis of the N-linked glycans were based on a biantennary digalactosylated (A2G2) core structure. There were varied levels of nongalactosylated and monogalactosylated structures (A2G0 and A2G1 respectively). A total of ~22% of structures displayed chitobiose cores that were fucosylated. Less than 10% of all structures showed a GlcNAc situated in an α 1,4 linkage to the trimannosyl core (normally called “bisecting GlcNAc”). A majority of the peaks were sialylated (65–75%). The change in glucose units after ABS digestions indicated that >95% of all sialylated structures were derived from an A2G2 structure, including the tri- and tetra-sialylated fractions, implying that there were multiple sialic acids linked to one or both of antennae of this structure. Between 40 and 45% of the overall N-glycome of FF were of the tri- or tetra-sialylated moiety. The majority of structures were hypersialylated of which sialic acids were bound to both the Gal and GlcNAc residues of a biantennary structure, as in the A2S1G2S2 structure shown in Fig. 2. The hypersialylated structures were confirmed by LC–MS² using exoglycosidase (NAN1 and BTG)-digested glycans (see Supplemental Information, see section on supplementary data given at the end of this article).

Glycosylation changes during dominant follicle selection

In order to identify FF N-glycans that change during dominant follicle selection, we compared the N-glycan profile of FF from subordinate and newly selected dominant follicles from heifers and lactating cows. The average peak areas and s.d. for GLY1–GLY17 are as given in Table 1. There was a significant change in the percentage area of GLY17 of the FF N-glycan profile

Figure 3 HILIC–HPLC chromatogram of N-glycans released from whole follicular fluid divided into 17 peaks with corresponding glucose unit values. Each peak is labelled GLY1–GLY17, and structures are presented in Fig. 4.

GLY	Structure	Nomenclature	GU
GLY1		A2G0	5.23
		FA2G0	5.70
GLY2		A2BG0	5.70
		A2G1	6.03
GLY3		A2BG1	6.16
		FA2G1	6.49
GLY4		FA2BG1	6.61
		A2G2	7.07
GLY5		A2G1S1	7.18
		FA2G2	7.40
GLY6		FA2BG2	7.50
		A2G2S1	7.65
GLY7		A2G2S1	7.98
		FA2G2S1	8.05
GLY7		A2G2Sg1	8.08
		A2G2S2	8.20
GLY7		A3G3	8.38
		FA2G2S1	8.38
GLY7		A2G2Sg1	8.34

Figure 4 (continued)

GLY8		FA2G2Sg1	8.48/8.74
		A2G2S2	8.53
GLY8		FA2G2S2	8.60
		A2G2S1Sg1	8.63
GLY8		A2G2S3	8.75
		FA3G3	8.77
GLY9		A3G3S1	8.95
		A2G2S2	8.86
GLY9		A2G2Sg2	9.06
		FA2G2S1Sg1	9.03
GLY10		A2G2S3	9.08
		A2G2S2Sg1	9.18
GLY10		A2G2S1Sg1	9.22
		FA2G2S2	9.26
GLY11		A2G2S4	9.30
		A2G2Sg2	9.32
GLY11		A3G3Sg1	9.38
		A2G2S3	9.41
GLY11		A2G2S2Sg1	9.44
		A4G4	9.60

Figure 4 (continued)

GLY12		A2G2S1Sg2	9.61
		FA2G2S1Sg1	9.62
		A3G3S2	9.50
		A2G2Sg2	9.58
		A3G3Sg1	9.64
		A2G2S3Sg1	9.73
GLY13		A2G2S1Sg2	9.87
		A2G2S1Sg2	9.87
GLY14		FA3G3S2	9.90
		FA2G2Sg2	9.98
		A2G2S1Sg2	9.94
		A2G2Sg3	10.04
		A3G3S3	10.05
		A2G2S2Sg2	10.16
GLY15		A2G2Sg3	10.30
		A2G2Sg3	10.44
GLY16		FA2G2S2Sg2	10.56
		A2G2S1Sg3	10.59
		A3G3Sg4	10.60
GLY17		A3G3S4	10.93
		A2G2Sg4	11.02

between subordinate and newly selected dominant follicles. Peak GLY17 is composed of A2G2Sg4 and A3G3S4. All other peaks were not statistically different between the dominant and subordinate FF N-glycans. However, the relative percentage areas for glycan peak GLY1 (A2G0), GLY3 (A2G1), GLY4 (FA2G1), GLY5 (A2G2, FA2G1S1), GLY6 (FA2G2, FA2BG2, A2G2S1), GLY8 (A3G3, FA2G2S1, A2G2Sg1, FA2G2Sg1, A2G2S2, FA2G2S2, A2G2S1Sg1, A2G2S3) and GLY17 (A3G3S4, A2G2Sg4) were higher ($P<0.05$), while GLY12 (A2G2Sg2, A3G3S2, FA2G2S1Sg1, A3G3Sg1, A2G2S3Sg1) and GLY16 (FA2G2Sg3, FA2G2S2Sg2, A2G2S13Sg3, A3G3Sg4) were lower ($P<0.05$) across the two stages in lactating cows compared with heifers.

