Studies relating to protein expression in the uterus of the cow

End of Project Report

Project 5236

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Summary

Embryo loss is a major cause of reproductive wastage in the cow. The majority of embryo loss occurs in the first 16 days after fertilisation when the embryo is critically dependent on the maternal uterine environment for survival. Despite the central role of uterine fluid in the normal growth and development of the embryo, there is limited information on the protein composition of these fluids. The main objectives of the studies in this thesis were to examine the protein composition of the bovine uterus during the oestrous cycle and to examine the relationship between the concentration of systemic progesterone and uterine protein expression in the cow.

In the first study, the concentration of retinol-binding protein (RBP) in the bovine uterus was found to vary across the cycle and was 5-15-fold higher \((P<0.001)\) on Day 15 than Days 3, 7 and 11. Additionally, the concentration of uterine RBP seems to be regulated in a local manner as the concentration in the uterine horn ipsilateral to the corpus luteum (CL) was more than 2-fold higher \((P<0.001)\) than the contralateral horn on Day 15. This indicates that RBP is possibly regulated by a local increase in the concentration of progesterone. Uterine and plasma concentrations of RBP were similar on days 3 to 11, however, uterine RBP concentrations were 6-15 fold higher than blood plasma RBP concentrations on day 15. This shows evidence of active transport and/or synthesis of proteins into the uterus which previously were thought to be due to transduction of plasma. There was no relationship between the concentration of systemic progesterone and concentration of RBP on day 7 \((P>0.05)\) of the cycle, which was surprising given that previous studies have indicated that uterine RBP gene expression was positively associated with the concentration of systemic progesterone.

In the second study, IGF binding protein 2 (IGFBP-2), IGFBP-3, IGFBP-4 and IGFBP-5 were identified in uterine fluid on days 3, 7, 11 and 15 of the oestrous cycle. There was a local effect on the concentration of IGFBPs where the concentration was greater on the ipsilateral side than that on the contralateral side for IGFBP-2 \((P<0.05)\), 3 \((P<0.01)\) and 5 \((P<0.01)\) on day 15. This difference is a further indication of a local controlling mechanism regulating proteins between the uterine horns. Similar to RBP expression this study could find no significant relationship between the concentration of systemic progesterone and IGFBP concentrations on Day 7 of the oestrous cycle.

In the third study, changes in the global pattern of uterine proteins between Days 3 and Day 15 of the oestrous cycle were examined using two-dimensional electrophoresis (2-DE). Six proteins were found to be upregulated on Day 15 compared to Day 3. Three proteins of these were identified as aldose reductase, plakoglobin and heat shock protein 27 while the other three proteins were identified as bovine serum albumin. Aldose reductase, an enzyme directly involved in the production of sorbitol and indirectly of fructose, was 10-fold higher \((P<0.0001)\) on Day 15 compared to Day 3. Plakoglobin \((Pg)\) was upregulated 2.3-fold \((P<0.0001)\) on Day 15 compared to Day 3. Pg is a component of cellular junctions and its up-regulation may have a role in the uterine glandular epithelium. Heat shock protein 27 \((Hsp27)\) was higher on Day 15 than on Day 3 \((P<0.01)\) and Hsp27 was 1.4-fold higher in the ipsilateral compared to the contralateral uterine horn \((P<0.01)\). Hsp27 may be secreted in response to potential stresses in the uterus or act as a molecular chaperone. On Day 7 there was no difference \((P<0.05)\) in the pattern of proteins secreted between cows with low \((2.7±0.10\text{ng/ml})\) and high \((4.8±0.13 \text{ ng/ml})\) concentrations of systemic progesterone on Day 7.

The results of these studies have shown that dramatic changes occur in protein expression across the bovine oestrous cycle. Additionally, it emphasises the need for gene studies to be followed with protein studies as an adjunct or complementary tool. Proteins have a wide range of essential roles in the uterus and together these studies provide novel information on protein expression in the uterus of the cow.
Introduction

Increased genetic selection combined with improvements in nutrition has increased milk production in dairy cattle over the past four decades (Darwash et al., 1999). This increase, however, has been accompanied by a decrease in fertility (Butler, 1998; Royal et al., 1999). Reproductive failure causes enormous economic losses to the dairy industry with lower milk sales and fewer calves born as well as resultant delays in genetic progress (Dunne et al., 1999). Embryo loss is recognised as the major cause of reproductive failure in the dairy cow and the majority of these losses occur within the first two weeks after AI (Sreenan et al., 2001). From Day 4 post fertilisation until implantation around Day 20, the embryo maintains a relatively free-floating existence surrounded by the fluid of the uterus. This represents a critical period in the development and differentiation of the embryo as it undergoes compaction, blastulation and elongation. The uterine fluid must provide any nutrients or growth factors necessary for its development, growth and ultimately its survival.

