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Associations between Circulating IGF-1 Concentrations, Disease Status and the Leukocyte Transcriptome in Early Lactation Dairy Cows

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Abstract: Peripartum dairy cows commonly experience negative energy balance (EB) and immuno-suppression together with high incidences of infectious and metabolic disease. This study investigated mechanisms linking EB status with immune defense in early lactation. Data were collected from multiparous Holstein cows from six herds and leukocyte transcriptomes were analyzed using RNA sequencing. Global gene expression was related to circulating IGF-1 (as a biomarker for EB) by subdividing animals into three groups, defined as IGF-1 LOW (<35 ng/mL, $n = 35$), MODERATE (35–100 ng/mL, $n = 92$) or HIGH (>100 ng/mL, $n = 43$) at 14 ± 4 days in milk (DIM). Differentially expressed genes between groups were identified using CLC Genomics Workbench V21, followed by cluster and KEGG pathway analysis, focusing on the comparison between LOW and HIGH IGF-1 cows. LOW cows were older and had significantly lower dry matter intakes and EB values, whereas HIGH cows produced more milk. During the first 35 DIM, 63% of LOW cows had more than one health problem vs. 26% HIGH cows, including more with clinical mastitis and uterine infections. Gene expression analysis indicated that leukocytes in LOW cows switched energy metabolism from oxidative phosphorylation to aerobic glycolysis (*PGM*, *LDH*, and *PDK4*). Many antimicrobial peptides were up-regulated in LOW cows (e.g., *PTX3*, *DMBT1*, *S100A8*, and *S100A9*) together with genes associated with inflammation, platelet activation and the complement cascade. HIGH cows had greater expression of genes regulating T and B cell function and the cytoskeleton. Overall, results suggested an ongoing cycle of poor EB and higher infection rates in LOW IGF-1 cows which was reflected in altered leukocyte functionality and reduced milk production.

1. Introduction

The requirement of the mammary gland for metabolizable energy and protein in the immediate post-calving period exceeds intake by approximately 25% [1]. This results in reduced availability of amino acids and glucogenic compounds, whereas ketogenic and lipogenic compounds are generally increased [2–4]. During this period, dairy cows mobilize both body fat and muscle in support of lactation, with higher rates of mobilization in animals carrying more body condition at calving [5]. The associated period of negative energy balance (NEB) may last for several weeks, extending into months in severe cases [6]. During this period, the liver co-ordinates the extensive changes in metabolic pathways required via up-regulation of genes involved in fatty acid oxidation and gluconeogenesis, and down-regulation of triacylglycerol synthesis [7,8]. This may be associated with the development of hepatic steatosis, which compromises glucose production and increases inflammatory responses [9].

Dairy cows also experience changes to their somatotropic axis at this time. Pituitary-derived growth hormone (GH) usually induces hepatic IGF-1 synthesis. When cows are in NEB, however, they become insulin resistant and down-regulation of the hepatic GH receptor causes IGF-1 synthesis to fall, so reducing circulating IGF-1 [10,11]. One effect of this uncoupling is to facilitate the indirect actions of GH on lipolysis and gluconeogenesis during early lactation [12]. At the same time, local support from IGF-1 in peripheral tissues towards cell metabolism and mitosis is reduced [13,14]. The IGF-1 concentration in lactating cows reflects their nutrient intake, showing a positive correlation with both energy and protein balance [15–17]. Such nutritionally driven changes occur over days rather than hours, so the IGF-1 concentration in blood is relatively stable [15,18]. This makes it a more reliable metabolic marker than non-esterified fatty acids (NEFA), beta-hydroxy butyrate (BHB) and glucose, whose concentrations all alter with respect to the time of feeding [18,19].

The somatotropic axis is also known to have an important relationship with the immune system. This is modulated via peripheral resistance and down-regulation of receptors, disruption in the GH/IGF-1 signaling pathways, and dysregulation of IGF-binding protein production, which in turn alters IGF bioavailability [20]. Immune cells including peripheral blood mononuclear cells (PBMC), T- and B-lymphocytes, and NK-cells all express the IGF-1R and are therefore responsive to the effects of IGF-1 [21,22]. This can influence innate immunity, for example by modulating differentiation and proliferation of myeloid lineage cells and influencing the responsiveness of mature immune cells to antigens. For example, experiments using peritoneal exudative cells in mice found that IGF-1 could enhance phagocytosis and their ability to kill *E. coli* [23]. More recently, IGF-1 was shown to potentiate pro-inflammatory cytokine production induced by microbial TLR ligands in PBMC [24]. IGF-1 also affects acquired immunity via proliferative, differentiating and functional effects on mature and maturing lymphocytes in primary and secondary lymphoid organs [21,22]. Circulating IGF-1 is thus a good indicator of metabolic status in the early postpartum period [6,15,25] and is also known to prime the response of immuno-competent cells to deal with infection and respond to sepsis and tissue injury [21,22]. IGF-1 was therefore chosen as a suitable candidate biomarker to investigate the links between metabolism and infectious disease in this study.

Immune function is known to be suppressed over the peripartum period. The numbers of circulating leukocytes, including all subset T cell populations, decrease in the first two weeks after calving followed by a gradual recovery [26–28]. The functional capacity of blood neutrophils is reduced, in particular their ability to generate reactive oxygen species (ROS), and this is strongly associated with the liver triacylglycerol content and

raised concentrations of NEFAs [29–32]. Genes important for granulocyte recruitment and interferon signaling were down-regulated in neutrophils from cows with elevated NEFA and BHB levels after calving [29,33]. There are also strong indications that cows are under increased oxidative stress at this time [34].

Mounting an effective immune defense is energetically demanding, as immune cells require an adequate supply of glucose, amino acids, fatty acids and cholesterol/oxysterols for proliferation and the production of immune molecules [35–37]. Neutrophils contain relatively few mitochondria, so are mainly reliant on glucose to fuel glycolytic energy production but, during an infection, pathogenic bacteria compete for a limited glucose supply [37]. Clonal expansion of T cells in response to antigen recognition requires dynamic changes in their metabolism [38,39]. As macrophages develop into a more pro-inflammatory phenotype, there is a shift towards enhanced glucose uptake and aerobic glycolysis, whereas anti-inflammatory M2 macrophages have lower glycolytic rates and higher levels of oxidative metabolism [36,40]. Both the innate and adaptive immune systems are thus affected, with reduced responsiveness of circulating T cells to mitogenic agents and less production of immunoglobulin by B cells [41]. The impairment in immune function in peripartum cows is, therefore, almost certainly linked to the reduction in nutrient availability [42].

Another contributing factor to the altered immune environment is the birth process itself. The postpartum uterus must undergo extensive remodeling to reduce in size, remove cellular debris, and restore normal architecture [43]. In 90% of cows, this early period of involution is accompanied by microbial contamination which results in uterine disease in up to half of animals [44]. The incidences of other infectious diseases including mastitis are also high in the early postpartum period [45–47]. During such an infection, neutrophils are rapidly recruited to the affected site(s) to kill the invading pathogens using a wide variety of mechanisms including degranulation, ROS production, phagocytosis and release of cytokines and chemokines to recruit additional leukocytes. If the local inflammatory response to an infection is insufficient to control it, then a systemic acute phase response is induced which may include fever, leukocytosis and altered expression of acute phase proteins [9,48]. This co-ordinated reaction is triggered by both pathogen-associated molecular patterns (PAMP) and host damage-associated molecular patterns (DAMP) [49,50].

The high disease incidence frequently found in early lactation adversely affects cow welfare, reduces milk production capacity and fertility and increases the likelihood of culling [1,35]. This has raised the need to improve our understanding of the links between nutrition, metabolism and the immune system during the transition period. Such adverse consequences are more common in multiparous cows [51]. In comparison with primiparous cows, these also exhibit differences in their metabolic profiles in early lactation, having lower circulating IGF-1 and higher BHB concentrations [52]. The aim of this study was thus to investigate the relationship between circulating IGF-1 (used as a biomarker of metabolic status), disease incidence and the transcriptomic profiles of leukocytes from high yielding multiparous dairy cows in early lactation.

2. Materials and Methods

2.1. Animals

Multiparous Holstein cows were recruited from six experimental farms located in the UK (Agri-Food and Biosciences Institute Hillsborough, Northern Ireland, *n* = 44); Denmark (Aarhus University, *n* = 23); Ireland (University College Dublin, *n* = 35); Germany (Leibniz Institute for Farm Animal Biology, FBN, *n* = 19); Belgium (Walloon Agricultural Research Centre, *n* = 18); and Italy (Consiglio per la Ricerca in Agricoltura a l'Analisi, 18 dell'Economica Agraria, CREA, *n* = 37). This provided a starting population of *n* = 176. All procedures had local ethical approval and complied with the relevant national and EU legislation under the European Union (Protection of Animals used for Scientific Purposes) Regulations 2012 (S.I. No. 543 of 2012). All cows were milked twice daily and their daily yields were recorded. Milk samples were collected twice weekly until 50 days in milk (DIM) and were analyzed for composition of protein, fat, and lactose. Body

weights (BW) were recorded at least twice weekly using weigh scales. Further details of the management of each herd are provided in [53]. The average milk yield for all six herds over the 7 week study period averaged 33.3 ± 9.3 kg/day, increasing from 25.8 ± 8.1 kg/day in week 1 to 36.1 ± 9.3 kg/day in week 7 [53]. For individual herds, the milk yield in week 2 (the time of leukocyte sample collection) ranged from 28.6 ± 1.10 kg/day (CREA) to 37.3 ± 2.01 kg/day (FBN) [33].

2.2. Measurement of IGF-1 and Metabolites in Blood

Blood samples were collected by jugular or tail vein venepuncture to obtain plasma (Na heparin tubes) and serum (plain tubes), separated by centrifugation and stored at −20 °C. Concentrations of IGF-1 were determined in serum by radioimmunoassay at University College Dublin, Ireland following acid-ethanol extraction [54]. Intra-assay coefficients of variation (CV) were 12.4, 7.5 and 9.9% for low, medium and high control samples, respectively. The corresponding inter-assay CV were 7.8, 3.9 and 9.4%. The sensitivity of the assay, defined as the lowest concentration detectable, was 4 ng/mL. NEFA, BHB, glucose and fructosamine analysis was performed at the Department of Animal Science, Aarhus University, Denmark [55]. Urea was determined in plasma according to standard procedures using an auto-analyzer (ADVIA 1800® Chemistry System, Siemens Medical Solutions, Tarrytown, NY, USA). Intra- and inter-assay CV for metabolite measurements were below 3% and 4%, respectively, for both low and high control samples.

2.3. Milk Sampling and Analysis for Metabolites and Enzymes

Additional milk samples (two × 8 mL) were collected twice weekly during morning milking and stored at −20 °C for subsequent analysis at the Department of Animal Science, Aarhus University, Denmark. The results from those taken closest to 14 DIM were used in this study. Fluorometric end point analyses were used to determine milk glucose and glucose-6-phosphate, uric acid, isocitrate and BHB as described by [53]. Urea was determined by spectrophotometry [56]. The indigenous enzymes lactic dehydrogenase (LDH, EC. 1.1.1.27) and N-acetyl-β-D-glucosaminidase (NAGase, EC 3.2.1.30) were analyzed by fluorometric assays [57,58]. Intra- and inter-assay CVs were in all cases below 5% and 8%, respectively, for both low and high control samples.