Glycosylation changes across the stage of follicle development

To determine whether the FF N-glycome profile changes during preovulatory follicle development, we compared the relative abundance of N-glycans in FF collected from follicles at three distinct stages of the follicle wave: Selection, Differentiation and Luteinisation (Table 1). The average peak areas and s.d. for GLY1–GLY17 and significantly different GLY peaks across the preovulatory follicle development stages and between cows and heifers at these stages are outlined in Table 1. Overall, the relative percentage areas of GLY1 (A2G0), GLY2 (FA2G0), GLY3 (A2G1), GLY4 (FA2G1), GLY7 (A2G2S1, FA2G2S1, A2G2Sg1, A2G2S2), GLY8 (A3G3, FA2G2S1, A2G2Sg1, FA2G2Sg1, A2G2S2, FA2G2S2, A2G2S1Sg1, A2G2S3), GLY12 (A2G2Sg2, A3G3S2, FA2G2S1Sg1, A3G3Sg1, A2G2S3Sg1), GLY14 (FA3G3S2, FA2G2Sg2, A2G2S1Sg2, A2G2Sg3, A3G3S3, A2G2S2Sg2), GLY15 (A2G2Sg3) and GLY16 (FA2G2Sg3, FA2G2S2Sg2, A2G2S1Sg3, A3G3Sg4) were altered in FF during preovulatory follicle development. The relative percentage areas of GLY1, GLY2, GLY3, GLY7 and GLY8 increased in FF during follicle development, while GLY12, GLY14, GLY15 and GLY16 decreased. In contrast, GLY4 increased in FF during follicle differentiation and decreased during luteinisation. Across follicle development, there was a decrease in sialylated GLY peaks and a corresponding increase in nonsialylated GLY peaks. GLY1, GLY2 and GLY3 consisted solely of nonsialylated structures, while GLY12, GLY14, GLY15 and GLY16 consisted mostly of tri- and tetra-sialylated forms and some disialylated forms. GLY7 and GLY8 although increased with follicle development consists of mostly monosialylated forms.

Figure 4 Structures of N-glycan present in bovine follicular fluid. The major structures in each peak are identified. There were structural isomers evident in each of the profiles. However, as the changes are due to sialic acid variants (Neu5Gc), the antennae and linkage at which each Neu5Gc is attached could not be confirmed.

Table 1 Average peak areas $\pm$ s.d. of the preovulatory follicle developmental stages (Selection, Differentiation and Luteinisation) and the subordinate follicle.

