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Abstracts

(Oral Presentations)

Tuesday, November 15th

Selection of dairy cattle based on genomic data

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Genomic selection of dairy cattle is being used widely in developed countries around the world. This technology uses the genotypes of cattle at many, densely packed single nucleotide polymorphisms (SNPs) to predict the genetic value of selection candidates for economically important traits such as milk production. The SNPs are not causal polymorphisms for these traits but are in linkage disequilibrium with the causal polymorphisms. The method relies on a large training set of animals that have been evaluated for the traits of interest and genotyped with the SNP chip. An equation to predict genetic value from SNP genotypes is derived from this training data and then applied to selection candidates such as young bulls who may have no phenotypic record or progeny of their own. This method is achieving high accuracy (<0.8) in Holsteins because they have the best training data but is less accurate in other breeds. To increase the accuracy, including in breeds other than Holstein, we are investigating methods that identify genes and polymorphic sites that cause variation in important traits. To do this we are using new statistical methods, genome sequence data, functional data such as gene expression and DNA signatures of past selection.

Understanding milk protein complexity to produce accurate phenotypes

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Milk is a complete and complex food containing a large number of biomolecules such as lipids, sugars and proteins. As far as proteins are concerned, they are found either at the colloidal state (micelles), soluble in the whey or associated with the fat globule membrane. For many years, milk has been considered as a raw material of which processing or “cracking” concentrated most of the added value. Therefore, the breeding target was over the past 30 years to produce larger amounts of milk with a high overall protein content, while controlling fat content, at the expense of quality and compromising health and fertility of high producing dairy cows. To reach such a goal, measurement and selection procedures have been developed and implemented based on milk yield, total fat and total protein in milk. More recently, emphasis has been put on milk elementary components since many of them, in particular fatty acids and peptides, have putative or actual positive effects on human health. It is now of major interest to accurately measure elementary milk components, including proteins, to identify genes affecting fine milk protein composition. Genetic variants of a number of milk proteins have been shown to impact the protein composition in milk and explain, at least in part, the genetic variation in milk protein composition.

Progress made in the field of comparative and functional genomics, as well as in proteomics, has highlighted how such genetic polymorphisms are responsible for the extreme complexity and the large variability (qualitative and quantitative) of the milk protein fraction. At the quantitative level, general mechanisms controlling gene expression act

both at the transcriptional and the post-transcriptional levels. Polymorphisms found in cis-regulatory elements, mainly within the 5'-flanking region of genes encoding several milk proteins (β-lactoglobulin, αs1- and αs2-caseins) have been shown to influence their transcription rate, in cattle. Polymorphisms found in the transcription unit, within intron as well as exon sequences, have been shown to be responsible for defects in processing of primary transcripts (exon skipping, usage of cryptic splice sites) impacting the amount and structure of messengers and consequently the primary structure of proteins. Such a situation, well-exemplified by the gene encoding αs1-casein in goats, may have dramatic biological consequences (protein and fat contents, casein micelle structure, secretion pathway, etc.).

Combining different proteomic approaches (liquid chromatography, electrophoresis and mass spectrometry) with DNA sequencing, we succeed in: i) accurately characterizing, quantitatively and qualitatively, the protein fraction of milk from different cattle breeds; ii) identifying and characterizing new genetic variants. Such a strategy was also effective to analyze milk from other species including mice for which a total of 34 SNP were identified in coding and 3' untranslated regions for 3 milk protein genes, between mouse species

Genomic Investigations in Dairy Cattle

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Use of high density Single Nucleotide Polymorphism (SNP) marker information allows for prediction of genetic merit via Genome Wide Selection (GWS) and for localisation of markers in gene regions of biological interest through Genome Wide Association (GWA) analyses. The former approach is now rapidly becoming a routine method for augmenting genetic evaluation of young dairy bulls worldwide. The latter is a popular approach for genomic analyses in human studies linking disease/complex traits to specific genetic variants.

Although both GS and GWAS can exploit similar resources in the case of dairy cattle where a large number of progeny tested sires are usually available as well as performance recorded cows, the experimental designs are often limited by total budget. Genotyping many 1000s of animals and the recording of new phenotypes (traits) most likely recorded in cows (unless routinely recorded traits are of interest), will require enormous resources to obtain genetic effect estimates with sufficient precision. Ideally we would like many 10,000s of animals with full genome content. Although this may seem unrealistic, alternative strategies may provide interim cost effective solutions. In the case of genomic selection, high accuracy of prediction of Direct Genomic Value (DGV or molecular breeding value) can be obtained with as few as 3000 markers per genome, with little difference in accuracy if random or trait-specific markers are chosen. The down side is that ongoing genetic evaluations of prediction equations will be required to overcome loss in accuracy of prediction over time. High density genetic markers increase the power of GWAS, but such scans come at a high cost. An interim solution maybe to impute (infer) high density genomic content in related animals from low density genetic marker information.

We recently assessed accuracies of different imputation strategies to impute very dense set of markers using data on 5000 animals genotyped with a 50K SNP array and 800 animals with a high density 610K SNP array. Various 2-tier and 3-tier imputation scenarios with reference panels of varying animal numbers and marker densities were generated, and compared by masking the known genotypes in the test panel. The implications for use of ultra-high density SNP panels and whole genome sequence content are discussed in context of GS and GWAS for an example of 5 dairy/milk traits ranging in heritability from low to through medium to highly heritable. The accuracy of genomic predictions and GWAS results in terms of size and location of SNP effects, reproducibility, and pleitrophic effects estimated from bull, cow, and combined bull and cow panels are presented for different SNP densities ranging from 3K through to 610K. Steps to improve the accuracy of GWAS through use of the false discovery rate (FDR) to identify relevant signals from the very high number of putative significant SNP which arise from such studies are presented.