2.4. Calculation of Energy Balance

Records required for energy balance (EB) calculations at approximately 14 DIM were available from four herds in this study. For these, daily dry matter intakes were recorded using electronic feeding systems. Weekly ration samples collected over the course of the sampling period were dried and shipped for NE_L analysis in a single run (Cumberland Valley Agricultural Services, Franklin County, PA, USA). The energy input was calculated by multiplying the weekly NE_L density of the ration with the daily dry matter intake (DMI) of the animal. The energy output was calculated according to the National Research Council guidelines [59] correcting for NE_M using the last recorded body weight of the animal and the most recent milk fat, protein and lactose content together with the daily milk yield for the NE_L. The energy corrected milk (ECM) yield was adjusted to 3.5% fat and 3.2% protein using the formula ECM = (0.327 × milk kg) + (12.95 × fat kg) + (7.65 × protein kg). The energy balance (in MJ/day) was determined as described previously [53].

2.5. Health Records

An illustrated scoring system covering uterine disease, mastitis and lameness was agreed in advance in order to promote consistency in diagnoses between herds. Uterine discharge and odor were assessed at approximately 14 and 35 DIM as described previously [60]. In brief, the discharge was sampled from the vagina and assessed for colour, proportion and volume of pus, and scored as: 0, clear or translucent mucus; 1, mucus containing flecks of white or off-white pus; 2, <50 mL exudate containing over 50% white or off-white mucopurulent material; or 3, >50 mL exudate containing purulent mate-

rial, usually white or yellow, but occasionally sanguineous. Discharge odor was given a score 0 for normal or 1 for fetid. Lameness was scored at the same time based on a nine point scale as previously described [61]. Milk quality was assessed based on the somatic cell count (SCC) and cows were subsequently categorized into three groups as described previously [33]. Healthy cows were defined as having a SCC <100,000 cells/mL milk with no clinical symptoms. Subclinically mastitic cows were defined as having a SCC between 100,000 and 400,000 cells/mL milk with no apparent clinical symptoms. In the cows diagnosed as having clinical mastitis, their SCC was >400,000 cells/mL milk and they displayed clinical symptoms. Other diseases were classified based on the ICAR Central Health Key (https://www.icar.org/index.php/publications-technical-materials/amendments-recording-guidelines/diseases-codes-for-cows/ last accessed on 15 November 2021). Clinical diagnoses were made and recorded by farm staff and any subsequent treatments followed the normal management practices in each herd.

2.6. RNA Extraction

Blood samples at approximately 14 DIM were also taken into Tempus blood collection tubes (Thermo Fischer, Loughborough, UK), which were shaken vigorously for 15–20 s immediately upon collection, then frozen and stored at −80 °C for RNA sequence analysis (RNA-seq). All chemicals were obtained from Sigma Aldrich (Poole, Dorset, UK) unless stated otherwise. Whole-blood extraction used Tempus Spin RNA isolation Kits (Thermo Fischer, Loughborough, Leics, UK) following the manufacturer's instructions as described previously [8]. RNA quantity and integrity were assessed using an Agilent BioAnalyzer 2000 (Agilent Technologies UK Ltd., Cheadle, UK) and Agilent RNA 6000 Nano Kit (Agilent Technologies UK Ltd., Cheadle, UK). RNA measurements were also validated using a NanoDrop 1000 (Thermo Fischer, Loughborough, Leics, UK). All selected RNA sample had a reasonable integrity (RIN number >7) and purity (260/280 between 1.8 and 2.3). The RNA was stored at −80 °C for subsequent sequencing.

2.7. RNA Sequencing

RNA-seq of the extracted whole-blood samples was performed at the University of Liege, GIGA Research Facility, Belgium. RNA-seq libraries were prepared using an epMotion liquid handling workstation (Eppendorf, Hamburg, Germany) with the Illumina TruSeq Stranded Total RNA Library Prep Ribo-Zero Gold kit (Illumina, San Diego, CA, USA). Pooled cDNA libraries were sequenced on an Illumina NextSeq 500 machine (4 lanes multiplexed) at 75 nucleotide length single-end reads to reach an average 30 million reads per sample. Raw data were stored as FASTQ files (Sanger/Illumina 1.9 [Phred + 33]) in ENA accession numbers ERP124149 and ERP122911. Sequence analysis was performed with a CLC Genomics Workbench V21 (Qiagen Digital Insights, Redwood City, CA, USA) based on the built-in workflows in its toolbox of RNA Sequencing Analysis. This included trimming the poor-quality reads, quality control and mapping the reads to a reference genome of *Bos taurus* assembly for both genes and transcripts (ARS-UCD1.2, Genbank). The sequencing was quantified as reads per genes and reads per kilobase of transcript per million mapped reads (RPKM) and stored in CLC Genomics Workbench as gene expression (GE) files.

2.8. Relationship between Gene Expression with Circulating IGF-1

The GE files derived from each sample were used for analysis of differential gene expression in leucocytes collected approximately 14 DIM using CLC Genomics Workbench V21 (Qiagen Digital Insights, Redwood City, CA, USA). Global gene expression was related to the circulating IGF-1 concentration measured in blood samples taken at the same time. For this, cows were subdivided into three groups defined as low IGF-1 < 35 ng/mL (LOW, $n = 35$), moderate IGF-1 35–100 ng/mL (MOD, $n = 92$) and high IGF-1 > 100 ng/mL (HIGH, $n = 43$). These thresholds were selected based on our previous work showing associations between the IGF-1 concentrations in early lactation with the likelihood of developing

endometritis [62] and subsequent fertility [63]. Four cows did not have an IGF-1 value, so they were removed from this study. Principal component analysis with normalized RNA-seq reads expressed as RPKM showed that the overall expression values of two cows were markedly different from the others. These were considered as outliers and excluded in the subsequent analysis. This left 170 cows in the analysis. The expression values were normalized with trimmed mean and Z-score normalizations across all samples. Gene expression levels were then compared between the IGF-1 groups using negative binomial generalized linear modeling. IGF-1 groups were set as the test variable and herd as the controlling variable to minimize the differences of gene expression arising from the differences of herds. False discovery rates (FDR) for multiple tests were controlled with the Benjamini–Hochberg (BH) adjustment and significance was considered at p(BH) < 0.05. Our previous studies of leucocyte gene expression [8] showed that a sample size of 15 cows per analytical group achieved 85% power at $p < 0.05$ with FDR correction to detect the true differential expression with fold changes > 1.2 between group pairs. Differentially expressed genes (DEG) with p(BH) < 0.05 and absolute fold change > 1.2 in pairwise comparisons between the three IGF-1 groups were selected for subsequent analysis. The DEG were taken forward for pathway analysis using DAVID bioinformatics resources version 6.8 (https://david.ncifcrf.gov/ accessed on 16 Aug 2021) [64,65] with *Bos taurus* as background. Fisher's exact test with BH adjustment was used and statistical significance was considered at p(BH) < 0.05. An overview of the study design and data analysis is provided in Figure 1.
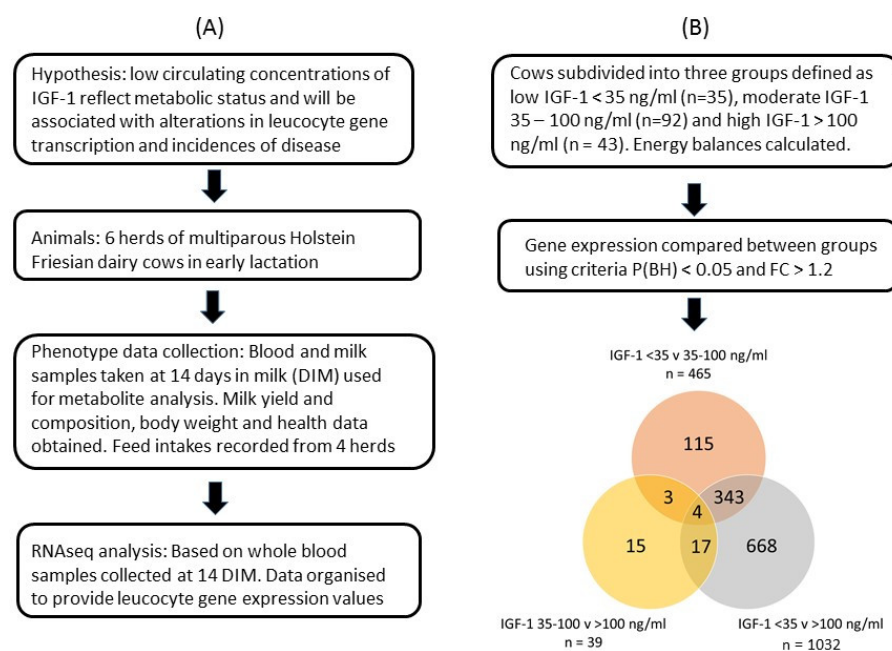

Figure 1. Overview of (**A**) the study design and (**B**) the data analysis. Venn diagram indicates numbers of differentially expressed genes (DEG) identified in each group and their overlap between groups.

2.9. Statistical Analysis

The values for milk yield and composition, blood and milk metabolites and EB at 14 DIM were expressed as the mean ± standard error of mean (SE). Differences between the IGF-1 groups were compared with one-way analysis of variance (ANOVA) using SPSS V27 software package (Chicago, IL, USA). The homogeneity of variance for each variable was assessed with Levene's test. Where homogeneity was not achieved, logarithmic transform was performed before ANOVA. Where ANOVA showed significance, multiple comparisons with Tukey's HSD test were performed to identify the source of differences. Health data

were tabulated and analyzed using Pearson's chi-squared test. In both cases significance was considered where $p < 0.05$.

3. Results

This paper relates to the results obtained from blood samples collected from each cow at 14 ± 4 days DIM and health records relating to calving and the first 5 weeks postpartum. The blood samples were used for: (i) measurement of circulating IGF-1 and metabolite concentrations and (ii) RNA-seq analysis of whole-blood leucocyte populations.

3.1. Cow Data

Cows were subdivided into three groups based on their circulating IGF-1 concentration. Those in the LOW group had significantly higher parity, averaging 3.9 ± 0.27 lactations in comparison with 2.6 ± 0.17 for the HIGH cows (mean $\pm$ SEM, $p < 0.001$, Table 1). Cows in the HIGH group had significantly higher milk yields by the second week after calving. Their milk also contained more protein, had a slightly lower fat:protein ratio and a higher lactose content compared to LOW group cows (Table 1). The energy corrected milk 7 day averages for week 2 did not, however, differ between IGF-1 groups (LOW, 38.0 ± 1.29 kg; MOD, 39.3 ± 0.95 kg; HIGH, 41.9 ± 1.34 kg; $p = 0.119$).