Peak	Dominant follicle stages				Subordinate follicle			
	Selection		Differentiation		Luteinisation		Significance	
	Cows	Heifers	Cows	Heifers	Cows	Heifers	Stage ^a	C vs H ^b
GLY1	0.54 $\pm$ 0.07	0.36 $\pm$ 0.16	0.68 $\pm$ 0.27	0.36 $\pm$ 0.14	0.84 $\pm$ 0.30	0.56 $\pm$ 0.19	#	*
GLY2	2.33 $\pm$ 0.04	2.11 $\pm$ 0.23	2.85 $\pm$ 1.11	2.91 $\pm$ 0.83	2.52 $\pm$ 0.48	3.86 $\pm$ 1.33	#	
GLY3	3.43 $\pm$ 0.49	2.78 $\pm$ 0.71	3.62 $\pm$ 0.88	3.08 $\pm$ 0.77	3.80 $\pm$ 0.82	4.41 $\pm$ 2.07	#	
GLY4	8.89 $\pm$ 0.66	7.40 $\pm$ 1.07	11.61 $\pm$ 3.14	9.24 $\pm$ 1.39	9.37 $\pm$ 1.40	11.66 $\pm$ 4.72	#	
GLY5	3.30 $\pm$ 0.45	2.42 $\pm$ 0.82	3.02 $\pm$ 0.79	2.02 $\pm$ 0.18	3.36 $\pm$ 1.03	2.75 $\pm$ 0.78		+
GLY6	10.18 $\pm$ 0.67	8.25 $\pm$ 1.32	12.08 $\pm$ 1.68	9.21 $\pm$ 1.27	9.50 $\pm$ 0.76	10.69 $\pm$ 3.49		#
GLY7	4.28 $\pm$ 0.61	3.88 $\pm$ 1.08	3.87 $\pm$ 0.58	5.64 $\pm$ 0.87	5.28 $\pm$ 0.24	6.18 $\pm$ 1.72	+	
GLY8	8.29 $\pm$ 0.43	7.32 $\pm$ 0.89	7.42 $\pm$ 0.71	8.78 $\pm$ 0.80	8.73 $\pm$ 0.62	9.19 $\pm$ 1.26	#	
GLY9	10.02 $\pm$ 2.24	9.81 $\pm$ 3.17	9.33 $\pm$ 0.99	12.95 $\pm$ 2.16	11.60 $\pm$ 0.38	11.86 $\pm$ 3.01		
GLY10	3.96 $\pm$ 1.56	4.66 $\pm$ 2.13	3.69 $\pm$ 0.67	6.06 $\pm$ 1.01	4.95 $\pm$ 0.52	4.93 $\pm$ 2.12		
GLY11	17.71 $\pm$ 1.24	17.63 $\pm$ 3.90	16.89 $\pm$ 1.94	20.12 $\pm$ 0.90	19.60 $\pm$ 1.36	17.56 $\pm$ 4.18		
GLY12	11.02 $\pm$ 1.83	11.93 $\pm$ 1.99	10.54 $\pm$ 2.20	9.65 $\pm$ 2.24	8.81 $\pm$ 0.98	7.58 $\pm$ 3.31	+	
GLY13	8.81 $\pm$ 1.18	10.04 $\pm$ 1.95	8.73 $\pm$ 2.18	6.35 $\pm$ 3.95	8.23 $\pm$ 0.89	6.00 $\pm$ 3.07		
GLY14	3.94 $\pm$ 1.56	6.37 $\pm$ 4.07	3.01 $\pm$ 1.54	1.72 $\pm$ 0.94	1.52 $\pm$ 0.19	1.36 $\pm$ 1.20	*	
GLY15	1.71 $\pm$ 0.49	2.28 $\pm$ 1.27	1.65 $\pm$ 0.60	1.20 $\pm$ 0.63	1.17 $\pm$ 0.15	0.89 $\pm$ 0.64	+	
GLY16	1.31 $\pm$ 0.43	2.55 $\pm$ 1.92	0.86 $\pm$ 0.56	0.57 $\pm$ 0.28	0.54 $\pm$ 0.06	0.39 $\pm$ 0.30	*	
GLY17	0.28 $\pm$ 0.06	0.21 $\pm$ 0.07	0.16 $\pm$ 0.06	0.17 $\pm$ 0.04	0.22 $\pm$ 0.04	0.14 $\pm$ 0.10		+

Peaks with statistical significance are indicated. Statistical significance: * $P \leq 0.001$, † $P \leq 0.01$, ‡ $P \leq 0.05$, § $P \leq 0.1$.

^aSignificance between stages across dominant follicle development (from Selection, Differentiation, Luteinisation). ^bSignificance between Cows and Heifers across dominant follicle developmental stages (from Selection, Differentiation, Luteinisation). ^cSignificance between subordinate follicle and dominant follicle at Selection. ^dSignificance between Cows and Heifers at Selection of dominant follicle (selected follicle vs subordinate follicle).

The relative percentage areas of GLY1 (A2G0), GLY5 (A2G2, A2G1S1) and GLY6 (FA2G2, FA2BG2, A2G2S1) were higher ($P < 0.05$) in FF from lactating cows compared with heifers. The major glycoforms within GLY5 and GLY6 are A2G2 and FA2G2, respectively, with the sialylated glycans comprising $< 5\%$ of each peak. Thus, the majority of structures within these three peaks are nonsialylated, which are observed to be higher in the cow vs the heifer. This indicates that the cow has more nonsialylated glycans than the heifer. Although, not statistically significant, the heifer appears to have higher averages of sialylated peaks when compared with cows.

Discussion

This study has defined, for the first time, the overall N-glycome of FF from dairy cattle. In addition, it has also revealed temporal changes in the profile of N-glycans of preovulatory dominant follicles through different stages of developmental progression and between heifers and lactating cows during preovulatory follicle development. No differences in glycan profile were identified between newly selected dominant follicles and subordinate follicles. However, a difference was evident over preovulatory follicle development between cow and heifer.

FF glycan characterisation

A major challenge in this study was to understand the detailed structural basis of the sialylation of the FF glycans. Each sialylated antennae of an N-glycan characteristically has a single sialic acid (Neu5Ac or Neu5Gc) linked ($\alpha 2,3$ or $\alpha 2,6$) to a Gal. However, the N-glycome of bovine FF consisted of tri- and tetra-sialylated but based on a biantennary structure. Unusually, this indicates that each antenna could have more than one sialic acid residue.