Dutch Milk Genomics Initiative: towards implementation

Johan Van Arendonk Wageningen University, The Netherlands

Dutch Milk Genomics Initiative: towards implementation Johan van Arendonk, Robert Demeter, Marc Rutten, Marleen Visker and Henk Bovenhuis Animal Breeding and Genomics Centre, Wageningen University, the Netherlands Business Economics Group, Wageningen University, the Netherlands In the Dutch Milk Genomics Initiative we have shown that there is substantial genetic variation in content and composition of both milk fat and milk protein. Part of the genetic variation in milk-fat content and composition can be attributed to polymorphisms in genes such as DGAT1 and SCD1, while part of the genetic variation in milk-protein composition can be attributed to milk-protein variants. In addition, variation in both milk-fat and milk-protein content and composition has been allocated to several regions of the bovine genome, without actually knowing the genes involved. Based on these results we have demonstrated opportunities to implement genetic selection to modify milk-fat composition as well as milk-protein composition. Implementation of genetic selection for improved milk quality has far-reaching consequences for the dairy production chain (farmers, breeding organization, milk recording organization and dairy company). To predict economic implications for the farmers, we have developed a bio-economic optimization and simulation model. Consequences of various genetic selection strategies for the ratio of saturated to unsaturated fatty acids and for relative amount of casein have been simulated, of which some show an increase in herd profit, while others show a decrease in herd profit. These results can be used by the dairy industry to identify the economically most optimal strategy and to quantify the changes in the milk payment scheme which are needed to compensate the financial consequences of the different selection strategies. Genetic selection for milk-fat or milk-protein composition would benefit from large-scale collection of phenotypes on these traits. Therefore, we have developed methods to predict detailed milk composition using infrared spectroscopy. Milk-fat composition can be predicted quite accurately, while prediction of milk-protein composition is less precise. Based on these results, infrastructure is currently being established for large-scale collection of infrared profiles from routinely collected milk recording data. Data will be used to provide dairy farmers with management information about relative amounts of unsaturated fatty acids in milk of their cows. In addition, data can be used to estimate breeding values for milk-fat or milk-protein composition of sires.

Combining gene expression data with SNP association studies to identify genes effecting bovine milk production traits

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(Student Travel Award Recipient)

It has been argued that microarray technology is becoming somewhat obsolete and being superseded by the more recent methodologies of next generation sequencing (in particular RNA-seq). However, there are still many instances where the use of microarrays is preferable. When transcript profiling in model organisms with well-annotated genomes, microarrays have the advantage of having low-cost, short turn-around time, quantitative accuracy, robust sample processing and well-established analysis-pipelines. In this study, we aim to identify genes critical for bovine milk production by examining a series of cDNA microarray experiments conducted in a collaboration between AgResearch (New Zealand) and the Department of Primary Industries (DPI) Victoria. The experiments performed involved investigating changes in bovine mammary gene expression under a number of experimental conditions (both *in vivo* and *in vitro*) and are briefly outlined below:

- *In vivo* bromocriptine-mediated prolactin loss
- *In vitro* bovine mammary epithelial cells in response to prolactin and extracellular matrix

- Extended lactation program in cows examining difference between:
    - Early and late lactation
    - Persistent and non-persistent dairy cows
    - Cows in extended lactation with low, medium or high nutritional status
- Genetic merit experiment comparing cows of high and low genetic merit under normal conditions, when fasted for 36 hrs and when on 50% rations for 7 days
- Time since milking: 6hrs, 12hrs, 18hrs and 36hrs post milking in cows

These experiments were analysed and Expressed Sequence Tags (ESTs) displaying significant ($p<0.05$) differential regulation of 2-fold or greater were identified and annotated. Gene Ontology terms and pathways linked significantly to these EST lists were used to generate a biological profile of the mammary gland under each condition. In addition, EST lists were compared between experiments and ESTs displaying differential regulation under multiple conditions were identified. Single Nucleotide Polymorphisms (SNPs) falling within corresponding genes or 50/500 kb on either side of each gene were identified. These SNPs had previously been tested for an association with bovine milk production traits in genotyping experiments using an 800k (Illumina) SNP-chip. It was found that a number of the candidate genes contained SNPs that were significantly associated with bovine milk production and/or milk composition traits. These results are discussed and potential roles in milk production hypothesised.