Table 1. Lactation number, milk yield and milk quality data from weeks one and two of lactation according to the IGF-1 group as measured in plasma at 14 days in milk.

| | IGF-1 Group (ng/mL) | | | |
	LOW < 35	**MOD 35–100**	**HIGH > 100**	p [‡]
Week 1				
n	32	84	38	
Lactation No.	3.9 ± 0.27 [b]	3.2 ± 0.11 [a]	2.6 ± 0.17 [a]	<0.001
Milk yield (kg/d)	29.2 ± 1.32	31.0 ± 0.76	31.7 ± 0.67	0.21 [#]
Fat (F%)	4.9 ± 0.20	4.8 ± 0.12	4.5 ± 0.10	0.40
Protein (Pr%)	3.5 ± 0.08 [a]	3.5 ± 0.04 [a]	3.9 ± 0.05 [b]	<0.001
F:Pr	1.4 ± 0.06 [b]	1.4 ± 0.04 [b]	1.2 ± 0.03 [a]	0.002
Lactose (%)	4.5 ± 0.05 [a]	4.5 ± 0.02 [ab]	4.6 ± 0.03 [b]	0.026
Week 2				
n	32	82	31	
Milk yield (kg/d)	30.2 ± 1.24 [b]	33.3 ± 0.79 [ab]	36.6 ± 1.13 [b]	0.001
Fat (F%)	4.4 ± 0.14	4.5 ± 0.11	4.2 ± 0.15	0.23
Protein (Pr%)	3.1 ± 0.05 [a]	3.2 ± 0.03 [a]	3.4 ± 0.05 [b]	<0.001
F:Pr	1.4 ± 0.04 [b]	1.4 ± 0.04 [b]	1.2 ± 0.03 [a]	0.009
Lactose (%)	4.6 ± 0.04 [a]	4.7 ± 0.02 [a]	4.8 ± 0.03 [b]	0.014

[‡] Different superscript letters in rows indicate a significant difference using Tukey's HSD test, a < b. [#] Data log-transformed for analysis to achieve homogeneity of variance.

Blood metabolite measurements made at the same time as the IGF-1, at approximately 14 DIM, showed that the HIGH cows also had higher circulating concentrations of glucose, fructosamine and urea than the other two groups (Table 2). NEFA concentrations were, however, greatest in the LOW group, with a trend towards raised concentrations of BHB. These findings were supported by milk metabolite measurements, which showed higher concentrations of free glucose, urea and uric acid in the HIGH group, whereas milk BHB and isocitrate were significantly raised in the LOW IGF-1 cows (Table 2). Cows from four of the six herds used in this study ($n = 117$) also had their feed intakes recorded. These together with cow weights and milk yield and content were then used to calculate energy balances. These data showed that the cows in all three IGF-1 groups had similar average body weights, but the LOW group cows consumed less feed in early lactation, with a DMI on average 3.4 kg/d lower than that of the HIGH group cows. This was associated with a significantly lower EB value in the LOW IGF-1 cows (Table 2).

Table 2. Blood and milk metabolites, feed intakes and calculated energy balance at 14 days in milk according to the IGF-1 group.

| Parameter [†] | IGF-1 Group (ng/mL) | | | |
	LOW < 35	MOD 35–100	HIGH > 100	*p* *
Blood				
n	34	88	41	
IGF-1 (ng/mL)	25 ± 1.3 [a]	63 ± 1.9 [b]	143 ± 5.3 [c]	<0.001
Glucose (mM)	3.2 ± 0.07 [a]	3.3 ± 0.06 [a]	3.5 ± 0.07 [b]	0.005
Fructosamine (μM)	246 ± 3.0 [a]	247 ± 2.0 [a]	258 ± 2.7 [b]	0.003
BHB (μM)	0.79 ± 0.082	0.75 ± 0.077	0.52 ± 0.044	0.082
NEFA (μekv/L)	884 ± 82 [b]	740 ± 47 [ab]	633 ± 57 [a]	0.046
Urea (mM)	3.0 ± 0.22 [a]	2.9 ± 0.12 [a]	3.7 ± 0.21 [b]	0.004
Milk				
n	32	83	41	
Glucose-6-phosphate (mM)	0.19 ± 0.010	0.18 ± 0.007	0.20 ± 0.009	0.080
Free glucose (mM)	0.16 ± 0.011 [a]	0.18 ± 0.007 [a]	0.26 ± 0.017 [b]	<0.001 [#]
BHB (μM)	77.9 ± 9.71 [b]	67.4 ± 5.11 [ab]	46.7 ± 3.28 [a]	<0.001 [#]
Isocitrate (mM)	0.20 ± 0.009 [b]	0.18 ± 0.006 [ab]	0.17 ± 0.006 [a]	0.045
Urea (mM)	2.83 ± 0.229 [ab]	2.90 ± 0.150 [a]	3.53 ± 0.242 [b]	0.041
Uric acid (μM)	161 ± 10.7 [a]	155 ± 6.7 [a]	194 ± 5.9 [b]	0.004 [#]
NAGase (units/L)	2.56 ± 0.194	2.66 ± 0.290	2.15 ± 0.152	0.428
LDH (units/L)	4.39 ± 0.658	4.54 ± 0.664	2.84 ± 0.222	0.485 [#]
Feed intake and Energy Balance				
n	18	61	38	
Weight (kg, 7 day average)	671 ± 18.9	670 ± 9.5	657 ± 10.4	0.288
DMI (kg/d, 7 day average)	16.4 ± 0.60 [a]	18.5 ± 0.38 [b]	19.8 ± 0.54 [b]	0.001
EB (MJoule/d 7 d average)	−10.7 ± 1.51 [b]	−7.5 ± 0.70 [ab]	−5.0 ± 1.09 [a]	0.005

[†] BHB, beta-hydroxybutyrate; NEFA, non-esterified fatty acids; NAGase, N-acetyl-β-D-glucosaminidase; LDH, lactic dehydrogenase; DMI, dry matter intake; EB, energy balance. * Different superscript letters in rows indicate a significant difference using Tukey's HSD test, a < b < c. [#] Data log-transformed for analysis to achieve homogeneity of variance.

3.2. Health

Among the LOW IGF-1 cows, only 3/35 (9%) had no recorded health issues during the first 5 weeks of lactation, 10/35 (29%) had one recorded health problem and 22/35 (63%) had more than one problem, with up to four recorded in total. This compared with 6/43 (14%) of the HIGH IGF-1 cows with no problems, 26/43 (60%) with one problem and 11/43 (26%) with two problems. Breaking this down further, there were no differences in the proportions of cows in each IGF-1 group according to various calving parameters (assisted calving, stillbirths, twins or retained placenta) (Table 3).

Table 3. Health data in the first 35 days in milk (DIM) according to the circulating IGF-1 concentration at 14 days in milk [†].

| | IGF-1 Group (ng/mL) | | | |
	LOW < 35	MOD 35–100	HIGH > 100	*p* (χ^2)
n	35	92	43	
Assisted calving	6 (17.1%)	7 (7.6%)	7 (16.3%)	0.187
Twins	4 (11.4%)	6 (6.5%)	1 (2.3%	0.267
Stillbirth	0 (0%)	2 (2.2%)	1 (2.3%)	0.672
Retained placenta	4 (11.4%)	5 (5.4%)	6 (13.9%)	0.221
Milk fever	2 (5.7%)	0 (0%)	0 (0%)	0.020
Displaced abomasum	4 (11.4%)	2 (2.2%)	0 (0%)	0.014
Lame [#]	10 (28.6%)	27 (29.3%)	13 (30.2%)	0.987

[†] Percentages calculated as the proportion of animals with that condition within each IGF group. [#] Lameness was assessed on a scale of 0 (movement free and easy) to 9 (disabled, not moving at all) at 14 DIM. Cows with a score > 3 had their movement defined as being mildly affected or worse and were classified as being lame.

Approximately 29% of all cows had a lameness score >3 at day 14, assessed on a scale of 0–9, implying some degree of lameness, but there were no differences in the proportions of lame animals between the three IGF-1 groups. However, LOW IGF-1 cows had a higher proportion of cases of displaced abomasum and the only two cases of milk fever reported in this study. Based on the herd records and measurements of SCC at 14 DIM, the LOW IGF-1 cows also had a higher proportion with clinical mastitis in early lactation and correspondingly fewer classified as having healthy milk (Figure 2).
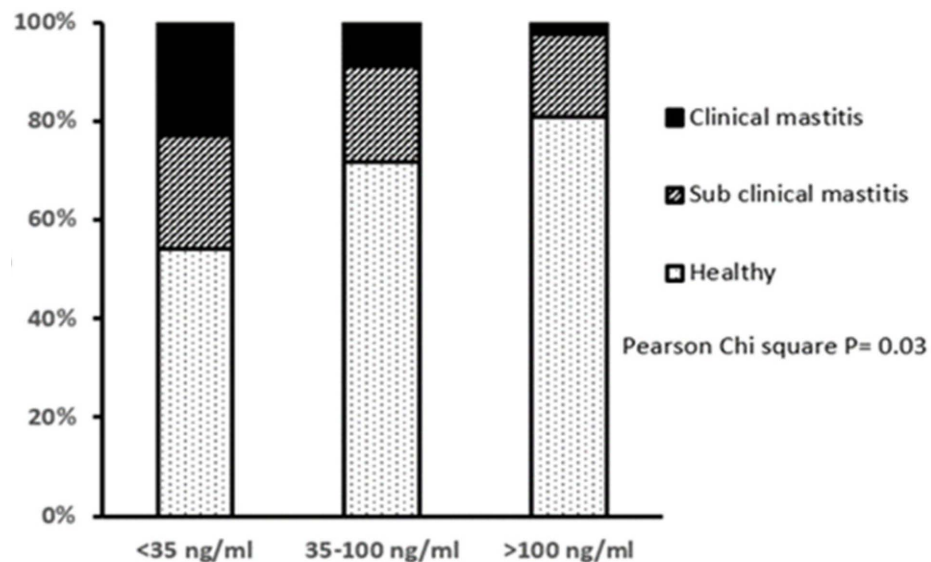
Figure 2. Relationship between circulating IGF-1 concentration at 14 days in milk and the incidence of mastitis in early lactation. Cows were grouped by their circulating IGF-1 concentration as LOW (<35 ng/mL, *n* = 35), MODERATE (35–100 ng/mL, *n* = 92) or HIGH (>100 ng/mL, *n* = 43). The mastitis classifications were: Healthy (SCC < 100,000 cells/mL milk, no clinical symptoms); subclinical mastitis (SCC 100,000 to 400,000 cells/mL milk, no clinical symptoms) or clinical mastitis (SCC > 400,000 cells/mL milk, with clinical symptoms). There was a significant difference in the proportions of cows with different mastitis classification between IGF-1 groups, with fewer healthy and more mastitic cows in the LOW IGF-1 group.

There were also differences noted in the character of the discharge from the reproductive tract (Figure 3). The LOW IGF-1 cows contained a significantly higher proportion with a fetid odor in their uterine discharge at day 14 (69% in IGF-1 < 35 ng/mL vs. 13.5% in cows with IGF-1 > 100 ng/mL). For 86% of cows in the HIGH IGF-1 category, the uterine discharge at day 35 was clear. This compared with only 38% of LOW IGF-1 cows having clear mucus at this time, with 62% classified as having mucus containing mucopurulent material (Figure 3).