By combining the HILIC and LC-MS² data (Supplemental Information, Figures S1 and S2, see section on supplementary data given at the end of this article), we determined that the majority of structures were hypersialylated, where sialic acids were linked to GlcNAc residues in the presence of sialic acids bound to the Gal residues. Hypersialylation has only recently been described in the bovine (Sumiyoshi *et al.* 2012) and mouse serum (Hua *et al.* 2013). However, similar linkages have previously been described in free oligosaccharides from bovine colostrums (Nemansky & Van den Eijnden 1992), human milk (Sumiyoshi *et al.* 2004) and mucin-type O-linked glycans from human mucosal tissues (Brockhausen 2006). Previous lectin histochemical studies have shown the increased expression of $\alpha 2,6$ sialic acids (in a Sia $\alpha 2,6$ Gal/GalNAc conformation) in porcine atretic follicle granulosa cells (Kimura *et al.* 1999). Furthermore, RT-PCR analysis demonstrated an upregulation of sialyl transferase

ST6GAL1 mRNA in these atretic follicles compared with non-atretic follicles (Kimura *et al.* 1999).

Another possible structural configuration that could account for the presence of multiple sialic acid residues on each antennae of biantennary glycans might be the presence of di- or oligo-sialic acids linked through an $\alpha 2,8$ linkage. However, our analysis failed to detect this linkage, although it has been detected in ovarian fluid from trout (Funakoshi *et al.* 1997). To the best of our knowledge, this is the first comprehensive report of the N-glycome of FF.

Changes in the FF N-glycome associated with dominant follicle selection

The comparison of FF glycans from dominant and subordinate follicles from lactating cows and heifers indicated that there was no difference (except GLY17) in the N-glycome of FF isolated from the dominant and subordinate follicles. However, the analysis indicated a higher proportion of nonsialylated glycans and a corresponding decrease in sialylated glycan structures in lactating cows when compared with heifers. An increase in uterine sialic acid, which was induced by oestrogen, has been reported in the rat (Rajalakshmi *et al.* 1969) and increases in sialic acid and sialyltransferase activity correlate with increased circulating progesterone concentrations in the bovine model (Sherblom *et al.* 1985). The concentrations of oestrogen and progesterone are also linked with fertility in heifers and lactating dairy cows, where heifers display higher circulating progesterone concentrations and this has been linked to their higher fertility in comparison with lactating dairy cows (Crowe 2008). In our study, the oestrogen levels of the dominant follicle was higher than that measured in the subordinate (data not shown); however, this difference did not relate to a difference in the glycosylation between these groups.

Changes in sialylation are widely understood to modify a range of protein-carbohydrate interactions; they are known to participate in the regulation of many cell biological events, including cell recognition, adhesion and disease progression (Varki 1997, 2007). Sialic acid-binding immunoglobulin-like lectins (Siglecs) represent protein families which have been adapted over evolution for the recognition of specific sialoside structures. For example, siglec-6, expressed in the human placenta, and siglec-5, expressed on myeloid cells of the hemopoietic system, are known to sequester leptin (Brinkman-Van der Linden *et al.* 2007). Interestingly, leptin is reduced in lactating cows compared with heifers (Clemenson *et al.* 2011). Siglec-11 detected in human and chimpanzee ovarian stromal cells (Wang *et al.* 2011) was speculated to create a unique microenvironment of cytokines or hormones for ovarian follicles and leukocytes.

The cytokines may be important in folliculogenesis, ovulation and in the process of follicular atresia and

corpus luteum regression through immune cell activation. In particular, TNF α is an example of a cytokine that initiates the apoptosis of the endothelial cells of granulosa cells and eventually leads to follicular atresia (Korzekwa *et al.* 2008, Glister *et al.* 2014). Although TNF α is not sialylated, it has been linked to changes in the sialylation of cells such as the increase in α 2,3-sialylation of human tracheal gland cells (Delmotte *et al.* 2001).

Temporal changes in FF N-glycome associated with the development of preovulatory follicle

Over development, there is an increase in the abundance of nonsialylated structures (GLY1–GLY4) with a corresponding decrease in tri- and tetra-sialylated structures (GLY12, GLY14–GLY16). As mentioned in the previous section, the oestrogen and progesterone have previously been shown to increase sialylation in the rat and bovine models (Rajalakshmi *et al.* 1969, Sherblom *et al.* 1985); thus, comparisons of the serum oestrogen levels in our model indicated that the oestrogen levels were low at selection, increased at differentiation and then decreased at luteinisation (Walsh *et al.* 2012b). This was evident in both the cow and heifer; however, the heifer exhibited approximately twofold higher serum oestrogen concentrations than the cow. The hormonal fluctuation could not be used to explain our results over the stages of follicle development; however, the cow-to-heifer difference was also evident across the follicular stages as stated earlier.

As previously described, the sialylation (hypersialylation or polysialylation) is a means to increase the hydrophilicity of the protein, thus inhibiting protein–protein interactions. It is therefore plausible to speculate that the loss of hypersialylation is relevant to the regulation of follicular development through increased interactions of functionally relevant proteins. Therefore, the differential expression of genes associated with immune function over follicle development may be linked to the changes in protein sialylation (Walsh *et al.* 2012b). The difference in sialylation observed between the heifer and cow model could also be attributed to the different expression of genes associated with immune function within the follicle, where a higher fold difference has been measured in the expression of a number of genes in the heifer during selection and lesser fold expression during luteinisation (Walsh *et al.* 2012b). However, in our model the increased expression of genes associated with immune function yielded higher sialylation; previous reports suggest that an activation of immune cells is correlated with a down-regulation of sialic acids (Liang *et al.* 2006, Varki 2008).