Effects of *in utero* exposure to dietary conjugated linoleic acid on mammary gland development in Balb/cJ mice

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(Student Travel Award Recipient)

Conjugated linoleic acids (CLA) are isomers of octadecadienoic acid (18:2 n6) found in ruminant-derived food products, predominantly as *cis*-9, *trans*-11 CLA (9,11 CLA). Spreadable animal fat substitutes and weight loss supplements are generally mixtures of 9,11 CLA and *trans*-10, *cis*-12 CLA (10,12 CLA). These isomers have markedly different biological effects. Notably, 10,12 CLA intake reduces adiposity while inducing hepatic steatosis and insulin resistance. Both 9,11 CLA and 10,12 CLA have been proposed to afford anti-cancer properties given their ability to inhibit the growth of experimental cancers *in vitro* and *in vivo*. However, 10,12 CLA also enhanced mammary tumorigenesis in mice over-expressing the ErbB2 oncogene and mixed isomer supplementation increased tumor burden in mice that express polyoma virus middle-T antigen. These findings demonstrate the importance of resolving the effects of dietary CLAs on mammary gland (MG) development, particularly given their effects on metabolism. We therefore examined the effect of maternal intake of CLAs by studying the MG of female pups born to dams fed CLA during gestation. In one study, females were fed 1% 9,11 CLA or 1% 10,12 CLA throughout pregnancy. A subset of females was fed 10,12 CLA only during the first or second half of gestation. At birth, female pups born to these dams were cross-fostered onto dams fed the control diet during gestation. The MG of females exposed to CLA isomers *in utero* were analyzed at 21d, 35d, and 55d of age. Females exposed to 10,12 CLA *in utero* showed decreased ($P<0.05$) MG mass compared to control animals at 21d whereas there were no differences between progeny at 35d or 55d of age. Mass of the liver and uterus did not differ between treatment progeny at any time point. We subsequently investigated the effects of CLA exposure *in utero* on MG responsiveness to estrogen (E) and progesterone (P) either alone or in combination. Dams were fed either a control diet or that supplemented with 1% 9,11 or 1% 10,12 CLA during some or all of gestation, and female pups born to these dams were cross-fostered at birth onto foster dams fed the control diet. Pups were ovariectomized at 21d prior to the onset of puberty, and one week post-ovariectomy were treated with E, P, or E+P for 5 days. Mice were euthanized and their MG collected at 33d of age. Of the various maternal treatments, only female progeny exposed to 10,12 CLA demonstrated an increase ($P<0.05$) in MG mass following treatment with E. Results from these studies are significant in that they highlight how maternal intake of animal products such as dairy foods and weight loss supplements during pregnancy may impact postnatal development and hormone responses.

Wednesday, November 16th

Mammary gland biology: lessons from evolution

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Lactation has evolved in monotremes, marsupials and eutherians. by exploiting a diverse range of strategies. For example the monotremes are egg laying mammals and the mother begins to produce milk when the immature young hatch. Marsupials have a very short gestation and a relatively long lactation. They give birth to an altricial young and change the composition of milk progressively during lactation to regulate growth and development of the young. Therefore there is greater postnatal investment in development of the young in both marsupials and monotremes. In contrast, eutherians have a long gestation relative to lactation and the majority of development of the young occurs in utero. However, there is considerable adaption to lactation in eutherians. For example, the fur seal has a lactation characterised by a repeated cycle of long at-sea foraging trips (up to 28 days) alternating with short suckling periods of 2-3 days ashore. Lactation almost ceases while the seal is off shore but the mammary gland does not progress to apoptosis and involution.

It is now becoming apparent that milk plays a central role not only in providing nutrition and programming signals to the young but also has a role in regulating the development and function of the mammary gland.

Comparative genomics is providing opportunities to identify key genes regulating mammary gland development, milk production and composition. The application of this technology to a range of species with extreme adaptation to lactation allows the identification and study of regulatory mechanisms that are present but not readily apparent in other species, and also allows the identification of novel molecules and processes for application in the biotechnology market.

In-silico mapping of quantitative trait loci for lactation-associated traits in inbred mice

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Significant variation exists for fecundity and maternal nurturing ability in inbred mice. Classical genemapping approaches in mice have identified several quantitative trait loci (QTL) that account for some this variation. Current studies in our laboratory are aimed at identifying QTL genes which underlie variation in lactation-associated traits in the inbred mouse. The recent generation of high-density SNP databases is facilitating this work. Quantitative data for a panel of lactation-associated traits were collected from females representing each of 32 inbred strains (N=8 - 19 dams/strain) during the first 10 days of their second lactation. Average daily weight gain of crossfoster litters served as the primary indicator of milk production and was analyzed both for the limited subset of dams that successfully reared 10 pups/litter (ADG10) and for all dams regardless of pup rearing ability (ADG_ALL). The number of pups successfully reared to 8 days (PNUM8) also served as a related indicator of maternal ability and/or milk production. Additional lactation-related traits studied included, pups born, maternal body mass, maternal food intake, and milk composition, as well as two traits related to mitochondrial biogenesis and function, and three traits related to maternal behavior. All three of the milk production traits were significantly affected by strain ($P<0.0001$). The range of strain means was -0.71±0.63 to 4.59±0.24 g/day and 1.48±0.29 to 4.75±0.20 g/day for ADG_ALL and ADG10, respectively. For PNUM8, the range of strain means was 6.4±1.1 to 10.0±0.0. Both ADG10 and PNUM8 were highly correlated with ADG_ALL ($r>0.90$, $P<0.0001$). Several of the related maternal traits listed above were also significantly correlated with ADG_ALL. Haplotype association using a false discovery rate of 5% detected haplotype blocks containing 70, 3513, and 387 genes for ADG10, ADG_ALL, and PNUM8, respectively. Comparison of gene enrichment among the three traits revealed only 7 common genes between ADG10 and PNUM8, while there were 40 genes in common between ADG10 and ADG_ALL, and 225 in common between PNUM8 and ADG_ALL. Ontology analysis of the 225 genes in common between ADG_ALL and PNUM8 using IPA produced networks linked to cell signaling, carbohydrate metabolism, small molecule biochemistry, cell morphology,