Many factors have been shown to influence embryo survival including the steroid hormone, progesterone, which is essential for establishment of pregnancy and low concentrations of progesterone have been associated with a low probability of embryo survival (Stronge et al., 2005; McNeill et al., 2006a). Studies have also shown that changes in the concentration of progesterone can affect gene expression in the bovine uterus thus potentially altering the protein composition of uterine fluid. Candidate gene studies have shown that genes encoding proteins essential for normal embryo development are sensitive to changes in the concentration of systemic progesterone on Day 7 of the oestrous cycle (McNeill et al., 2006b). Changes in gene expression, however, do not always translate into corresponding changes in protein expression and gene expression studies do not take account of post-translational modifications or protein-protein interactions subsequent to translation. Ultimately mRNA must be thought of as intermediate product in the synthesis of functional proteins and mRNA expression alone is not sufficient to predict the concentration of the encoded protein (Wolf et al., 2003). Thus in order to determine the effects of progesterone on the uterine fluid it is essential to also characterise functional protein expression in the uterus of the dairy cow. There is little published information on the protein composition of the bovine uterus or the relationship between the uterine proteome and the concentration of systemic progesterone. This study aimed to redress this lack of knowledge. The main objective of this study was to examine protein expression in the uterus of the cow on specific days of the oestrous cycle and to determine the relationship between the concentration of systemic progesterone and uterine protein expression in the dairy cow and if these concentrations differ from plasma.
Study 1: Retinol-binding protein in the bovine uterus

Objective

The objective of this study was to determine the concentration of RBP, beta-carotene and retinol in both uterine horns, ipsilateral and contralateral to the corpus luteum, on days 3, 7, 11 and 15 of the oestrous cycle and to determine the relationship between the concentration of RBP and the concentration of systemic progesterone on day 7 of the oestrous cycle.

Materials and Methods

Animals

Spontaneously cycling, reproductively normal lactating Holstein-Friesian dairy cows were used. The animals were at least 50 days post-partum with at least one normal oestrous cycle prior to use in this study. Two experimental groups of animals were used in this study. In Experiment 1, uterine flushings were collected non-surgically from both uterine horns, ipsilateral and contralateral to the corpus luteum on days 3, 7, 11 and 15 of the oestrous cycle (n=6 per day). In Experiment 2, UF were collected from 27 cows on Day 7 of the oestrous cycle. Blood plasma samples were collected before and after flushing in both experiments and were analysed by radioimmunoassay for concentrations of progesterone and oestradiol.

Measurement of Protein in uterine flushings and plasma

Total protein was measured in UF and plasma by Bradford assay. Retinol binding protein (RBP) was not commercially available for use in this study and so it necessary to purify RBP from bovine plasma. The concentration of plasma and uterine retinol-binding protein was measured by an enzyme-linked immunosorbent assay developed in-house. Retinol and beta-carotene was measured in uterine flushings and plasma following extraction using high-performance liquid chromatography.

Statistical Analysis

Data were analysed using a REML based mixed effects model using PROC MIXED in SAS (SAS, 2003). Pearson correlation coefficients were determined using PROC CORR. Significant differences were compared using Tukey’s option. A probability of P<0.05 was considered significant.

Results

Experiment 1

Total Uterine Protein

Total uterine protein was found to vary in a quadratic manner across the cycle as shown in Figure 1. There was no difference in total uterine protein content between the ipsilateral and contralateral uterine horns across the cycle (P>0.05). The total uterine protein concentrations were lower on day 11 than days 3 or 15 (P<0.01).