The major glycoproteins in FF are transferrin and α -macroglobulin; these proteins have been reported to play a role in maturation of oocytes. The concentrations of γ -globulin, transferrin, α -1-antitrypsin,

haptoglobulins, apolipoproteins and albumin were found to increase with follicular size (Spitzer *et al.* 1996, Wen *et al.* 2009). The function of these proteins may be modulated through changes in their glycosylation as the follicular size increases. It was beyond the scope of the current study to map these changes in specific proteins. However, future glycoproteomic studies could illuminate such phenomena.

Conclusions

In conclusion, the present study has defined, for the first time, the overall N-glycome of FF from dairy cattle. Temporal changes in the profile of N-glycans of preovulatory dominant follicles through different stages of development progression and differences between heifers and lactating cows during preovulatory follicle developmental were identified. In addition, differences in cow and heifer preovulatory follicle development were evident in N-glycans between newly selected dominant follicles and subordinate follicles. This study was a preliminary observation to establish the FF glycan profile and establish any trends in follicle development. Future glycoproteomic analyses would be required to map these changes to specific proteins in the FF.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-14-0035>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

The work was supported by Science Foundation Ireland under grant no. 07/SRC/B1156 (the opinions, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the Science Foundation Ireland).

Acknowledgements

The authors thank Dr Rebecca M Duke for thoroughly reading this manuscript.

References

- Aardema H, Vos PL, Lolicato F, Roelen BA, Knijn HM, Vaandrager AB, Helms JB & Gadella BM 2011 Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. *Biology of Reproduction* **85** 62–69. (doi:10.1095/biol-reprod.110.088815)