cellular movement, and cellular assembly and organization. The most highly enriched cannonical pathway for this gene set was Erk5 signaling (P=10-5). With a more conservative genome-wide threshold of (10-5), haplotype associations to ADG10 were detected on 9 chromosomes with two strong associations on MMU13. The strongest (P=10-8) was with a block of 94 kbp which contained the gene, namely Emb. For ADG_ALL, There were also associations on 9 chromosomes. All but two of these were unique in comparison to ADG10. The strongest associations were detected using PNUM8. For this trait, associations were detected on 11 chromosomes. The strongest of these were found on MMU11, which contained a total of 11 associated (P=10-6 to 10-10) blocks. Genes within these blocks included Sec61g, Egfr, Krt17, and Krt42. A previously identified fecundity QTL (Pregq2) was also present. A block on MMU8 (P=10-9) contained the gene Nr3c2, which encodes for the mineralocorticoid receptor. A cluster of blocks on MMU9 overlapped with the previously described litter gain QTL, Neogq1 and contained 3 genes. Lastly, a block (P=10-7) on mMU19 contained the gene Gnaq, which encodes a quanine nucleotide binding protein that interacts with the Erk5 signaling pathway. These results suggest that in-silico haplotype association mapping is a useful tool for identifying the genes and possible even pathways which contribute to natural variations in milk production and other lactation-related traits. This work was supported by funds from the NICHD (#1R21HD059746-01A1), and from USDA cooperative agreement #58-6250-6001.

Matrigel signals abnormal development of Cape fur seal primary mammary cells, possibly through the activation of the TGFß pathway.

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(Student Travel Award Recipient)

The Cape fur seal is a sea mammal, belonging to the Pinnipedia family, who presents an unusual model of lactation. Indeed, contrary to the other sea mammals, the body of the fur seal is not big enough to store bubbler layer allowing it to remain on-shore for a long period of time without eating. Thus, this animal feeds its pup on-shore only for few days before foraging alone at sea for up to 4 weeks (cycle carrying out for about 10 months). Interestingly, during this foraging time, the mammary gland does not enter into the involution process despite the absence of sucking, which normally induces in all the other species the signalling of programmed death of the epithelial cells, bringing about to the return of the mammary gland to a virgin-like stage. This ability to switch “on/off” its milk production makes this animal an original model for the study of mammary gland biology, especially for the transition lactation to involution. Nevertheless, this study requires the adjustment of an in vitro model because of the limits on in vivo studies due to ethical considerations. Currently, MatrigelTM, a gelatinous protein mixture secreted by mouse sarcoma, is a commonly used biomatrix for the growth and differentiation of bovine and mouse primary mammary cells into mammospheres; a 3-dimensional acinar structure resembling the secretory alveolus in the mammary gland. These cells grow as a monolayer when cultured on plastic. In contrast, mammary cells from the Cape fur seal produce their own matrix when cultured on plastic to form mammospheres. However, when these cells are cultured on Matrigel they develop a stellate-like phenotype. In order to better understand the extracellular matrix mediated signaling that leads to correct polarization of mammary cells and mammosphere formation and investigate the effect of Matrigel on seal mammary cell differentiation, we conducted a microarray analysis on mammary cells before culture, mammospheres and stellate structures. The results show that the stellate phenotype is characterized by an activation of genes for the TGF-ß pathway which is well known for its role in cell development, migration and matrix formation. Surprisingly, these results contrast data using the mouse model in which the TGF-ß pathway was up-regulated in mammary cells on plastic, presumably to stimulate the cells to produce a matrix required for mammosphere formation. The results suggest there is a factor in Matrigel, which signals abnormal development of seal mammary cells resulting in a loss of polarization which is probably due to an epithelial-mesenchymal transition known to be stimulated by TGF-ß pathway. Thus, cape fur seal represents a new model to study the specificity of the matrix in normal cellular development and better understand cell-matrix interactions and signaling, mediating cell polarization and differentiation.

Market and Technology Opportunities in Gut Health

A novel strategy for functional synbiotics

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The probiotic story has been good for dairy sales and for the public perception of dairy as a healthy product category. However, a number of other market channels are surpassing dairy in terms of sales volume and in meeting diverse customer needs. For example, the supplements market continues to grow rapidly, in part because of its identification of market segments with specific gut health concerns and needs. Dairy growth has slowed partly due to a lack of claims, partly due a one size fits all product strategy. Generic probiotics are no longer enough - the technology must evolve to meet the diverse needs of a segmented market. Plus we now have tools to confirm efficacy for specific market segments and needs.

Recent scientific breakthroughs and tools will change how probiotic strains are selected and delivered. The genomic characterization of the human microbiome is a key to the rapid acceleration in research findings and proof of efficacy. Benchmarking of healthy versus dysbiotic microbiomes will occur independent of dairy industry investment. Dairy probiotic researchers should be prepared to use this new information to create a next generation of pro- and prebitics that can be delivered in dairy formats.

The global dairy industry is well positioned to support and even lead innovation in this field, with its ongoing support of cutting edge science in gut health. Dairy products also have great potential to be an optimal delivery system for functional probiotic solutions. To be participate in this opportunity, the industry must be willing to segment its markets, and support a wider range of products and claims. Technology companies, such as DSM, and academic researchers are ready to collaborate with the dairy industry to create the next generation of functional synbiotics.