Concentration of retinol-binding protein

Due to changes in total protein with day, the RBP concentrations were normalised and expressed per unit of total uterine protein. The RBP concentration of the ipsilateral and contralateral uterine horns across the cycle is shown in Figure 2. RBP concentrations were 5-15-fold higher (P<0.001) on day 15 than on all other days and were lower on day 11 than day 3 (P<0.05). There was a significant day and day by side interaction on uterine RBP concentration when expressed per unit of total uterine protein, with the ipsilateral side more than 2-fold higher (P<0.001) than the contralateral side on day 15. When compared, uterine and blood plasma RBP concentrations were similar on days 3 to 11 of the oestrous cycle however uterine RBP concentrations were 6-15 fold higher than blood plasma RBP concentrations on day 15 as shown in Figure 2.
Figure 1 Total uterine protein (mg) on different days of the oestrous cycle. Data are presented as backtransformed least square means and standard errors (bLSM±SEM). * = Day 11 was lower than days 3 and 15 (P<0.01).

Figure 2 RBP concentration (ng per µg protein) (bLSM±SEM) in the ipsilateral and contralateral uterine horns on days 3, 7, 11 and 15 of the oestrous cycle.
Experiment 2

There was no relationship between either total RBP or RBP concentration and the concentration of systemic progesterone on day 7 ($P>0.05$) as shown in Figure 3.

![Figure 3](image)

**Figure 3** The relationship between total RBP concentration (pg/µg) and progesterone concentrations (ng/ml) on day 7 of the oestrous cycle.

Concentration of retinol in the uterus

In order to allow for the differences in total protein between days, the concentrations of retinol and beta-carotene were standardised and expressed per unit of protein. The concentrations of retinol in the ipsilateral and contralateral uterine horns across the oestrous cycle are shown in Figure 4.

![Figure 4](image)

**Figure 4** Concentration of retinol (ng per µg protein) (bLSM±SEM) in the uterine horns on days 3, 7, and 15 of the oestrous cycle. The mean concentration of retinol (ng/µg protein) in plasma is represented by the dashed line.

There was a significant effect of day on the concentration of retinol per unit of protein in uterine flushings, with concentrations on Day 15 up to 7-fold higher compared to the other days ($P<0.05$). The ipsilateral concentrations of retinol were also higher than the contralateral side on day 15 ($P<0.01$). There was a linear ($P<0.0001$) and quadratic relationship between day and retinol concentration ($P<0.0007$). When uterine and blood plasma concentrations of retinol were compared,
retinol was higher in plasma on days 3 to 11 of the oestrous cycle, however, uterine concentrations of retinol were up to 2-fold higher than blood plasma concentrations on day 15 as shown in Figure 4. A significant correlation of $r^2=0.46$ was found between the concentration of retinol and RBP in the uterine flushings across the cycle ($P<0.01$).

**Study 2: Insulin-like growth factor binding proteins in the bovine uterus**

**Objective**
The objectives of this study was to examine the insulin-like growth factor system (IGF) in the bovine uterus on Days 3, 7, 11 and 15 of the oestrous cycle and to determine the relationship between the IGFBP concentration and the concentration of systemic progesterone on Day 7 of the oestrous cycle.

**Materials and Methods**

*Animals*
Spontaneously cycling, reproductively normal lactating Holstein-Friesian dairy cows were used. The animals were at least 50 days post-partum with at least one oestrous cycle prior to use in this study. Two experimental groups of animals were used in this study. In Experiment 1, uterine flushings were collected non-surgically from both uterine horns, ipsilateral and contralateral to the corpus luteum on days 3, 7, 11 and 15 of the oestrous cycle (n=6 per day). In Experiment 2, UF were collected from 27 cows on Day 7 of the oestrous cycle. Blood plasma samples were collected before and after flushing in both experiments and were analysed by radioimmunoassay for concentrations of progesterone and oestradiol.

*Characterisation of IGFBPs*
IGFBPs were characterised and quantified using western blotting using biotinylated IGF-II. Uterine flushings were analysed by non-reducing SDS-PAGE followed by Western ligand blotting onto a PVDF membrane using a semi-dry system. The blotted membrane was probed with biotinylated IGF-II followed by extravidin-peroxidase. The IGFBPs were detected by chemiluminescence using the ECL-plus system. The blots were imaged using a calibrated CCD system. IGFBP-4 and IGFBP-2 were identified in uterine flushings using antibodies specific to IGFBP-4 and IGFBP-2.