3.3. Gene Expression Data in Leucocytes

Gene expression data in circulating leukocytes were compared between the three IGF-1 groups. A comparison of gene expression levels between the LOW and HIGH IGF-1 groups generated a list of 1032 DEG. There were 465 DEG between the LOW and MOD groups but only 39 DEG between the MOD and HIGH groups (Figure 1). These results indicated that most of the differences in gene expression lay between the cows with LOW IGF-1 and the remainder. Subsequent analysis as reported here therefore focused on the comparison between cows with LOW and HIGH IGF-1. This included 817 genes which were more highly expressed in the LOW cows (i.e., up-regulated with low IGF-1) and 215 which were more highly expressed in the HIGH cows (i.e., down-regulated in cows with low IGF-1). Only four genes were common between all three comparisons. Three of these were most highly expressed in the LOW IGF-1 cows (*CFAP69*, cilia and flagella-associated

protein 69; *DMBT1*, deleted in malignant brain tumors 1 and *MERTK*, MER proto-oncogene, tyrosine kinase) and one was most highly expressed in the HIGH IGF-1 cows *(SEMA6B,* semaphorin 6B).
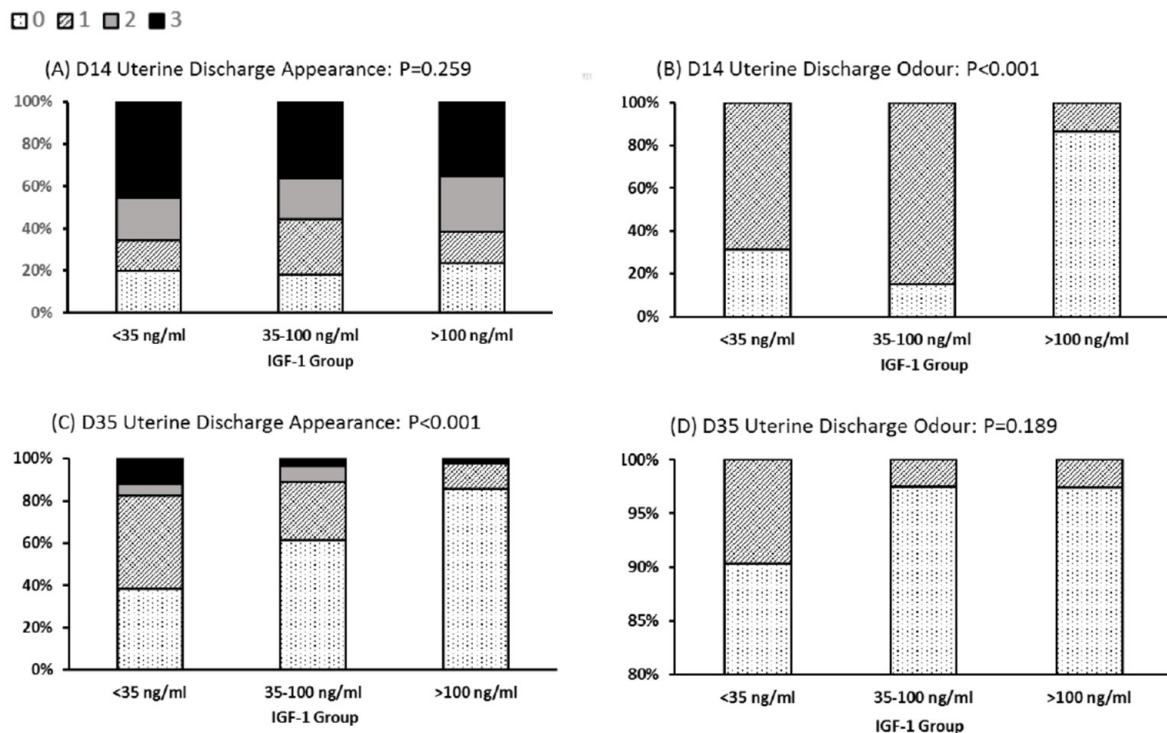

Figure 3. Relationships between IGF-1 category and uterine discharge appearance and odor. Cows were grouped by their circulating IGF-1 concentration as LOW (<35 ng/mL, *n* = 35), MODERATE (35–100 ng/mL, *n* = 92) or HIGH (>100 ng/mL, *n* = 43). Uterine discharge was sampled from the vagina at 14 and 35 days in milk (D14, D35) and scored as: 0, clear or translucent mucus; 1, containing flecks of white or off-white pus; 2, <50 mL exudate containing over 50% white or off-white mucopurulent material; or 3, >50 mL exudate containing purulent material, usually white or yellow, but occasionally sanguineous (**A,C**). Discharge odor was scored 0 for normal or 1 for fetid (**B,D**). The graphs are plotted to show the percentage of animals in each IGF-1 category with each score. Note the difference in *y*-axis scale used for (**D**). There was a significant difference in classification (Pearson's chi-squared test) among IGF-1 groups with respect to discharge odor at Day 14 and discharge appearance at D35.

3.4. Genes Up-Regulated in LOW IGF-1 Cows

The full list of 817 genes is provided in Supplementary Table S1, with the top genes summarized in Table 4. *PTX3* and *DMBT1*, which both have antimicrobial activity, were the two most highly up-regulated genes and were also the most highly significant. Other up-regulated genes encode proteins with a role in inflammation and coagulation (*FGA, LOC112445470, LOC511106, LOC107131803*) and *TF* encodes transferrin, an iron transport protein. Two genes with important immunological functions were highly significant: *DCSTAMP* is involved in osteoclastogenesis and myeloid differentiation, while *PROK2* acts on prokineticin receptors on monocytes, macrophages and dendritic cells to induce chemotaxis and release of inflammatory cytokines. The three up-regulated genes with the highest expression levels all encode S100 calcium-binding proteins (*S100A8, S100A9* and *S100A12*) and other highly expressed genes were *HP, RNAse6* and *CTSB*. Haptoglobin plays a key role in iron metabolism and all three of these genes encode proteins with antimicrobial activity. Two other highly significant genes, *GATM* and *PDK4*, have important roles in metabolism. Of the highly expressed genes, *PFM* encodes a pyruvate kinase important in glycolysis and *TALDO1* is involved in the pentose phosphate pathway.

Table 4. Topmost significantly up-regulated genes in leucocytes of cows with LOW (<35 ng/mL, *n* = 35) in comparison with HIGH (>100 ng/mL, *n* = 43) circulating IGF-1 at 14 days in milk.

Gene Symbol	Gene Name	FC ‡	*p*(BH)
Up-regulated genes in LOW IGF-1 cows, ranked by fold change			
PTX3	Pentraxin 3	28.9	<0.001
DMBT1	Deleted in malignant brain tumors 1	16.2	<0.001
ALB	Albumin	12.5	0.001
LOC112445470	Serpin B4-like	10.0	<0.001
FGA	Fibrinogen alpha chain	9.95	0.006
KRT1	Keratin 1	9.37	0.010
LOC100297334	Armadillo repeat containing X-linked 6	9.07	0.005
LOC511106	Serpin peptidase inhibitor, clade B like	9.06	<0.001
TF	Transferrin	9.02	0.008
LOC107131803	Serpin B3-like	8.84	<0.001
Up-regulated genes in LOW IGF-1 cows, ranked by FDR *p*-value [#]			
PTX3	Pentraxin 3	28.9	<0.001
DMBT1	Deleted in malignant brain tumors 1	16.2	<0.001
ZCCHC14	Zinc finger CCHC-type containing 14	1.48	<0.001
ATP13A3	ATPase 13A3	1.41	<0.001
NRG1	Neuregulin 1	2.03	<0.001
DCSTAMP	Dendrocyte expressed seven transmembrane protein	2.70	<0.001
GATM	Glycine amidinotransferase	1.83	<0.001
PROK2	Prokineticin 2	3.02	<0.001
DGKH	Diacylglycerol kinase eta	1.74	<0.001
PDK4	Pyruvate dehydrogenase kinase 4	2.10	<0.001
Up-regulated genes in LOW IGF-1 cows, ranked by RPKM values [#]			
S100A12	S100 calcium-binding protein A12	1.57	<0.001
S100A8	S100 calcium-binding protein A8	1.71	<0.001
S100A9	S100 calcium-binding protein A9	1.93	<0.001
HP	Haptoglobin	2.16	<0.001
RNASE6	Ribonuclease A family member k6	1.23	0.028
TSPO	Translocator protein	1.21	0.022
PKM	Pyruvate kinase M1/2	1.26	<0.001
CTSB	Cathepsin B	1.23	0.008
TALDO1	Transaldolase 1	1.28	<0.001
C10H15orf48	Chromosome 10 open reading frame, human C15orf48	1.29	0.003

‡ FC, fold change; [#] FDR, false discovery rate; RPKM, reads per kilobase of transcript per million mapped reads.

3.5. Genes Down-Regulated in LOW IGF-1 Cows

Comparison of gene expression levels between the LOW and HIGH IGF-1 groups generated a list of 215 DEG which were down-regulated in cows with low IGF-1. The full list is provided in Supplementary Table S2 and the top genes are summarized in Table 5. A number of genes with roles in immune signaling (*ST6GAL1*, *CXXC5*, *BLA-DQB*, *CD52*, *MS4A1*, *KIAA0040*, *SPIB*, *BANK1*, *PTPRCAP*, *CD79B*, *SEMA6B*) and autoimmunity (*MYH6*, *LBH*) were less highly expressed in the cows with LOW IGF-1. Two further genes encoded proteins identified as being multidrug resistance-associated protein 4-like (*LOC112449072*, *LOC100847574*). Of these, *LOC112449072* featured in both the greatest fold change and the most significant lists. The purinergic receptor *P2RY10* is involved in ATP release from cells and inflammation. *LTB* and *LBH*, encoding lyphotoxin beta and limb bud and heart development, respectively, also have potential roles in inflammation. Several other less highly expressed genes in LOW IGF-1 cows are involved in the cytoskeleton and cell motility (*DMTN*, *REM1*, *MYLIP*, *ARPC5L*). *HBZ* had the highest fold change. This gene encodes zeta-globin, an alpha-like hemoglobin normally expressed during embryonic development while *ALAS2* encodes an erythroid-specific mitochondrially located enzyme

which catalyzes the first step in the heme biosynthetic pathway. Highly down-regulated genes encoding two other mitochondrial enzymes were *COQ2* and *BCAT2*.

Table 5. Top most significantly down-regulated genes in leucocytes of cows with LOW (<35 ng/mL, *n* = 35) in comparison with HIGH (>100 ng/mL, *n* = 43) circulating IGF-1 at 14 days in milk.

Gene Symbol	Gene Name	FC ‡	*p*(BH)
Down-regulated genes in LOW IGF-1 cows, ranked by fold change			
HBZ	Hemoglobin subunit zeta	−7.95	0.001
LOC112449072	Multidrug resistance-associated protein 4-like	−6.85	<0.001
SEMA6B	Semaphorin 6B	−6.45	0.001
LOC112441635	DNA-directed RNA polymerase III subunit RPC9-like	−5.88	0.028
MYH6	Myosin, heavy chain 6, cardiac muscle, alpha	−5.52	0.011
DMTN	Dematin actin-binding protein	−3.79	0.005
C14H8orf88	Chromosome 14 C8orf88 homolo	−3.15	0.029
ALAS2	5′-aminolevulinate synthase 2	−3.04	<0.001
REM1	RRAD and GEM like GTPase 1	−2.91	0.027
LOC100847574	Multidrug resistance-associated protein 4-like	−2.88	<0.001
Down-regulated genes in LOW IGF-1 cows, ranked by FDR *p*-value #			
LBH	Limb bud and heart development	−1.32	<0.001
COQ2	Coenzyme Q2, polyprenyltransferase	−1.26	<0.001
LOC112449072	Multidrug resistance-associated protein 4-like	−6.85	<0.001
P2RY10	P2Y receptor family member 10	−1.23	<0.001
MYLIP	Myosin regulatory light chain interacting protein(MYLIP)	−1.24	<0.001
PLXDC1	Plexin domain containing 1	−1.57	<0.001
ST6GAL1	ST6 beta-galactoside alpha-2,6-sialyltransferase 1	−1.22	<0.001
ARPC5L	Actin related protein 2/3 complex subunit 5-like amyloid beta precursor-like protein 1(APLP1)	−1.23	<0.001
CXXC5	CXXC finger protein 5	−1.36	<0.001
BCAT2	Branched-chain amino acid transaminase 2(BCAT2)	−1.33	<0.001
Down-regulated genes LOW IGF-1 cows, ranked by RPKM values #			
BLA-DQB	MHC class II antigen	−1.20	0.014
CD52	CD52 molecule	−1.22	<0.001
MS4A1	Membrane spanning 4-domains A1	−1.25	0.006
KIAA0040	KIAA0040	−1.23	0.008
LTB	Lymphotoxin beta	−1.21	0.003
LBH	Limb bud and heart development	−1.32	<0.001
SPIB	Spi-B transcription factor	−1.24	0.014
BANK1	B cell scaffold protein with ankyrin repeats 1	−1.25	0.003
PTPRCAP	Protein tyrosine phosphatase, receptor type C-associated protein (PTPRCAP)	−1.21	0.001
CD79B	CD79B molecule	−1.27	0.008

‡ FC, fold change; # FDR, false discovery rate; RPKM, reads per kilobase of transcript per million mapped reads.