- Adamczyk B, Tharmalingam T & Rudd PM 2012 Glycans as cancer biomarkers. *Biochimica et Biophysica Acta* **1820** 1347–1353. (doi:10.1016/j.bbagen.2011.12.001)
- Apweiler R, Hermjakob H & Sharon N 1999 On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochimica et Biophysica Acta* **1473** 4–8. (doi:10.1016/S0304-4165(99)00165-8)
- Barnes R, Cha K, Lee D & Lobo R 1986 Modulation of luteinizing hormone immunoreactivity and bioactivity by dopamine but not norepinephrine in women. *American Journal of Obstetrics and Gynecology* **154** 445. (doi:10.1016/0002-9378(86)90688-5)
- Bender K, Walsh S, Evans ACO, Fair T & Brennan L 2010 Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. *Reproduction* **139** 1047–1055. (doi:10.1530/REP-10-0068)
- Brinkman-Van der Linden EC, Hurtado-Ziola N, Hayakawa T, Wiggleton L, Benirschke K, Varki A & Varki N 2007 Human-specific expression of Siglec-6 in the placenta. *Glycobiology* **17** 922–931. (doi:10.1093/glycob/cwm065)
- Brockhausen I 2006 Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions. *EMBO Reports* **7** 599–604. (doi:10.1038/sj.embor.7400705)
- Carson DD, DeSouza MM, Kardon R, Zhou X, Lagow E & Julian J 1998 Mucin expression and function in the female reproductive tract. *Human Reproduction Update* **4** 459–464. (doi:10.1093/humupd/4.5.459)
- Clempson A, Pollott G, Brickell J, Bourne N, Munce N & Wathes D 2011 Evidence that leptin genotype is associated with fertility, growth, and milk production in Holstein cows. *Journal of Dairy Science* **94** 3618–3628. (doi:10.3168/jds.2010-3626)
- Crowe M 2008 Resumption of ovarian cyclicity in *post-partum* beef and dairy cows. *Reproduction in Domestic Animals* **43** 20–28. (doi:10.1111/j.1439-0531.2008.01210.x)
- Delmotte P, Degroote S, Merten MD, Van Seuningen I, Bernigaud A, Figarella C, Roussel P & Périni J-M 2001 Influence of TNF α on the sialylation of mucins produced by a transformed cell line MM-39 derived from human tracheal gland cells. *Glycoconjugate Journal* **18** 487–497. (doi:10.1023/A:1016038219183)
- Ellsworth L, Balmaceda J, Schenken R, Silverman A, Prihoda T & Asch R 1984 Human chorionic gonadotropin and steroid concentrations in human follicular fluid in relation to follicle size and oocyte maturity in stimulated ovarian cycles. *Acta Europaea Fertilitatis* **15** 343–346.
- Eppig J 2001 Oocyte control of ovarian follicular development and function in mammals. *Reproduction* **122** 829–838. (doi:10.1530/rep.0.1220829)
- Evans AC & Walsh SW 2011 The physiology of multifactorial problems limiting the establishment of pregnancy in dairy cattle. *Reproduction, Fertility, and Development* **24** 233–237. (doi:10.1071/RD11912)
- Funakoshi Y, Taguchi T, Sato C, Kitajima K, Inoue S, Morris HR, Dell A & Inoue Y 1997 Occurrence of terminal alpha 2 $\rightarrow$ 8-linked disialylated poly-N-acetylactosamine chains with Le(X) and I antigenic glycotopes in tetraantennary arms of an N-linked glycoprotein isolated from rainbow trout ovarian fluid. *Glycobiology* **7** 195–205. (doi:10.1093/glycob/7.2.195)
- Ginther OJ, Knopf L & Kastelic J 1989 Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. *Journal of Reproduction and Fertility* **87** 223–230. (doi:10.1530/jrf.0.0870223)
- Ginther OJ, Beg MA, Bergfelt DR, Donadeu FX & Kot K 2001 Follicle selection in monovular species. *Biology of Reproduction* **65** 638–647. (doi:10.1095/biolreprod65.3.638)
- Glister C, Hatzirados N, Hummitzsch K, Knight PG & Rodgers RJ 2014 The global effect of follicle-stimulating hormone and tumour necrosis factor α on gene expression in cultured bovine ovarian granulosa cells. *BMC Genomics* **15** 72. (doi:10.1186/1471-2164-15-72)
- Gornik O, Royle L, Harvey DJ, Radcliffe CM, Saldova R, Dwek RA, Rudd P & Lauc G 2007 Changes of serum glycans during sepsis and acute pancreatitis. *Glycobiology* **17** 1321–1332. (doi:10.1093/glycob/cwm106)
- Harvey DJ, Merry AH, Royle L, Campbell MP, Dwek RA & Rudd PM 2009 Proposal for a standard system for drawing structural diagrams of N- and O-linked carbohydrates and related compounds. *Proteomics* **9** 3796–3801.
- Hua S, Jeong HN, Dimapasoc LM, Kang I, Han C, Choi J-S, Lebrilla CB & An HJ 2013 Isomer-specific LC/MS and LC/MS/MS profiling of the mouse serum N-glycome revealing a number of novel sialylated N-glycans. *Analytical Chemistry* **85** 4636–4643. (doi:10.1021/ac400195h)