A COMPARISON OF THE MAMMARY GLAND TRANSCRIPTOME AND MILK PROTEOME

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The global market for functional foods and nutraceuticals is growing substantially, with an estimated value in excess of $100bn. The development of these products is largely dependent on identification of naturally occurring bioactive molecules. Milk is a complex food and a rich source of biologically active proteins that provide an opportunity to develop functional dairy products.

Recent advances in bovine genome sequencing, and the development of post-genomic tools, has provided a mechanism to annotate the mammary gland transcriptome and identify potential novel protein-derived milk bioactives. We surveyed gene expression profiles from dairy cow mammary tissue collected during lactation, to classify expressed genes and to evaluate the capacity to translate the findings for milk protein detection.

Expressed genes from the mammary glands of lactating dairy cows were collated by analysis of microarray data. Bovine Affymetrix Gene Chips were used to probe ~20K genes and from these a permissive set of over 4,000 were selected for further consideration. The genes were then analysed through annotation pipeline that identified gene products with putative bioactive potential. Excluding caseins, a total of 175 genes were then classified for evaluation based on physiological properties. The greatest proportion were related to immunity and defense, or digestion and metabolism.

Milk proteins were analysed for comparison with genes detected in mammary glands, and annotated as physiologically active and potential bioactives. Whey proteins were extracted from public data bases or analysed directly from a commercial source. Direct analysis was performed using a combination of chromatography, electrophoresis and mass spectrometry. This analysis identified proteins with abundance down to 3% of total whey protein. These were

complemented by inclusion of more abundant proteins, and a total of 90 proteins were selected for evaluation and comparison with those annotated from expressed genes.

The comparison of 175 proteins predicted from the mammary gland gene expression data and 90 proteins from milk analysis identified 19 that were common to both sets. These were predominantly derived from abundant milk proteins but also included minor proteins or expressed The results suggest that there is some predictive value in transcriptomic-based approaches to detection of milk proteins, and that it provides a useful method for annotation. However with increased sensitivity of proteomic tools, direct approaches may provide sufficient levels of sensitivity for bioactive screening.

In-silico **approach to generating and protecting milk bioactives.**

*Nora Khaldi University College Dublin**, Ireland***

The extent of which bioinformatics approaches aid in detecting novel bioactive peptides is limited in many areas such as the food area. As part of Food for Health Ireland (FHI) we set out to predict bioactive peptides found in bovine milk proteins. The bioactivity of these predicted peptides was tested on 4 health pillars (such as metabolic health consisting in weight management & glycaemic control. And inflammation and antimicrobial activities) with the aim of developing new functional foods. We developed new techniques and methods to enable researchers to focus on the most meaningful areas in the sequences of milk whey and casein proteins. Currently there are no set methods to computationally predict bioactive peptides. We thus had to develop in-house techniques and methods for the bovine milk proteins. We used a combination of three techniques. Firstly, an evolutionary approach compared different mammalian milk proteins and cues in amino acid conservation and position in the sequence, relating these to protein structural constraints. Secondly, we used a similarity approach to other known bioactive peptides and combined this with amino acid composition and electro-properties of regions within the milk proteins. And finally, we examined the evolutionary enrichment of certain peptides in the evolution of milk proteins. Using these techniques we predicted 36 bioactive peptides in bovine milk proteins. Two of these displayed anti-microbial activity, and 2 had an impact on cellular signaling relating to Diabetes;3 inhibited ACE and 2 had effects on immune signaling. The success of these methods in identifying bioactive peptides from milk proteins indicates that computational predictions appear beneficial in accelerating the discovery of bioactive peptides from food.

Differences in pathogen-sugar interactions between human and bovine milk glycoproteins.

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Human milk is composed of a potent mixture of protective agents including free sugars, specific antibiotics and anti-microbial proteins that form the innate immune system to protect against microbial pathogens and disease. Bovine milk and formula-based products do not contain the same components. Milk contains sugar epitopes that are similar to the receptors on the gut epithelial surface that can competitively bind to and remove disease-causing microorganisms before they are able to attach to the gut and infect infants. An example of this includes the free oligosaccharide (α1,2)-fucosylated glycan in human milk, which is protective against *E.coli* induced diarrhoea in humans. However, we have shown that cow's milk contain a very different subset of glycan structures on its milk fat globule membrane glycoproteins when compared to human milk. Such differences in the glycosylation of different sources of milk potentially offer a variation in the microbes that the milk can protect against.

We will review the existing knowledge of bacterial binding to milk (cow and human) glycoproteins, glycolipids and oligosaccharides. We have further investigated how milk confers innate immune protection to the infant gut through binding of gastrointestinal-associated bacteria to human and bovine milk glycoconjugates and compare the difference in

binding of specific bacteria. We show that the sugars attached to breast milk glycoproteins change over lactation time and display many specific sugar antigens that are known to be involved in bacterial binding. We found that specific bacteria bind differently to human and bovine milk glycoconjugates and sugars are integral to that binding. These findings indicate the importance of milk glycoconjugates in binding to gastrointestinal-associated bacteria to provide innate immune protection to the infant's gut.