3.6. Cluster Analysis

Cluster analysis was performed in DAVID using a gene list which combined all the up-regulated and down-regulated DEG between LOW and HIGH IGF-1 cows. This analysis identified five significant annotation clusters with enrichment scores between 7.69 and 2.77 (Table 6). The genes in each cluster are listed in Supplementary Tables S3 and S4. Clusters 1 and 2 were both large clusters with considerable overlap, with 82 genes (19%) common to both. These focused around disulfide bonds, glycoproteins, glycosylation sites and transmembrane proteins. Out of the total of 425 genes in these two clusters, 340 (80%) were up-regulated in the LOW IGF-1 cows and 85 (20%) were down-regulated. Based on the GO term cellular components, most were associated with either a membrane (plasma, Golgi, endoplasmic reticulum, lysosome) or the extracellular space, but they had a very

wide variety of molecular functions. As the majority of proteins encoded by immune-related genes are located in or at the plasma membrane, this is expected from an immune cell population.

Table 6. Functional cluster analysis of all genes which showed significant differential expression in leukocytes collected from dairy cows with LOW (<35 ng/mL) or HIGH (>100 ng/mL) circulating IGF-1 concentrations, sampled at approximately 14 days in milk.

Category [#]	Term	Count	Fold Enrich	p(BH)
Annotation Cluster 1: Enrichment Score: 7.69				
UP_SEQ_FEATURE	Disulfide bond	85	2.37	3.60×10^{-12}
UP_SEQ_FEATURE	Glycosylation site:N-linked (GlcNAc...)	93	2.18	1.01×10^{-11}
UP_KEYWORDS	Disulfide bond	154	1.85	1.33×10^{-11}
UP_SEQ_FEATURE	Signal peptide	88	2.01	4.37×10^{-9}
UP_KEYWORDS	Glycoprotein	104	1.77	1.29×10^{-6}
UP_SEQ_FEATURE	Topological domain:Extracellular	48	2.06	3.61×10^{-4}
UP_SEQ_FEATURE	Topological domain:Cytoplasmic	57	1.73	0.005
UP_SEQ_FEATURE	Transmembrane region	79	1.44	0.047
UP_KEYWORDS	Secreted	66	1.50	0.029
Annotation Cluster 2: Enrichment Score: 4.95				
UP_KEYWORDS	Transmembrane helix	318	1.27	3.00×10^{-5}
UP_KEYWORDS	Transmembrane	318	1.26	3.39×10^{-5}
UP_KEYWORDS	Membrane	354	1.23	5.02×10^{-5}
Annotation Cluster 3: Enrichment Score: 3.84				
GOTERM_MF_DIRECT	GO:0005044~scavenger receptor activity	12	4.66	0.026
INTERPRO	IPR001190:Speract/scavenger receptor	9	5.55	0.048
INTERPRO	IPR017448:Speract/scavenger receptor related	9	5.21	0.048
SMART	SM00202:SR	9	4.82	0.027
Annotation Cluster 4: Enrichment Score: 3.38				
GOTERM_BP_DIRECT	GO:0042742~defense response to bacterium	17	3.85	0.008
INTERPRO	IPR006080:Beta defensin/Neutrophil defensin	6	9.56	0.048
UP_KEYWORDS	Antimicrobial	11	4.01	0.012
SMART	SM00048:DEFSN	6	8.31	0.027
Annotation Cluster 5: Enrichment Score: 2.77				
UP_KEYWORDS	Inflammatory response	12	3.88	0.009
UP_KEYWORDS	Innate immunity	15	3.02	0.013
UP_KEYWORDS	Immunity	19	2.34	0.029

[#] Category terms were derived from DAVID bioinformatics resources version 6.8 (https://david.ncifcrf.gov (accessed on 16 Aug 2021).

Cluster 3 identified genes associated with scavenger receptor activity. Of these *CD163*, *MARCO* and *SCARF1* were all up-regulated in LOW IGF-1 cows. *CD163* encodes a protein which is important in the clearance of hemoglobin/haptoglobin complexes by macrophages, *MARCO* encodes a receptor which may bind both Gram-negative and Gram-positive bacteria, while *SCARF1* regulates the uptake of chemically modified low density lipoproteins. The gene list also included four members of the WC1 co-receptor family (*LOC786796*, *WC1*, *WC1.3*, *WC-7*). These are group B scavenger receptors which are found exclusively on gamma delta T cells, all of which were less highly expressed in the LOW IGF-1 cows.

Cluster 4 identified 20 genes involved in defense responses to bacteria, of which all but one were up-regulated in LOW IGF-1 cows. These mainly encoded antimicrobial proteins (*CATHL4*, *DEFB1*, *DEFB13*, *DEFB4A*, *DEFB7*, *DEFB10*, *HP*, *LAP*, *S100A8*, and *S100A9*). The cluster also included genes encoding two components of the IgE receptor (*FCGR1A* and *FCER1G*), the TNFα receptor (*TNFRSF1A*) and *BCL3* and *NOD2*, which are both involved in the NFκB pathway. *ELANE* was the only down-regulated gene. This encodes an elastase which hydrolyzes proteins in azurophil granules, specialized lysosomes found in neutrophils.

Cluster 5 contained 28 genes relating to "Inflammatory response" and "Innate immunity", of which all but four were up-regulated in LOW IGF-1 cows and six were common to Cluster 4. In addition to the antimicrobial proteins already mentioned, this cluster included additional antimicrobial genes (*PTX3*, *S100A12*), genes encoding key cytokine and chemokine signaling molecules (*TLR4*, *CD14*, *CCL8*, *CXCL8*, *IL2RA*), two members of the complement system (*C2*, *CFB*) and the scavenger receptor *CD163*. Other genes involved in inflammation included two components of the coagulation factor fibrinogen (*FGA*, *FGB*). The down-regulated genes were *BOLA-DQB*, *BLK*, *LGR4*, and *TLR10*.

3.7. KEGG Pathway Analysis

KEGG pathway analysis using all identified DEG between LOW and HIGH IGF-1 cows initially revealed four pathways significant at p(BH) < 0.05. These are summarized in Table 7 and the full gene lists are provided in Supplementary Tables S5 and S6.

Table 7. KEGG pathway analysis based on all genes which showed significant differential expression in leukocytes collected from dairy cows with LOW (<35 ng/mL) vs. HIGH (>100 ng/mL) circulating IGF-1 concentrations, sampled at approximately 14 days in milk.

Term	Gene Count	Fold Enrich	p(BH)
bta04611:Platelet activation	24	3.61	3.23×10^{-5}
bta04640:Hematopoietic cell lineage	19	3.95	1.19×10^{-4}
bta04610:Complement and coagulation cascades	16	4.13	4.09×10^{-4}
bta04060:Cytokine–cytokine receptor interaction	26	2.28	0.010
bta04015:Rap1 signaling pathway	23	2.05	0.064
bta04978:Mineral absorption	9	3.91	0.064
bta04512:ECM-receptor interaction	13	2.86	0.064

The pathway "bta04640:Hematopoietic cell lineage" is again to be expected in a leukocyte cell population. Three other highly significant pathways were "bta04611:Platelet activation", "bta04610:Complement and coagulation cascades" and "bta04060:Cytokine–cytokine receptor interaction". A further three pathways were identified at p(BH) = 0.064. These were "bta04015:Rap1 signaling pathway", "bta04978:Mineral absorption", and "bta04512:ECM-receptor interaction".

No significant metabolic pathways were identified in the initial analysis. As this was unexpected, a manual search of the entire list of 1,032 DEG was therefore made, which identified 149 genes (14.4%) which had some known metabolic function (Supplementary Table S7). These were then input into DAVID as a new gene list. Of these, 44 genes were present in KEGG pathway "bta01100:Metabolic pathways" of which 36 were up-regulated and 8 down-regulated in LOW IGF-1 compared to HIGH cows (Supplementary Tables S8 and S9). A further five significant metabolic pathways were identified, as summarized in Table 8: "bta01230:Biosynthesis of amino acids", "bta01200:Carbon metabolism", "bta00410:beta-Alanine metabolism", "bta00620:Pyruvate metabolism" and "bta00770:Pantothenate and CoA biosynthesis". All of these pathways participate in energy generation. In addition the pathway "bta02010:ABC transporters" was significant. ABC transporters are integral membrane proteins that allow ATP-dependent movement of various molecules across the plasma membrane.

Table 8. KEGG pathway analysis of DEG [#] which showed significant differential expression in leukocytes collected from dairy cows with LOW (<35 ng/mL) vs. HIGH (>100 ng/mL) circulating IGF-1 concentrations, sampled at approximately 14 days in milk.

Term	Gene Count	Fold Enrich	p(BH)
bta01100:Metabolic pathways	44	2.72	1.78×10^{-8}
bta01230:Biosynthesis of amino acids	8	8.59	0.003
bta01200:Carbon metabolism	9	6.30	0.005
bta02010:ABC transporters	6	10.89	0.009
bta00410:beta-Alanine metabolism	5	11.22	0.033
bta00620:Pyruvate metabolism	5	10.03	0.039
bta00770:Pantothenate and CoA biosynthesis	4	16.95	0.039

[#] Based on sublist of 149 DEG with metabolic functions.

4. Discussion

4.1. Gene Expression in Peripheral Blood

Whole peripheral blood has been widely used in gene expression studies due to its initial ease of collection and because it can be processed directly without the requirement to separate out specific cell types. The whole-blood leucocyte population includes T and B lymphocytes, natural killer cells, platelets, monocytes and granulocytes (neutrophils, eosinophils and, basophils). In dairy cows the average leucocyte population is between 5–12 cells per ml of blood, predominantly consisting of lymphocytes and neutrophils [66]. The transcriptional changes measured between different cows in this study therefore represent changes in gene expression within particular cell types together with changes in their relative proportions. This proportion will change as cells of different lineages may proliferate, undergo apoptosis or migrate to or from tissues into blood in response to different signals [66,67]. It was not possible to collect additional blood samples for analysis of leukocyte populations within this study. Despite this limitation, many previous studies in both human and livestock have now reported that transcriptional signatures of whole blood can reliably differentiate individuals with a variety of diseases [68,69].