*Statistical Analysis*
Data were analysed using a REML based mixed effects model using PROC MIXED in SAS (SAS, 2003). Pearson correlation coefficients between IGFBPs were determined using PROC CORR. A probability of $P<0.05$ was considered significant.

**Results**

*Detection and characterisation of IGFBPs*
IGFBP-2, IGFBP-3, IGFBP-4 and IGFBP-5 were detected in uterine fluid on Days 3, 7, 11 and 15 of the oestrous cycle as shown in Figure 6.

*IGFBPs*
IGFBP-2, -3 and -5 were detected in bovine plasma on all days of the oestrous cycle was examined. While IGFBP-2, -3, -4 and -5 were detected in uterine flushings across the oestrous cycle however only IGFBP-2, -3 and -5 were in quantities sufficient for statistical analysis.

*Uterine IGFBP-3*
The concentration of IGFBP-3 in uterine flushings across the cycle is shown in Figure 7.
Figure 6 Western ligand blot of IGFBP-2, 3, 4 and 5 in uterine flushings and IGFBP-2, 3 and 5 in plasma following SDS-PAGE. The IGFBPs were detected with biotinylated IGF-II and chemiluminescence using ECL+. Approximate molecular weights of IGFBPs are shown.

Figure 7 Uterine IGFBP-3 concentrations (bLSM ± SEM) in the ipsilateral and contralateral uterine horns on days 3, 7, 11 and 15 of the oestrous cycle. The mean plasma IGFBP-3 concentration is represented by the dashed line.

There was no significant effect of day or side on uterine IGFBP concentrations however there was a significant day by side interaction with the concentration of IGFBP-3 on the ipsilateral side greater than that on the contralateral side with (P<0.01). Concentrations of uterine IGFBP-3 were up to 10-fold higher in the uterine flushings compared to plasma.

Uterine IGFBP-2
The concentration of IGFBP-2 in uterine flushings across the cycle is shown in Figure 8. There was no difference in the concentration of IGFBP-2 between the days of the cycle or between the sides of the uterus. However, the concentration of IGFBP-2 on the ipsilateral side was greater than that on the contralateral side (P<0.05). There was a difference in the concentrations of uterine IGFBP-2 between plasma and the uterus with uterine concentrations up to 4-fold higher in the uterine flushings compared to plasma.
Uterine IGFBP-5
There was no difference in the uterine IGFBP-5 concentrations across the cycle, however, the concentration of IGFBP-5 was greater on the ipsilateral side than the contralateral side (P<0.01).

Experiment 2
In experiment 2, uterine IGFBP expression on Day 7 of the oestrous cycle was examined. IGFBP-2, -3, -4 and -5 were detected in bovine uterus flushings. Uterine flushings were recovered from animals with a wide range of endogenous progesterone concentrations. There was no relationship between any of the other binding proteins and the concentration of systemic progesterone on Day 7 (P>0.05).

Study 3: Global protein expression in the bovine uterus
Objective
The objective was to characterise differentially expressed proteins in the uterus on Days 3 and 15 of the oestrous cycle and to determine the relationship between the concentration of systemic progesterone and global protein expression on day 7 of the oestrous cycle.

Materials and methods
Animals
Spontaneously cycling, reproductively normal lactating Holstein-Friesian dairy cows were used. The animals were at least 50 days post-partum with at least one oestrous cycle prior to use in this study. Two experimental groups of animals were used in this study. In Experiment 1, uterine flushings were collected non-surgically from both uterine horns, ipsilateral and contralateral to the corpus luteum on days 3 and 15 of the oestrous cycle (n=6 per day). In Experiment 2, UF were collected from cows with low (2.7±0.10ng/ml, n=6) or high (4.8±0.13ng/ml, n=6) on Day 7 of the oestrous cycle. Blood plasma samples were collected before and after flushing in both experiments and were analysed by radioimmunoassay for concentrations of progesterone and oestradiol.
Two-dimensional gel electrophoresis

UF were rehydrated on a 24 cm 3-10 pH non-linear Immobiline DryStrip gel and isoelectric focused for 90kVh. The second dimension separation was carried out on a 12% SDS-PAGE gel. Separated proteins were detected using silver staining and imaged using a calibrated CCD system. Image analysis and statistical analysis was carried out using Same Spots software (Progenesis;Nonlinear Dynamics, UK). The differentially expressed proteins were excised from a preparative gel stained with Colloidal Coomassie Blue. The excised proteins underwent LC/MS on LTQ (Thermo-Finnegan) and Sequest database search for identification.