Sialic acid is involved in the differential binding of streptococcal species to milk and salivary glycoproteins

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The flow of human saliva constantly bathes the mouth and is thought to provide several mechanisms of innate immune protection against the huge number of micro-organisms to which the oral cavity is exposed; a similar mechanism is postulated for the oligosaccharides in milk as a protection against infant infection. *Streptococcus gordonii* is an early coloniser of human tooth enamel, whereas *S. mutans* colonises at a later stage in the development of the biofilm involved in the formation of dental caries. The glycoprotein component of saliva and milk is known to have antimicrobial properties and the attached sugar residues have been implicated in the interactions of salivary glycoproteins with bacteria. The binding of oral streptococci to salivary and milk glycoproteins and the involvement of terminal sialic acid residues in this pathogen-sugar interaction was investigated.

Binding of fluorescently-labelled *Streptococcus gordonii* F552 and *S. mutans* LT11 to saliva-coated hydroxyapatite crystals visually showed a stronger *S. gordonii* adherence to salivary glycoproteins. This difference in interaction of the two streptococci was confirmed and extended by quantitative measurement of the binding of these bacteria to both salivary and milk glycoproteins. Sialic acid residues on these proteins were demonstrated to be integral to streptococcal adherence to both human secreted fluids by i) altered binding of the two strains with pre-incubation of the bacteria with sialic acid, ii) washing of the bound bacteria with sialic acid and iii) enzymatic removal of sialic acid before bacterial binding. The two streptococcal strains demonstrated differential responses to the changes in glycoprotein sialylation in both saliva and milk. A rapid, facile assay of bacterial adherence to glycoproteins in the secreted fluids of saliva and milk allowed the contribution of terminal sialylation to be quantitatively determined. Our findings indicate that human saliva and milk bind similarly to common oral pathogens. *S. gordonii* binding to both milk and salivary glycoproteins was shown to rely on the presence of sialic acid residues on the glycoproteins, whereas *S. mutans* did not appear to use sialic acid epitopes for binding and bound more strongly with a decrease in sialylation.

The Glycobiology of Milk

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Human milk has been found to contain a high concentration and diversity of soluble oligosaccharides, carbohydrate polymers formed from a relatively small number of different monosaccharides that remarkably infants cannot digest. The structures and diversity of these oligosaccharides follow the general pattern of mammalian and primate evolution. The function that provides a selective advantage to their presence in milk has been shown to be the carbon source to a select group of intestinal symbiotic bacteria, notably Bifidobacteria longum biovar infantis. This bacterium is able to grow extensively on HMO as sole carbon source due to a unique genetic capability. The genomic sequence of this strain revealed a variety of co-regulated glycosidases, relative to other Bifidobacteria, implying a co-evolution of human milk oligosaccharide synthesis and the genetic capability of select intestinal bacteria to utilize them. Ongoing research has now addressed the genetic capabilities in mammalian mothers necessary to make them. Breakthroughs in methodology are driving this research including epithelial cell isolation from milk, sequencing methods capable of cataloguing the entire repertoire of expressed genes in these cells and mass spectrometry methods now able to identify all of the oligosaccharides produced by the cells. Analyses of the expressed genes mapped onto human and bovine genomes reveal that mammalian lactation has elaborated a complex system of glycan biochemistry to produce the abundant and complex group of glycan conjugates on proteins, lipids and as free oligosaccharides. The genetic diversity in humans and bovine has considerable potential to explain variation in human health and the translation of this knowledge to the industrialization to a new group of health-promoting components.

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Evolution of lactation: Nutrition versus protection

Peter Hartman- University of Western Australia, Australia

The evolutionary origin of the mammary gland has been difficult to establish because little knowledge can be gained on the origin of soft tissue organs from fossil evidence. One approach to resolve the origin of lactation has compared the anatomy of existing primitive mammals to skin glands, whilst another has examined the metabolic and molecular synergy between mammary gland development and the innate immune system. We have reviewed the physiology of lactation in five mammalian species with special reference to these theories. In all species, milk fulfils dual functions of providing protection and nutrition to the young and furthermore, within species the quality and quantity of milk are highly conserved despite maternal malnutrition or illness. There are vast differences in birth weight, milk production, feeding frequency, macronutrient concentration, growth rate and length of lactation between rabbits, quokkas, pigs, cattle and humans. The components that protect the neonate against infection do so without causing inflammation. Many protective components are not unique to the mammary gland and are shared with the innate immune system. In contrast many of the macronutrients in milk are unique to the mammary gland, have evolved from components of the innate immune system, and have either retained or developed multiple functions including the provision of nourishment and protection of the hatchling/neonate. Thus, there is a strong argument to suggest that the mammary gland evolved from the inflammatory response, however, the extensive protection that has developed in milk to actively avoid triggering inflammation seems to be a contradiction.

Reference:

McClellan, H.L., Miller, S.J. and Hartmann, P.E. Evolution of lactation: Nutrition v. protection with special reference to five mammalian species. *Nutrition Research Reviews* **21**(02): 97 – 116 (2008)

Thursday, November 17th

Proteomic profiling of defence-related proteins in bovine milk during an experimentally induced *Streptococcus uberis* infection

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Besides the major proteins, milk also contains other proteins of lesser abundance. In recent years a series of studies have applied a proteomic approach to characterising these low abundance proteins, revealing that milk contains as many as 200 distinct gene products, many of which have a function that is associated with host defence. We have now used a combination of proteomics technologies to characterise the repertoire of bovine milk proteins whose abundance is altered during the inflammatory response occurring during a mammary infection.