4.2. Phenotypic Changes

Milk production capacity should increase progressively until approximately the fifth lactation [70]. However, the older LOW IGF-1 cows, with a mean lactation number of 3.9, produced less milk than the HIGH cows by the second week of lactation and their milk contained less protein. This was associated with reduced feed intakes, with DMIs being on average approximately 3 kg/d lower in the LOW cows. Voluntary DMIs during late pregnancy fall to a nadir around calving then increase again in early lactation, during which time a variety of metabolic and humoral signals and environmental factors affect appetite [3,5]. Blood and milk metabolite measurements showed that the LOW IGF-1 cows had lower glucose, fructosamine and urea concentrations together with raised BHB and NEFA. These indices show that the LOW IGF-1 cows were experiencing a shortage of both protein and energy, which would cause them to mobilize more body tissue. The higher NEFA concentration may itself be one factor reducing feed intake [3]. In addition, endotoxemia and sepsis cause acute phase inflammatory responses, which drive a shift towards catabolism [48]. During this process a number of cytokines can cause hypophagia, in particular tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) [71,72]. Treatment of cattle steers with lipopolysaccharide (LPS, an endotoxin produced by Gram negative bacteria) to induce an acute phase response reduced their feed intake by 60% within 24 h and this was accompanied by a reduction of 24% in circulating IGF-1 [73].

In terms of health, the LOW IGF cows experienced higher incidences of displaced abomasum and uterine infections, confirming our earlier studies [28,74]. The LOW IGF-1 cows were also more likely to be affected by either clinical or subclinical mastitis in early

lactation. Based on the SCC data, it is possible that some cows may have had a mammary infection during the dry period which was carried over into the next lactation. This has been shown to influence inflammatory responses during the transition period [75], but was not monitored in our study. These results therefore support earlier work cited in the Introduction establishing a link between health and metabolism. It should be noted, however, that our experimental design could not distinguish cause from effect. A two-way interaction seems likely with nutrient deficiency compromising the immune system while infections and inflammation reduce appetite, so worsening the situation.

4.3. Markers of Energy Metabolism

Immunometabolism encompasses both the immediate response to an infection and the longer term inflammatory responses triggered by cell injury or infection [76]. As mitochondria are the main organelles of energy metabolism, the overall ability of a cell to respond appropriately becomes compromised if any sort of stressor causes mitochondrial dysfunction. Important genes and pathways which were differentially affected between LOW and HIGH IGF-1 cows in our study are illustrated in Figure 4.

Three genes which were highly up-regulated in leucocytes of LOW IGF-1 cows encode enzymes which are absolutely central to the reprogramming of metabolism associated with mounting an immune response, namely *PKM2*, *PDK4*, and *GATM*. In resting macrophages, most pyruvate is directed to the tricarboxylic acid (TCA) cycle via acetyl-CoA to generate ATP via oxidative phosphorylation (OXPHOS), although some is converted to lactate. In activated immune cells, such as M1 macrophages and dendritic cells stimulated by LPS, their energy metabolism switches from OXPHOS to aerobic glycolysis, similar to the "Warburg effect", causing conversion of the majority of the pyruvate generated into lactate, with glycolytic intermediates providing the source of adenosine triphosphate (ATP) [77,78]. This is associated with up-regulation of pyruvate kinase M2 (PKM2) the rate-limiting enzyme in the glycolytic pathway [79]. At the same time, the mitochondrial enzyme pyruvate dehydrogenase kinase isozyme 4, encoded by *PDK4*, is up-regulated in response to an increased lipid supply in the form of free fatty acids, inactivating the pyruvate dehydrogenase complex and helping to conserve glucose by limiting the conversion of pyruvate to acetyl-CoA [80] (Figure 4A,B). Under anaerobic conditions, lactate dehydrogenase promotes the NADH-dependent reduction of pyruvate to lactate. Another mitochondrial enzyme *GATM* encodes glycine amidinotransferase (AGAT) which is central to creatine biosynthesis [81]. Creatine then combines with a phosphoryl group to generate phosphocreatine, which can donate its phosphate group to convert adenosine diphosphate (ADP) into ATP, so acting as a rapidly available source of energy.

Key genes contributing to the pentose phosphate shunt (PPS) were also up-regulated in LOW IGF-1 cows (Figure 4C). This provides an alternative pathway for oxidation of glucose and generates both nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and ribulose-5-phosphate. Glucokinase (GK) converts glucose to glucose-6-phosphate, the starting point for the PPS, while phosphogluconate dehydrogenase (PGD) is the rate-limiting enzyme in this pathway. Some ribose-5-phosphate is used for nucleotide synthesis but unused glycolytic intermediaries can be recycled by transaldolase 1 (TALDO1). The relative production of NADPH varies with metabolic demand and it subsequently acts as a reducing agent in many biosynthetic pathways including fatty acid synthesis, detoxification and ROS generation. For example, the PPS is enhanced in macrophages stimulated with LPS and this is crucial in providing anti-oxidant cellular defense [76,82].
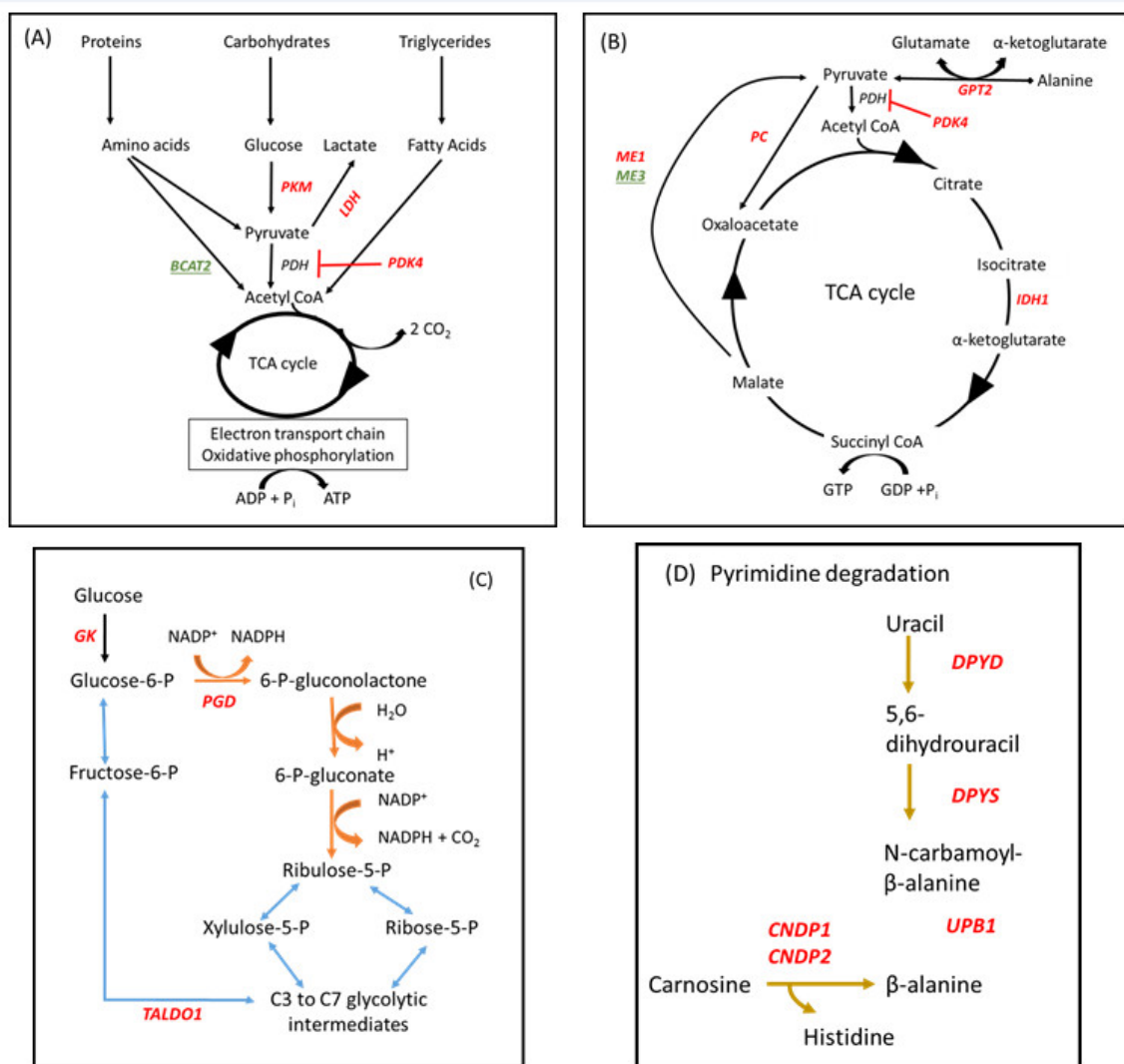
Figure 4. Metabolic pathways which were differentially influenced in leucocytes between cows with LOW (<35 ng/mL) and HIGH (>100 ng/mL) IGF-1 in early lactation [#]. [#] Genes encoding enzymes which were up- or down-regulated in LOW IGF-1 cows are shown in red or <u>green</u> font, respectively. (**A,B**) Energy sources used for adenosine triphosphate (ATP) and guanine triphosphate (GTP) synthesis via the tricarboxylic acid (TCA) cycle. In LOW IGF-1 cows *PKM2*, *PDK4*, *LDH*, *GPT2*, *PC*, *ME1*, *IDH1* were all up-regulated whereas *BCAT2* and *ME3* were down-regulated. (**C**) The pentose phosphate shunt (PPS) provides an alternative pathway for oxidation of glucose. It has two phases, an oxidative phase (shown in brown), in which NADPH oxidase and ribulose-5-phosphate (a pentose sugar) are generated, and a non-oxidative phase (shown in blue), in which unused ribulose-5-phosphate is converted to other sugar intermediates and returned to the glycolytic pathway. *GK*, *PGD*, and *TALDO1* were all up-regulated in LOW IGF-1 cows. (**D**) Part of pathway bta00410:beta-alanine metabolism. *DPYD*, *DPYS*, *UPB1*, *CNDP1* and *CNDP2* were all up-regulated in LOW IGF-1 cows. See text for further information.

Two of the KEGG pathways identified in this study centered on amino acid metabolism. A number of identified DEG *(GPT2, BCAT2, PC, IDH1*, and *PKM)* are involved in regulating the balance of usage between proteins and carbohydrates to provide energy for cell metabolism via the TCA cycle (Figure 4A,B). Glutamic-pyruvic transaminase 2 (GPT2) catalyzes the reversible transamination between the glucogenic amino acid alanine and 2-oxoglutarate to generate pyruvate and glutamate. Pyruvate carboxylase (PC) converts pyruvate to oxaloacetate and is activated by acetyl CoA during fasting. Isocitrate dehydrogenase (IDH1) is one of three regulated enzymes contributing to the TCA cycle, catalyzing an oxidative decarboxylation reaction which generates NADH. Malic enzyme catalyzes the oxidative decarboxylation of malate to pyruvate. *ME1* encodes a cytosolic,

NADP(+)-dependent isoform which was up-regulated whereas *ME3* encodes a mitochondrial NADP(+)-dependent isoform which was down-regulated. This reaction links the glycolytic and citric acid cycles and generates NADPH. *BCAT2* encodes a branched-chain aminotransferase which catalyzes the first step in the breakdown of branched-chain amino acids (valine, leucine and isoleucine, BCAAs). Intermediary branched-chain α-keto acids are subsequently degraded further to generate propionyl CoA (valine and isoleucine) or acetyl CoA (leucine and isoleucine), which can then enter the TCA cycle. As *BCAT2* was down-regulated in LOW IGF-1 cows, this would be expected to increase circulating BCAA. Multiple studies have found a positive correlation between circulating concentrations of BCAA, obesity and insulin resistance in human populations [83,84]. Other DEG included *ASS1* and *CPS1*, which both encode enzymes involved in the urea cycle. *DPYD*, *DPYS* and *UPB1* encode three consecutive enzymes required for the degradation of uracil to β-alanine, while *CNDP1* and *CNDP2* both encode carnosine dipeptidases, which convert carnosine to β-alanine (Figure 4D). In LOW IGF-1 cows this would reduce availability of carnosine, a dipeptide which has beneficial antioxidant effects including acting as a ROS scavenger [85]. β-alanine itself may, however, be involved in synaptic transmission [86].