Results

This is the first study to examine bovine uterine flushings on Day 3 and Day 15 of the oestrous cycle using 2DE as shown in Figure 10.

![2-DE map of proteins from uterine flushings on Day 15 of the oestrous cycle (n=12). Proteins were separated over pH range 3-10 and 12% SDS-polyacrylamide gel. Proteins were visualised by silver staining.](image)

In experiment 1, six proteins were found to be upregulated on Day 15 compared to Day 3. Three proteins of these were identified as aldose reductase plakoglobin and heat shock protein 27 while the other three proteins were identified as bovine serum albumin. Aldose reductase was 10-fold higher (P<0.0001) on Day 15 compared to Day 3. Plakoglobin (Pg) was upregulated 2.3-fold (P<0.0001) on Day 15 compared to Day 3. Heat shock protein 27 (Hsp27) was higher on Day 15 than on Day 3 (P<0.01) as shown in Figure 11 and 12, and Hsp27 was 1.4-fold higher in the ipsilateral compared to the contralateral uterine horn (P<0.01).
Figure 11 Comparison of the normalised protein spot area of Hsp27 between Day 3 and Day 15 shows that the indicated protein is upregulated on Day 15.

Figure 12 Normalised volume (±SEM) of Hsp27 on Day 3 and Day 15 (n=12).

In Experiment 2, we could find no significant relationship between the concentration of systemic progesterone and global uterine protein expression in the bovine uterus on Day 7 of the cycle.

Overall Discussion
Embryo loss is now established as a major cause of reproductive failure in the dairy cow with 70-80% of embryo loss occurring in the first two weeks of pregnancy. There are many factors involved in embryo loss, however, one factor known to be involved in the establishment and maintenance of a successful pregnancy is the concentration of systemic progesterone. Low concentrations of progesterone in the early luteal phase associated with a low probability of embryo survival. Progesterone may affect the embryo through indirect actions on the uterine environment as previous studies have shown small changes in the concentration of systemic progesterone were associated with significant changes in uterine gene expression. Therefore, progesterone is capable of altering the uterine environment which may ultimately affect embryo survival. However, gene expression does not always mean protein expression and translation into functional proteins may not occur. The overall objective of this study was to investigate the expression of proteins in the bovine uterus and determine the relationship between the concentration of systemic progesterone and expression...
of uterine proteins. The results of this study demonstrated the characterisation and measurement of proteins in the bovine uterus.

Retinol-binding protein (RBP) was purified from bovine serum and subsequently the purified RBP was used in the development of an enzyme-linked immunosorbent assay to measure RBP in bovine uterine flushings and plasma. The concentration of uterine RBP was to be found to stage dependent with a dramatic increase on Day 15 compared to Days 3, 7 and 11 of the oestrous cycle. These results concur with previous reports that RBP protein expression decreases from metestrus to mid dioestrus and subsequently increases in late dioestrus (MacKenzie et al., 1997). The concentration of RBP in the ipsilateral uterine horn was over 2-fold higher than the contralateral concentration on Day 15 of the cycle. This is a novel finding showing that uterine RBP is regulated in a local manner, most likely by progesterone. The comparison of the concentration of RBP between plasma and the uterus revealed another novel finding in that the concentration of RBP was found to be similar in the uterus and plasma across Days 3 to 11, however, on Day 15 RBP was significantly higher in the uterine fluid compared to plasma. This difference between the uterus and plasma would suggest active transportation and/or synthesis of RBP into the uterus.

As the physiological role of RBP is to bind and transport retinol throughout the system, the concentration of retinol was measured by high performance liquid chromatography in the bovine uterus across the oestrous cycle. To the authors knowledge this is the first time that the concentration of retinol has been measured in bovine flushings. The concentration of retinol was correlated with RBP concentrations across the cycle with a dramatic increase in retinol on Day 15 compared to the other days of the cycle. Furthermore, similar to RBP, the concentration of retinol was higher in the ipsilateral uterine horn compared to the contralateral uterine horn.