Milk was collected from infected and uninfected udder quarters of five cows with clinical mastitis experimentally induced by infusion of *S. uberis* into the gland via the teat. Whey, milk fat globule membrane and fraction enriched for basic proteins were prepared. Pools of these preparations were analysed using two different technical approaches; 2DE/MALDI-TOF and 1DE-LC-MS/MS (GeLC). The 2DE analysis resulted in a set of 182 spots that were altered in abundance between control and infected quarters. Of these, 136 were identified by MALDI-TOF. These correspond to 52 distinct gene products, of which 24 have functions related to host defence. The GeLC analysis of the fractions identified 189 proteins, which included all but six of the proteins identified by 2DE. In total, the two analyses identified 195 distinct gene products in the three fractions, of which 77 were found in whey, 113 in the milk fat globule membrane and 95 in the basic fraction. Over a third of the identified proteins (70 out of 195) have functions that are associated with host defence. In total, 45 % of the proteins identified (88 out of 195) were apparently altered between control and infected samples. These proteins may have a role in responding to the intramammary infection. To validate these results, the relative abundance of five of these infection-responsive proteins (lactoferrin, cathelicidin, CG39, S100A9, and S100A12) was estimated in the individual milk samples contributing to each pool using quantitative western blotting.

Taken together the results reveal the complexity and dynamic nature of the proteome of bovine milk as well as the degree of responsiveness to infection. Thus, they underscore the function of milk as a host defence secretion.

Characterization of the differences in the host defense proteome of human and bovine milk

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Milk is the single source of nutrients for the newborn mammal. During evolution, the composition of milk of different mammals has been adapted to fulfill the needs of the newborn. Milk not only provides nutrients, but also serves as a medium for transfer of host defense components to the newborn. The host defense proteome of milk from different mammalian species is expected to reveal signatures of evolution. Because bovine milk is used as a substitute for human milk, it is important to know the differences in host defense proteome between human and bovine milk. Despite the description of several differences between human and bovine milk, there is limited knowledge on differences in the host defense proteome. In addition to knowledge on the qualitative and quantitative differences in host defense proteome, it is also important to understand the biological implications of these differences. For studying the qualitative and quantitative differences, human and bovine milk samples were divided in two protein fractions: milk serum and milk fat globule membrane. For qualitative experiments, the proteins were separated using SDS-PAGE, followed by in gel digestion of the proteins. For quantitative experiments, the samples were prepared in five-fold using FASP [1]. The peptides obtained after both sample preparation methods were studied using LC/MSMS (FT-MS Orbitrap). Proteins were identified using the human and bovine IPI database. For quantification, the peak height of the 3 most abundant peptides

were summed for all proteins. The main results of this study have recently been published [2]. In short, 268 proteins in human milk and 269 proteins in bovine milk were identified. Of these, 44 from human milk and 51 from bovine milk are related to the host defense system. Proteins involved in the mucosal immune system (immunoglobulin A, CD14, lactoferrin, and lysozyme) were present in high concentrations in human milk. On the other hand, antimicrobial proteins (5 cathelicidins and lactoperoxidase) were abundant in bovine milk. These differences in the host defense proteome are thought to be related to developmental differences between human and bovine newborns. This first detailed comparison of the host defense proteome of human and bovine milk is an important first step in understanding the function of milk in immune system development. However, to better understand the implications of these proteomics findings, it is necessary to further characterize the host defense proteome. To start with, we studied the variability in the concentration of these proteins in both human and bovine milk. This determination of the variability of the host defense proteome may help in understanding which proteins are evolutionary conserved: these proteins are probably more important to the newborn. The function of selected host defense proteins will next be elucidated using biological assays followed by in-vivo studies. Finally, the physical-chemical stability of these proteins will be determined. Knowledge on the stability is especially useful if these proteins are to be used as bioactive ingredient. In my presentation, I would like to discuss the proteomics results we have obtained up till now, as well as our plans for further characterization of the host defense proteome in milk. [1]Wiśniewski JR, Zougman A, Nagaraj N, Mann M. 2009. Universal sample preparation method for proteome analysis. Nature Methods 6(5): 359-362 [2]Hettinga K, van Valenberg H, de Vries S, Boeren S, van Hooijdonk T, et al. (2011) The Host Defense Proteome of Human and Bovine Milk. PLoS ONE 6(4): e19433

Long-term effects of nutrition on mammary gland development and milk composition leading to offspring predisposition to obesity

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Mammary epithelial growth and differentiation are tightly modulated by several hormonal and metabolic signals (Hennighausen and Robinson, 2005). Altered nutrition, leading to differences in the body weight, during the major developmental steps of this organ may be of critical importance (Sejrsen, 1994). In order to understand the impact of nutrition on mammary gland development and lactation, changes to the nutritional status of various animal models have been made in order to alter the metabolic environment. In cattle, an increased growth rate due to a high feeding level during puberty has been shown to reduce mammary epithelial cell proliferation in areas of active ductal expansion and thus limit mammary development and subsequent milk potential (Davis Rincker et al., 2008; Sejrsen et al., 2000). The effects of obesity on lactogenesis have also been demonstrated in the rat, where this pathology was shown to affect the chances of a successful outcome to pregnancy and lactation (Rolls et al., 1984). Similarly, in mice, diet-induced obesity resulted in lactation failure. More specifically, obese mice displayed marked abnormalities in alveolar development of the mammary gland during pregnancy, together with a marked decrease in major milk protein expression (Flint et al., 2005). Moreover, during earlier stages of development (puberty), obesity was shown to disrupt mammary ductal growth by reducing the branching frequency and width of ducts (Kamikawa et al., 2009). In humans, obesity is considered to be a major worldwide health issue and a predisposing risk for the morbidity of type 2 diabetes, hypertension and cardiovascular diseases. Obesity has also been strongly correlated with an increased risk of mammary tumorigenesis (Stoll 2000). Furthermore, in the United States, Chapman and Pérez-Escamilla noted that women who were overweight or obese at the time of childbirth were at significant risk of failing to initiate successful lactation, or were no longer breastfeeding at two days postpartum (Chapman and Perez-Escamilla, 1999). Similarly, a European study of obese women (BMI >26 kg/m2) found an association between obesity and an early cessation of breastfeeding (Riva et al., 1999) and a decrease in the normal prolactin response to suckling (Rasmussen and Kjolhede, 2004). We have further investigated the impact of obesity on mammary gland development. During this study, we used a model of rabbits