COQ2 was a highly down-regulated gene in LOW IGF-1 cows. This encodes coenzyme Q2, a polyprenyltransferase that forms part of the coenzyme Q10 pathway. CoQ10 is an obligate component of the mitochondrial electron transport chain and CoQ depletion in MCF-7 cells was shown to decreases the ATP level and increase oxidative stress. It also induced a glycolytic phenotype causing an increased glucose uptake rate, increased lactate production, and led to mitochondrial dysfunction [87,88]. Overall, these results provide strong evidence that the energy and protein deficiency experienced by LOW IGF-1 cows leads to significant alterations in their leucocyte gene expression, indicative of a switch from OXPHOS to aerobic glycolysis and an increased reliance on glucogenic carbon. This is likely to be associated with increased oxidative damage in comparison with leucocytes from HIGH IGF-1 cows.

4.4. ABC Transporters

Two genes (*LOC112449072* and *LOC100847574*) more highly expressed in HIGH IGF-1 cows encode multidrug resistance-associated protein 4-like proteins which belong to the superfamily of ATP-binding cassette (ABC) transporters. These are integral membrane proteins which allow ATP-dependent movement of various molecules across the plasma membrane. ATP, UTP, ADP, and NAD+ are all released into the extracellular space by damaged and dying cells, but also by regulated mechanisms which include ABC transporters. It is therefore possible that these transporter proteins are involved in secretion of ATP into the extracellular space [89] where it acts as an inflammatory mediator [90]. Our study also identified the ABC transporter pathway, which included six up-regulated (*ABCA3*, *ABCA5*, *ABCA6*, *ABCA9*, and *ABCA10)* and one down-regulated *(ABCG1)* gene in LOW IGF-1 cows. Some of the encoded proteins have been implicated in cholesterol efflux to high density lipoprotein, which reduces accumulation of cholesteryl esters in macrophages. Combined deletion of *ABCA1* and *ABCG1* in mice leads to excessive lipid accumulation in tissue macrophage foam cells causing inflammation [91,92]. The precise roles of many of these individual ABC transporters do, however, need further investigation.

4.5. Antimicrobial Activity

There was strong evidence for up-regulation of many genes which encode proteins with antimicrobial activity in the LOW IGF-1 cows. Pentraxin 3, encoded by *PTX3*, is a member of the long pentraxin family, which plays an essential role in innate immunity and is an established biomarker for several inflammatory conditions [93]. Expression in dairy cows increased in mammary tissue following experimental infection with *Staphylococcus aureus* [94] and also in endometrium of postpartum cows with metritis [95]. *DMBT1* encodes a multifunctional glycoprotein known as deleted in malignant brain tumors 1 or salivary scavenger and agglutinin (SALSA). This protein is able to bind both bacteria and viruses

directly and can also interact with various endogenous ligands, including IgA, lactoferrin, fibrin and components of the complement system [96,97]. It plays an important role in both immune defense and inflammation while deposition in the vascular epithelium assists with vascular repair [98]. S100A8, S100A9 and S100A12 are all calcium-binding proteins belonging to the S100 protein family. They act as antimicrobials and in wound healing [99–101] and can amplify endotoxin-induced up-regulation of cytokines, chemokines and other inflammatory mediators [102,103]. Their respective genes are amongst the most highly expressed in endometrium of early postpartum cows in severe NEB [28] or with endometrial inflammation [104]. S100A8 and S100A9 proteins were localized to epithelial and stromal cells in addition to leucocytes [105] and gene expression in bovine endometrium in vitro was up-regulated in response to LPS [106].

Additional antimicrobial genes were up-regulated in LOW IGF-1 cows, contributing to Cluster 4 "defense response to bacteria" (*CATHL4*, *DEFB1*, *DEFB4A*, *DEFB7*, *DEFB10*, *DEFB13* and *LAP*). These encode cathelicidin 4 and several β-defensins, which belong to a large group of cationic peptides with amphipathic properties, enabling them to permeate pathogen membranes and cause cell death [107,108]. They also act as DAMPs to activate host responses to cell damage [109]. All of the genes identified here apart from *DEFB13* have previously been associated with bovine mastitis [33,110,111] and/or endometrial infection and inflammation in postpartum cows [28,104]. Neutrophil expression of DEFB1, -4 and -10 also increased on the day of calving [112].

RNAse4 and *CTSB* were highly expressed in leucocytes of LOW IGF-1 cows. *RNAse4* encodes a protein with broad-spectrum antimicrobial activity which is also secreted into milk, so contributing to host defense in the mammary gland [113]. *CTSB* encodes cathepsin B, an endogenous cysteine protease with both endopeptidase and exopeptidase activity, providing the ability to kill pathogens [114]. The protein is abundant in milk from cows with subclinical mastitis associated with *Staphylococcus aureus* or *Streptococcus uberis* infection [115].

Two other highly up-regulated genes were *TF* and *HP*, which encode transferrin (also known as lactoferrin) and haptoglobin, respectively, which are principally involved in iron homeostasis. The pathway "Mineral absorption" included *CYBRD1*, *STEAP1* and *STEAP2*, which all encode proteins that participate in iron uptake, while *HMOX1* encodes heme oxygenase, an essential enzyme in heme catabolism. Sufficient iron is essential for many cellular processes but iron in excess is harmful as it catalyzes free radical formation and also increases availability for microbial proliferation [116]. Macrophages play a key role in maintaining iron homeostasis by phagocytosing senescent erthyrocytes and recycling the iron [117]. Transferrin is able to sequester free iron from the environment to inhibit bacterial growth [118] and it also has direct bactericidal actions and can prevent viral entry into cells [119]. Haptoglobin binds free plasma hemoglobin, which is then captured by CD163, a ligand-specific transmembrane scavenger receptor found on monocytes [48], which was also up-regulated in LOW IGF-1 cows. This may help to protect tissues from free hemoglobin-mediated oxidative damage.

Although hemoglobin is mainly associated with erythrocytes, it can also be found in PBMC in which it localizes to the mitochondria with the dual function of increasing mitochondrial respiratory rate and protecting from H_2O_2-induced cytotoxicity and mitochondrial DNA damage [120]. *HBZ* expression is normally found in embryonic erythroid cells but the gene can be partially re-activated postnatally by acetylation and inhibition of histone de-acetylases [121]. *ALAS2* encodes a mitochondrial enzyme 5'-aminolevulinate synthase 2 that regulates the initial step of heme biosynthesis and the gene is up-regulated in blood leukocytes of human patients with sepsis [122]. In contrast, both *HBZ* and *ALAS2* in the present study were more highly up-regulated in cows with HIGH IGF-1, possibly providing a greater level of cytoprotection.

4.6. Vascular Damage and Inflammation

Two of the most significant KEGG pathways identified were "Platelet activation" and "Complement and coagulation cascades", which have overlapping components. Following vascular injury, platelets initially adhere to the extracellular matrix (ECM), with the glycoprotein von Willebrand factor (VWF) forming a bridge between exposed collagen and the platelet glycoprotein receptor complex on the platelet membrane [123]. This process can be triggered through the activation of multiple pathways by the binding of several agonists to their receptors, such as thromboxane A2, adenosine diphosphate (ADP), and thrombin. Platelets express many adhesion molecules to facilitate their interactions between each other, leukocytes and the endothelium, causing aggregation. Almost all the genes identified which play a role in vasodilation and platelet aggregation were up-regulated in the LOW IGF-1 cows. They included *VWF* itself, *F5*, *FGA FGB*, *ITGA2*, *ITGB3*, *P2RY1*, *PLA2G4A*, *PRKG1*, *PTGIR*, *PTGS1*, *SELP*, *TBXAS1* and *TFPI*.

The complement system proteins are involved in opsonization of pathogens, lysis of infectious organisms, phagocytosis of microbes and damaged cells and induction of an inflammatory response. DEG directly involved in the complement cascade in the present study included *CFI*, *C1R*, *C2*, *C3AR1*, *C8G*, *CD55*, *CFB*, *CFI*, and *CR2*. All except *CR2* were up-regulated in LOW IGF-1 cows. This is supported by the finding that blood complement was increased in periparturient cows with a high liver activity index, shown to be associated with inflammatory and immune parameters [124]. A proteomic analysis identified nine complement proteins in PBMC from postpartum dairy cows [125].

Three highly up-regulated genes in LOW IGF-1 cows were *LOC107131803*, *LOC112445470* and *LOC511106*, which were identified as Serpin B3-like, Serpin B4-like and Serpin peptidase inhibitor, clade B like, respectively. SERPINB3 and SERPINB4 are serine/cysteine protease inhibitors and in humans their expression is up-regulated in association with a number of inflammatory conditions [126]. Other serpin family members play key functions as serine protease inhibitors in thrombotic and fibrinolytic cascades in response to infection and vascular injury [127].

The Rap1 signaling pathway was also identified in the analysis. Rap1 is a small GTPase that couples extracellular signals to intracellular responses to control a large array of cellular-signaling pathways. Based on the DEG identified in our study, it is likely to be playing an important role in the inflammatory processes described above including cell-matrix interactions and angiogenesis. A variety of growth factors, adenosine, dopamine and lysophosphatidic acid (LPA) all contribute to the control of Rap1 cycling between an inactive GDP-bound and an active GTP-bound conformation (Supplementary Figure S1). Activated Rap1 then influences adhesion and migration by regulating the function of integrins and other adhesion molecules and it also affects cell proliferation and survival via altered MAP kinase (MAPK) activity [128]. Rap1 has also been implicated in regulating glucose homeostasis and lipid metabolism during heat stress in Holstein cows [129].

4.7. Cytokine and Chemokine Signaling Pathways

Kegg pathway analysis identified "Cytokine–cytokine receptor interaction" as significant. The gene list included transforming growth factor-beta (TGF-beta) superfamily receptors (*ACVR1*, *ACVR1B*, *ACVR2B*, and *BMPR2*); chemokines and their receptors (*CCL8*, *CXCL8*, *CCR1*, and *CCR4*); cytokines and their receptors (*CSF1R*, *IL12B*, *TSLP*, *IL1RAP*, *IL2RA*, *IL3RA*, *IL15RA*, and *IL18R1*) and members of the TNF-receptor superfamily (*LTB*, *LTBR*, *RELT*, *TNFRSF1A*, *TNFRSF1B*, and *TNFRSF9*). Feeding cows high energy diets during the dry period is known to increase circulating concentrations of pro-inflammatory mediators such as IL1B, TNF and CXCL2 [130]. TNFα is a key inflammatory cytokine which has a direct effect on the bovine liver to promote triacylglycerol accumulation and decrease glucose production [131], while *TNFSF13* and *TNFSF13B* encode proteins termed APRIL and BAFF which are important for B cell activation. The influence of TNFα on appetite was discussed above. Colony stimulating factor 1 is another important cytokine

which controls the production, differentiation, and function of macrophages via its receptor CSF1R [132].