- Jorritsma R, Cesar M, Hermans J, Kruitwagen C, Vos P & Kruij T 2004 Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes *in vitro*. *Animal Reproduction Science* **81** 225–235. (doi:10.1016/j.anireprosci.2003.10.005)
- Kimura Y, Manabe N, Nishihara S, Matsushita H, Tajima C, Wada S & Miyamoto H 1999 Up-regulation of the α 2, 6-sialyltransferase messenger ribonucleic acid increases glycoconjugates containing α 2, 6-linked sialic acid residues in granulosa cells during follicular atresia of porcine ovaries. *Biology of Reproduction* **60** 1475–1482. (doi:10.1095/biolreprod60.6.1475)
- Komar C, Berndtson A, Evans A & Fortune J 2001 Decline in circulating estradiol during the periovulatory period is correlated with decreases in estradiol and androgen, and in messenger RNA for P450 aromatase and P450 17 α -hydroxylase, in bovine preovulatory follicles. *Biology of Reproduction* **64** 1797–1805. (doi:10.1095/biolreprod64.6.1797)
- Kornfeld R & Kornfeld S 1985 Assembly of asparagine-linked oligosaccharides. *Annual Review of Biochemistry* **54** 631–664. (doi:10.1146/annurev.bi.54.070185.003215)
- Korzekwa A, Murakami S, Woclawek-Potocka I, Bah MM, Okuda K & Skarzynski DJ 2008 The influence of tumor necrosis factor α (TNF) on the secretory function of bovine corpus luteum: TNF and its receptors expression during the estrous cycle. *Reproductive Biology* **8** 245–262. (doi:10.1016/S1642-431X(12)60015-1)
- Leroy J, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruij A, Genicot G & Van Soom A 2005 Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes *in vitro*. *Reproduction* **130** 485–495. (doi:10.1530/rep.1.00735)
- Leroy J, Rizos D, Sturmey R, Bossaert P, Gutierrez-Adan A, Van Hoek V, Valckx S & Bols P 2011 Intrafollicular conditions as a major link between maternal metabolism and oocyte quality: a focus on dairy cow fertility. *Reproduction, Fertility, and Development* **24** 1–12. (doi:10.1071/RD11901)
- Liang F, Seyrantepe V, Landry K, Ahmad R, Ahmad A, Stamatatos NM & Pshezhetsky AV 2006 Monocyte differentiation up-regulates the expression of the lysosomal sialidase, Neu1, and triggers its targeting to the plasma membrane via major histocompatibility complex class II-positive compartments. *Journal of Biological Chemistry* **281** 27526–27538. (doi:10.1074/jbc.M605633200)
- López-Gatius F 2003 Is fertility declining in dairy cattle?: a retrospective study in northeastern Spain. *Theriogenology* **60** 89–99. (doi:10.1016/S0093-691X(02)01359-6)
- Mariño K, Bones J, Kattla JJ & Rudd PM 2010 A systematic approach to protein glycosylation analysis: a path through the maze. *Nature Chemical Biology* **6** 713–723. (doi:10.1038/nchembio.437)
- Mihm M, Baker PJ, Fleming LM, Monteiro AM & O'Shaughnessy PJ 2008 Differentiation of the bovine dominant follicle from the cohort upregulates mRNA expression for new tissue development genes. *Reproduction* **135** 253–265. (doi:10.1530/REP-06-0193)
- Miller DJ 2011. The roles of carbohydrate binding in fertilization. In *Carbohydrate Recognition: Biological Problems, Methods, and Applications*, pp. 107–132.
- Miller DL, Jones C, Aplin J & Nardo L 2010 Altered glycosylation in peri-implantation phase endometrium in women with stages III and IV endometriosis. *Human Reproduction* **25** 406–411. (doi:10.1093/humrep/dep401)
- Nemansky M & Van den Eijnden DH 1992 Bovine colostrum CMP-NeuAc: Gal beta (1 $\rightarrow$ 4) GlcNAc-R alpha (2 $\rightarrow$ 6)-sialyltransferase is involved in the synthesis of the terminal NeuAc alpha (2 $\rightarrow$ 6) GalNAc beta (1 $\rightarrow$ 4) GlcNAc sequence occurring on N-linked glycans of bovine milk glycoproteins. *Biochemical Journal* **287** 311.
- Okajima T, Matsuura A & Matsuda T 2008 Biological functions of glycosyltransferase genes involved in O-fucose glycan synthesis. *Journal of Biochemistry* **144** 1–6. (doi:10.1093/jb/mvn016)
- Rajakoski E 1960 The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical and left-right variations. *Acta Endocrinologica* **34** S7–S68.
- Rajalakshmi M, Prasad M & Mohla S 1969 Effect of estrogen and clomiphene on uterine sialic acid in the rat during delayed implantation. *Steroids* **14** 47–54. (doi:10.1016/0039-128X(69)90092-0)

- Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M & Rinaudo P 2009 Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reproductive Biology and Endocrinology* **7** 40. (doi:10.1186/1477-7827-7-40)
- Rodgers RJ & Irving-Rodgers HF 2010 Formation of the ovarian follicular antrum and follicular fluid. *Biology of Reproduction* **82** 1021–1029. (doi:10.1095/biolreprod.109.082941)
- Royle L, Dwek RA & Rudd PM 2006 Determining the structure of oligosaccharides N- and O-linked to glycoproteins. *Current Protocols in Protein Science* **43** 12.6.1–12.6.45. (doi:10.1002/0471140864.ps1206s43)
- Royle L, Campbell MP, Radcliffe CM, White DM, Harvey DJ, Abrahams JL, Kim Y-C, Henry GW, Shadick NA & Weinblatt ME 2008 HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. *Analytical Biochemistry* **376** 1–12. (doi:10.1016/j.ab.2007.12.012)
- Savio JD, Keenan L, Boland MP & Roche JF 1988 Pattern of growth of dominant follicles during the oestrous cycle of heifers. *Journal of Reproduction and Fertility* **83** 663–671. (doi:10.1530/jrf.0.0830663)
- Sherblom A, Smagula R, Moody C & Anderson G 1985 Immunosuppression, sialic acid, and sialyltransferase of bovine serum as a function of progesterone concentration. *Journal of Reproduction and Fertility* **74** 509–517. (doi:10.1530/jrf.0.0740509)
- Spiro RG 2002 Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* **12** 43R–56R. (doi:10.1093/glycob/12.4.43R)
- Spitzer D, Murach K, Lottspeich F, Staudach A & Illmensee K 1996 Different protein patterns derived from follicular fluid of mature and immature human follicles. *Human Reproduction* **11** 798–807. (doi:10.1093/oxfordjournals.humrep.a019257)
- Suchanek E, Simunic V, Macas E, Kopjar B & Grizelj V 1988 Prostaglandin F_{2a} progesterone and estradiol concentrations in human follicular fluid and their relation to success of *in vitro* fertilization. *European Journal of Obstetrics Gynaecology and Reproductive Biology* **28** 331–339. (doi:10.1016/0028-2243(88)90019-6)
- Sudik R, Chari S, Pascher E & Sturm G 1996 Human follicular fluid levels of endothelins in relation to oocyte maturity status. *Experimental and Clinical Endocrinology & Diabetes* **104** 78–84. (doi:10.1055/s-0029-1211426)
- Sumiyoshi W, Urashima T, Nakamura T, Arai I, Nagasawa T, Saito T, Tsumura N, Wang B, Brand-Miller J & Watanabe Y 2003 Sialyl oligosaccharides in the milk of Japanese women: changes in concentration during the course of lactation. *Journal of Applied Glycoscience* **50** 461–468. (doi:10.5458/jag.50.461)
- Sumiyoshi W, Nakakita S-i, Miyaniishi N, Yamada K, Hasehira K, Nakakita Y & Hirabayashi J 2012 Hypersialylated type-I lactosamine-containing N-glycans found in Artiodactyla sera are potential xenoantigens. *Glycobiology* **22** 1031–1041. (doi:10.1093/glycob/cws069)
- Sunderland S, Crowe M, Boland M, Roche J & Ireland J 1994 Selection, dominance and atresia of follicles during the oestrous cycle of heifers. *Journal of Reproduction and Fertility* **101** 547–555. (doi:10.1530/jrf.0.1010547)
- Telford JE, Bones J, McManus C, Saldova R, Manning G, Doherty M, Lewke FM, Rothermundt M, Guest PC & Rahmoune H 2012 Antipsychotic treatment of acute paranoid schizophrenia patients with olanzapine results in altered glycosylation of serum glycoproteins. *Journal of Proteome Research* **11** 3743–3752. (doi:10.1021/pr300218h)
- Tharmalingam T, Mariño K & Rudd PM 2010 Platform technology to identify potential disease markers and establish heritability and environmental determinants of the human serum N-glycome. *Carbohydrate Research* **345** 1280–1282. (doi:10.1016/j.carres.2010.04.012)
- Van Hoesck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PE & Leroy JL 2011 Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *PLoS ONE* **6** e23183. (doi:10.1371/journal.pone.0023183)
- Vanholder T, Leroy J, Soom AV, Opsomer G, Maes D, Coryn M & de Kruif A 2005 Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation *in vitro*. *Animal Reproduction Science* **87** 33–44. (doi:10.1016/j.anireprosci.2004.09.006)
- Varki A 1997 Sialic acids as ligands in recognition phenomena. *FASEB Journal* **11** 248–255.
- Varki A 2007 Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. *Nature* **446** 1023–1029. (doi:10.1038/nature05816)
- Varki A 2008 Sialic acids in human health and disease. *Trends in Molecular Medicine* **14** 351–360. (doi:10.1016/j.molmed.2008.06.002)
- Walsh SW, Williams EJ & Evans AC 2011 A review of the causes of poor fertility in high milk producing dairy cows. *Animal Reproduction Science* **123** 127–138. (doi:10.1016/j.anireprosci.2010.12.001)
- Walsh SW, Mehta JP, McGettigan PA, Browne JA, Forde N, Alibrahim R, Mulligan F, Loftus B, Crowe MA & Matthews D 2012a Effect of the metabolic environment at key stages of follicle development in cattle: focus on steroid biosynthesis. *Physiological Genomics* **44** 504–517. (doi:10.1152/physiolgenomics.00178.2011)
- Walsh S, Fair T, Browne J, Evans A & McGettigan P 2012a Physiological status alters immunological regulation of bovine follicle differentiation in dairy cattle. *Journal of Reproductive Immunology* **96** 34–44. (doi:10.1016/j.jri.2012.07.002)
- Wang X, Chow R, Deng L, Anderson D, Weidner N, Godwin AK, Bewtra C, Zlotnik A, Bui J & Varki A 2011 Expression of Siglec-11 by human and chimpanzee ovarian stromal cells, with uniquely human ligands: implications for human ovarian physiology and pathology. *Glycobiology* **21** 1038–1048. (doi:10.1093/glycob/cwr039)
- Wen X, Perrett D, Patel P, Li N, Docherty SM, Tozer AJ & Iles RK 2009 Capillary electrophoresis of human follicular fluid. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences* **877** 3946–3952. (doi:10.1016/j.jchromb.2009.09.046)
- Wu YT, Tang L, Cai J, Lu XE, Xu J, Zhu XM, Luo Q & Huang HF 2007 High bone morphogenetic protein-15 level in follicular fluid is associated with high quality oocyte and subsequent embryonic development. *Human Reproduction* **22** 1526–1531. (doi:10.1093/humrep/dem029)
- Yanaihara A, Mitsukawa K, Iwasaki S, Otsuki K, Kawamura T & Okai T 2007 High concentrations of lactoferrin in the follicular fluid correlate with embryo quality during *in vitro* fertilization cycles. *Fertility and Sterility* **87** 279–282. (doi:10.1016/j.fertnstert.2006.06.025)
- Zachara NE & Hart GW 2006 Cell signaling, the essential role of O-GlcNAc. *Biochimica et Biophysica Acta* **1761** 599–617. (doi:10.1016/j.bbali.2006.04.007)

Received 11 July 2014

First decision 14 August 2014

Revised manuscript received 20 August 2014

Accepted 10 September 2014