receiving an obesogenic diet (OD rabbits), starting before puberty and extending until mid-pregnancy. At mid pregnancy we have shown that the body weight of OD animals was significantly higher than that of animals fed the control diet (C rabbits) and their mammary glands displayed a precocious and abnormal development at mid-pregnancy. These results support the critical influence of nutrition on mammary growth and differentiation, which may be deleterious to subsequent lactation. We have recently studied lactation of those animals. Milk production was equivalent between the two groups. However, lipid content of milk was 6-fold increased in OD rabbits. Moreover milk leptin concentration was significantly higher in OD animals. The growth of the offspring was not altered until weaning, but pups fed with OD milk and receiving an OD diet (OD/OD rabbits) exhibit a further increase in the body weight as compared to those fed control milk and the same OD diet (C/OD rabbits). Moreover, OD/OD females had on Day 8 of pregnancy an abnormal development of the mammary gland. Secretory tissue was scarce, embedded in adipose tissue and presented huge luminal structures filled with dense products. Lactation of these females is currently under study. Results will provide an insight on effects of maternal obesity on lactation and on growth of their offspring. They might thus lead to breastfeeding recommendations to obese women.

NutriChip – a technological platform for the selection of healthy dairy products

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Advances in food transformation have dramatically increased the diversity of products on the market and, consequently, exposed consumers to an increasingly complex spectrum of bioactive nutrients whose potential risks and benefits have mostly not been confidently demonstrated. Tools are therefore needed to efficiently screen food products for selected physiological properties before these products enter the market.

NutriChip is an interdisciplinary modular project funded by the Swiss program Nano-Tera that bundles scientists from several research institutions with the aim of developing analytical strategies that will enable a functional screening of foods.

We selected dairy products as a demonstrator for this project because milk benefits from the advantage of selective evolution (*i.e.* it possesses well recognized nutritive and immunomodulatory properties), is easily amenable to technological and microbial transformation, and is a natural vector for the delivery of bacteria and fermented metabolites to the gut. We also focus our efforts on the postprandial inflammatory stress induced by food as this phenomena potentially contributes to the development of chronic inflammatory diseases and can be satisfactorily modeled *in vitro*. As diets rich in saturated fat promote an inflammatory response in humans, in particular in subjects with a metabolic deregulation, a high-fat meal was selected as a pro-inflammatory diet against which dairy products can be compared.

The first module of the NutriChip project is composed of a series of biochemical *in vitro* steps that mimic the digestion, intestinal absorption and subsequent modulation of immune cells by the bioavailable nutrients. To this end, we first characterized undigested dairy products at the molecular level, focusing on their proteomes. We also developed a method to identify the bacterial proteins present in fermented dairy products. The dairy products where then digested using a three-steps model (oral, gastric, intestinal) and the molecules resulting from this digestion where extensively characterized. This analysis underscored the striking capacity of the digestive system to efficiently extract essential amino acids from the pool present in dairy proteins. The digested dairy products were subsequently exposed to the apical side of a confluent layer of intestinal cells to allow a molecular characterization of the transport of the nutrients present in the digested food matrix, such as bioactive peptides and minerals, across the gastrointestinal barrier. The

modulation of the immune system underlying the gastrointestinal tract is finally modeled by measuring the inflammatory response of differentiated monocytic cells grown on the basolateral side of the intestinal cells.

The second module is the development of a Lab-on-a-Chip that integrates engineering technologies, e.g. microfluidics, CMOS optical detection nanotechnology, and superresolution software algorithms, to the co-culture system to allow a sensitive, high throughput, and real time analysis of the immunomodulatory properties of the dairy products to be screened *in vitro*.

The third module aims at validating the *in vitro* screening model by assessing the metabolic (glucose, insulin, lipoproteins, triglycerides) and immunomodulatory (inflammatory cytokines) properties of milk in humans. To this end, a series of nutritional intervention studies are underway that compare fasting and postprandial blood parameters after single and repeated ingestions of increasing doses of milk and the high-fat meal in healthy subjects as well as in subjects with a metabolic disorder.

To allow an iterative development of the NutriChip, the classical molecular parameters measured in humans and in the *in vitro* studies will be complemented by omics technologies (e.g. transcriptomics, proteomics and metabolomics) on immune cells, plasma and cell culture media in order to identify bioavailable nutrients (nutrikinetics) and biological pathways (functional genomics) that are shared between the *in vivo* and *in vitro* models, thus allowing the future screening of dairy products with a set of physiologically relevant biomarkers.