The relationship between the concentration of systemic progesterone and RBP expression on Day 7 of the cycle was investigated. Although a wide range of concentrations of progesterone were used in this study, the results of the study suggest that there is no significant relationship between the concentration of systemic progesterone and RBP concentrations on Day 7 of the oestrous cycle. This is in contrast to previous gene studies that found that small changes in the concentration of progesterone were associated with significant increases in RBP gene expression (McNeill et al., 2006b). Translation into proteins may be delayed or may not occur at all and these results stress the need for protein studies as an adjunct or complementary tool to gene studies.

The results suggests that active synthesis and/or transportation of RBP together with its bound retinol into the uterus are most likely under the control of progesterone. Furthermore, it highlights the importance and need for RBP and retinol in the preimplantation environment specifically on Day 15 of the oestrous cycle.

In study 2, a number of components of the insulin-like growth factor system were determined in the bovine uterus. IGFBP-2, IGFBP-3, IGFBP-4 and IGFBP-5 were determined by western blotting in the bovine uterus across the cycle. Like RBP, the concentration of uterine IGFBP-2, IGFBP-3 and IGFBP-5 were found to be regulated locally with differences in the concentrations of IGFBPs between uterine horns, ipsilateral and contralateral to the CL, on Day 15. This difference could indicate a local controlling mechanism with progesterone possibly playing a role in regulating the concentration of IGFBPs between the uterine horns. The physiological role of the binding proteins in the uterus is not fully established however, they can have IGF dependent or independent roles. The IGFBPs can modulate activity the IGFs ligands by binding to them. Additionally, the IGFBPs can interact with components such as cell surface glycosaminoglycans (Wathes et al., 1998) and the extracellular matrix to increase the local concentrations of IGFs in the uterus or transport locally produced or circulating IGFs (Keller et al., 1998).
Significant differences in the concentration of IGFBP-2 and IGFBP-3 were found between plasma and uterine fluid. The uterine concentration of these proteins was higher than concentrations in plasma suggesting synthesis and/or transportation of the IGFBPs into the uterus. The relationship between the concentration of systemic progesterone and IGFBPs or IGF-I expression on Day 7 the cycle was investigated, however, this study could find no significant relationship between the concentration of systemic progesterone and IGFBP concentrations on Day 7 of the oestrous cycle. Overall, the results of this study show significant changes in IGFBPs during a time of major change in the developing of the embryo.

In study 3, two-dimensional gel electrophoresis and silver staining were used to separate and visualise proteins in bovine uterine flushings. This is the first study to examine bovine uterine flushings on Day 3 and Day 15 of the oestrous cycle using 2DE. When proteins from Day 3 and 15 were compared, six proteins were found to be differentially expressed, being upregulated on Day 15 compared to Day 3. Following colloidal Coomassie Blue staining, the proteins were identified by mass spectrometry as aldose reductase, plakoglobin and heat shock protein 27 while other three were identified as related to bovine serum albumin which may indicate significant interference from albumin in the sample or may indicate a physiological role for changes in the concentration of albumin on day 15 such as the transport of lipids or steroids.

Aldose reductase, an enzyme involved in the direct production of sorbitol and indirectly production of fructose, was 10-fold higher on Day 15 compared to Day 3. Plakoglobin, a component of cellular junctions and its upregulation may have a role in the uterine glandular epithelium. Heat shock protein 27 was higher on Day 15 and a local effect was found with Hsp27 being higher in the ipsilateral than the contralateral uterine horn. This protein may respond to potential stresses in the uterus or act as a molecular chaperone. In Experiment 2, we could find no significant relationship between the concentration of systemic progesterone and global uterine protein expression in the bovine uterus on Day 7 of the cycle.

Overall, a number of proteins were found to be upregulated on Day 15 compared to Day 3 but the exact nature of their function or regulation in the bovine uterus is not clear at this moment. However, it is possible that progesterone influences their expression as they are found in the uterus at a time of high progesterone concentrations.

The results of this study have shown that dramatic changes occur in protein expression in the bovine uterus on Day 15 of the oestrous cycle. Although it is thought that proteins occur in the uterus as transducate of plasma the results of study 2 and 3 provide evidence of active transport into or synthesis of proteins by the uterus. These studies also emphasise the need for caution in using temporal gene expression studies as sole predictions of cellular function. Therefore, adjunct or complementary proteomic studies should be followed by confirmatory proteomic studies where possible.
References


Publications arising from this study