4.8. Adaptive Immunity

Several prominent genes with lower expression in LOW IGF-1 cows (and conversely up-regulated in HIGH IGF-1 cows) are known to be involved in T and B cell activation. The major histocompatibility complex (MHC, known as BoLA in cattle) is a set of highly polymorphic genes that bind pathogen-derived peptides and present them for recognition by specialized T cells, resulting in T and B cell activation and expansion. BOLA-DQB plays a role in antigen presentation [133]. *CD52* encodes a glycoprotein widely expressed on the cell surface, which can modulate T cell activation [134]. *MS4A1* encodes membrane spanning 4-domains A1, also known as CD20, which plays a role in the development and differentiation of B cells into plasma cells. *SPIB* encodes a transcriptional activator which acts as a lymphoid-specific enhancer and regulates the expression of many components of the B cell receptor signaling pathway [135]. BANK1 (B cell scaffold protein with ankyrin repeats 1) is an adaptor protein which is primarily expressed in B cells where it is also involved in many signaling pathways. These include TLR signaling, B cell receptor-induced calcium mobilization and tyrosine phosphorylation of inositol 1,4,5-trisphosphate receptors. BANK1 also negatively regulates CD40-mediated B cell activation, so preventing hyperactive B cell responses [136]. Other genes encoding proteins playing key roles in T and B cell activation and function included *PTPRCAP* [137], *CD79B* [138] and *ST6GAL1* [139]. *ST6Gal* knock out mice are severely immunosuppressed [140]. These results corroborate the idea that HIGH IGF-1 cows have a greater capacity to mount an effective adaptive immune response.

4.9. Cytoskeleton and Cell Motility

Cell migration is crucial for leucocyte functionality and two of the four genes which discriminated between all three IGF-1 groups (*CFAP69* up-regulated and *SEMA6B* down-regulated in LOW IGF-1 cows, respectively) have roles related to the cytoskeleton and cell motility. A number of the other genes most down-regulated in LOW IGF-1 cows (*DMTN*, *REM1*, *MYLIP*, *ARPC5L*) also came into this functional category. *CFAP69* encodes cilia and flagella-associated protein 69, which forms part of the cytoskeletal scaffold, with knockout resulting in loss of motility [141]. Semaphorin signaling results in changes to the cytoskeletal and adhesive machinery that modulate cellular adhesion and influence cell shape, morphology and motility. Some semaphorins can influence vascular permeability and are involved in establishing interactions between platelets that allow thrombus formation during hemostasis [142]. Within immune cell populations, they have roles in initiation of T cell mediated antibody responses, B cell function and macrophage activation by antigen-specific T cells at sites of inflammation [143]. Semaphorin 6B encoded by *SEMA6B* is a transmembrane protein which is a key target gene for vitamin D in PBMC [144]. It was one of the most highly down-regulated genes in LOW IGF-1 cows (−6.4 fold) but there is currently little information available regarding its specific functions. Several other SEMA genes (*SEMA4F*, *SEMA4D*, and *SEMA3C*) were up-regulated in the LOW IGF-1 cows. Of these, *SEMA4D* was very highly expressed.

The cytoskeleton is composed of actin and tubulin networks and intermediate filaments. These are involved in the development of adhesive contacts, establishment of polarity and cell motility. Cytoskeleton remodeling involves cycles of actin polymerization and depolymerization, which are regulated by a variety of proteins and requires tight coordination [145]. The Arp2/3 complex is involved in generating branched actin filament networks [146]. The small GTPases Rac and Rho antagonize each other at multiple levels and control cell polarization and different migration modes: Rac activity dominates at the leading edge during mesenchymal migration, while amoeboid movement is characterized by high levels of active Rho [147]. Actomyosin interactions are also closely involved in the regulated assembly and disassembly of the actin network [148]. The genes more highly

expressed in cows with HIGH IGF-1 included *ARPC5L* (encoding actin related protein 2/3 complex subunit 5 like), *REM1* (encoding a member of the RAS-like GTP-binding protein family) and *DMTN (*encoding dematin actin-binding protein). All of these are known to be involved in regulating the actin cytoskeleton, while myosin regulatory light chain interacting protein (encoded by *MYLIP)* is a cytoskeletal effector protein that links actin to membrane-bound proteins at the cell surface. The differential expression of these various genes between leukocytes derived from HIGH and LOW IGF-1 cows therefore suggests that there are likely to be differences in adhesive properties and migratory capacity between their respective cell populations.

4.10. Potential Links to Health and Fertility

Our own previous work has indicated that the IGF-1 concentration in the immediate post-calving period is strongly linked to subsequent fertility, a relationship which was much more pronounced in multiparous than primiparous cows [63]. Key genes of the somatotropic axis regulating the production and bioavailability of IGF-1 showed differential hepatic expression in cows with high or low genetic merit for fertility traits [149] and circulating concentrations of IGF-1 were greater throughout the gestation-lactation cycle in the more fertile cows [150]. This relationship could in part be caused by a direct effect on the reproductive tract. The IGF system is important in promoting the repair of the uterine endometrium after calving [28,151]. Circulating IGF-1 is also considered critical for follicular maturation as it amplifies the gonadotropin stimulus [152,153]. Cows with low IGF-1 after calving therefore remain anovulatory for longer [154–156]. Overall, cows with a low nadir in IGF-1 in the first 2 weeks after calving subsequently take longer to conceive in both high yielding [63] and pasture based [156] systems.

There is also a more indirect relationship between the somatotropic axis and fertility via compromised immunity, as evidenced here. LOW IGF-1 cows are clearly more likely to succumb to infections after calving, particularly affecting the uterus and mammary gland. The association between low IGF-1 and clinical endometritis supports an earlier study which showed that multiparous cows in which the plasma IGF-I concentration fell below 40 ng/mL in the week after calving were nearly three times more likely to suffer from a subsequent uterine infection [62]. The data presented here show an increased likelihood that they may also develop clinical or subclinical mastitis. In addition, previous studies have shown associations between low IGF-1 pre-calving with clinical ketosis [157] and between low IGF-1 postpartum with both displaced abomasum and an increased risk of culling [74].

These diseases may well contribute to persistent inflammatory changes, which have an adverse effect on the cow's ability to conceive some months later. In this respect, it is interesting to compare our results with a study of fertility in beef heifers which measured transcriptional profiles in white blood cells collected at the time of insemination [158]. Their analysis revealed 18 DEG between heifers which did or did not conceive. *ALAS2*, *BOLA-DQB* and *DMBT1* were common to the lists of highly differentially expressed genes identified in our study. Of these *DMBT1* was one of the most highly up-regulated genes in both studies and therefore warrants further investigation as a biomarker for fertility. *FCER1A* and *P2RY12* were also on their list and we found genes for two other components of the IgE receptor, *FCGR1A* and *FCER1G* were up-regulated in LOW IGF-1 cows while three other purinergic receptors were identified, with *P2RY1* and *P2RY2* up-regulated and *P2RY10* down-regulated.

5. Conclusions

A growing number of studies have measured selected inflammatory mediators in peripartum cows but few to date have used a similar global approach or based it on such a sizeable population as that used here. Our study has linked transcriptomic analysis of circulating leukocytes to the IGF-1 concentration, used as a biomarker of metabolic function. This has confirmed changes in metabolic pathways in LOW compared to HIGH IGF-1 cows,

with immune cells switching their energy metabolism from oxidative phosphorylation to aerobic glycolysis, and making greater use of the pentose phosphate shunt. Our analysis has also extended the range of candidate genes and pathways regulating immunity which were differentially affected. Many antimicrobial peptides were up-regulated in LOW vs. HIGH IGF-1 cows. On the other hand, the HIGH IGF-1 cows had higher expression of more genes regulating T and B cell function and the cytoskeleton. A large number of genes associated with inflammation were also differentially expressed, particularly those associated with platelet activation and the complement cascade. Overall, the results suggest that the LOW IGF-1 cows are engaged in an energetically demanding battle against ongoing infection(s) involving the innate immune system. This reduces their appetite and milk yield and is likely to have an adverse effect on their subsequent fertility. On the other hand, the HIGH IGF-1 cows have higher nutrient availability and can use this to promote use of their adaptive immune system to better resist infection. Our results therefore revealed several mechanisms which help to explain why and how innate and adaptive immunity can become compromised in postpartum dairy cows with low circulating IGF-1 levels in early lactation. The data presented here thus support other available evidence in suggesting that the metabolic status of individual cows is linked directly to the high incidence of health disorders during this critical period [1,3,51,159]. Furthermore, 35/170 (20%) of all cows in this study fell into the LOW IGF-1 category, indicating the need for improved peripartum management to reduce the incidence of such animals.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ruminants1020012/s1, Table S1: List of DEG with FC $\geq$ 1.2 and FDR p < 0.05 with higher expression in cows with LOW IGF-1 (<35 ng/mL, n = 35) in comparison with HIGH IGF-1 (>100 ng/mL, n = 43) in leucocytes of cows at 14 days in milk, based on the global transcriptome; Table S2.: List of DEG with FC $\geq$ 1.2 and FDR p < 0.05 with lower expression in cows with LOW IGF-1 (<35 ng/mL, n = 35) in comparison with HIGH IGF-1 (>100 ng/mL, n = 43) in leucocytes of cows at 14 days in milk, based on the global transcriptome; Table S3: Cluster analysis using DAVID based on list of 1,032 DEG with FC $\geq$ 1.2 and FDR p < 0.05 between cows with LOW IGF-1 (<35 ng/mL, n = 35) and HIGH IGF-1 (>100 ng/mL, n = 43) in leukocytes of cows at 14 days in milk, based on the global transcriptome; Table S4: Lists of genes in clusters 4–6 from analysis using DAVID based on list of 1,032 DEG with FC $\geq$ 1.2 and FDR p < 0.05 between cows with LOW IGF-1 (<35 ng/mL, n = 35) and HIGH IGF-1 (>100 ng/mL, n = 43) in leukocytes of cows at 14 days in milk, based on the global transcriptome; Table S5: Kegg pathway analysis using DAVID based on list of 1,032 DEG with FC $\geq$ 1.2 and FDR p < 0.05 between cows with LOW IGF-1 (<35 ng/mL, n = 35) and HIGH IGF-1 (>100 ng/mL, n = 43) in leukocytes of cows at 14 days in milk, based on the global transcriptome; Table S6: Lists of genes in significant KEGG pathways from analysis using DAVID based on list of 1032 DEG with FC $\geq$ 1.2 and FDR p < 0.05 between cows with LOW IGF-1 (<35 ng/mL, n = 35) and HIGH IGF-1 (>100 ng/mL, n = 43) in leukocytes of cows at 14 days in milk, based on the global transcriptome; Table S7: Sub list of metabolic-related DEG with FC $\geq$ 1.2 and FDR p < 0.05 in comparison between cows with LOW (<35 ng/mL, n = 35) and HIGH IGF-1 (>100 ng/mL, n = 43) in leucocytes collected at 14 days in milk, based on the global transcriptome; Table S8: Kegg pathway analysis using DAVID based on sublist of 149 metabolic-related DEG with FC $\geq$ 1.2 and FDR p > 0.05 between cows with LOW IGF-1 (<35 ng/mL, n = 35) and HIGH IGF-1 (>100 ng/mL, n = 43) in leukocytes of cows at 14 days in milk, based on the global transcriptome; Table S9: Lists of genes in significant KEGG pathways from analysis using DAVID based on list of 149 metabolic-related DEG with FC $\geq$ 1.2 and FDR p > 0.05 between cows with LOW IGF-1 (<35 ng/mL, n = 35) and HIGH IGF-1 (>100 ng/mL, n = 43) in leukocytes of cows at 14 days in milk, based on the global transcriptome; Figure S1: Modified representation of Kegg pathway bta04015: Rap1 signaling pathway, highlighting DEG identified in this study.

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