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Biomarker metabolite mating of viable frozen-thawed IVP bovine embryos with pregnancy-competent recipients leads to improved birth rates

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ABSTRACT

Selection of competent recipients before embryo transfer (ET) is indispensable for improving pregnancy and birth rates in cattle. However, pregnancy prediction can fail when the competence of the embryo is ignored. We hypothesized that the pregnancy potential of biomarkers could improve with information on embryonic competence. In vitro produced (IVP) embryos cultured singly for 24 h (from d 6 to 7) were transferred to d 7 synchronized recipients as fresh or after freezing and thawing. Recipient blood was collected on d 0 (estrus; n = 108) and d 7 (4–6 h before ET; n = 107) and plasma was analyzed by nuclear magnetic resonance ($1H^{+}$NMR). Spent embryo culture medium (CM) was collected and analyzed by UHPLC-MS/MS in a subset of n = 70 samples. Concentrations of metabolites quantified in plasma (n = 35) were statistically analyzed as a function of pregnancy diagnosed on d 40, d 62 and birth. Univariate analysis with plasma metabolites consisted of a block study with controllable fixed factors (i.e., embryo cryopreservation, recipient breed, and day of blood collection; Wilcoxon test and *t*-test). Metabolite concentrations in recipients and embryos were independently analyzed by iterations that reclassified embryos or recipients using the support vector machine (SVM). Iterations identified some competent embryos, but mostly competent recipients that had a pregnancy incompetent partner embryo. Misclassified recipients that could be classified as competent were reanalyzed in a new iteration to improve the predictive model. After subsequent iterations, the predictive potential of recipient biomarkers was recalculated. On d 0, creatine, acetone and L-phenylalanine were the most relevant biomarkers at d 40, d 62, and birth, and on d 7, L-glutamine, L-lysine, and ornithine. Creatine was the most representative biomarker within blocks (n = 20), with a uniform distribution over pregnancy endpoints and type of embryos. Biomarkers showed higher abundance on d 7 than d 0, were more predictive for d 40 and d 62 than at birth, and the pregnancy predictive ability was lower with frozen-thawed (F-T) embryos. Six metabolic pathways differed between d 40 pregnant recipients for fresh and F-T embryos. Within F-T embryos, more recipients were misclassified, probably due to pregnancy losses, but were accurately identified when combined with embryonic metabolite signals. After recalculation, 12 biomarkers increased ROC-AUC (>0.65) at birth, highlighting creatine (ROC-AUC = 0.851), and 5 new biomarkers were identified. Combining metabolic information of recipient and embryos improves the confidence and accuracy of single biomarkers.

Key words: bovine embryo, recipient, pregnancy, metabolism

INTRODUCTION

Optimizing the selection of recipients and in vitro produced (**IVP**) embryos for transfer is essential to improve pregnancy and birth rates in cattle. Pregnancy rates, in particular with cryopreserved IVP embryos, remain far from meeting the needs of the market, and efforts have been made to identify markers and develop strategies allowing selection of the most appropriate embryos and recipients to obtain successful pregnancies (Diskin et al., 2016). Within recipients, because the common selection procedures for reproductive assessment often excludes fertile animals from transfer (Dickinson et al., 2019), there is a demand for biomarkers based on simple and logistically feasible methods (Kanazawa et al., 2016; Daly et al., 2020; Demetrio et al., 2020). Although the genomic selection of fertile recipients is efficient (Geary et al., 2016), genomic

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tools do not allow the selection of specific reproductive cycles in which pregnancy will proceed, as management, nutrition, and environmental factors also affect pregnancy (Robles and Chavatte-Palmer, 2017; Caton et al., 2020).

Omics are gaining relevance as sophisticated methods to explain the biological processes inherent to bovine reproduction (Canovas et al., 2014; Rabaglino et al., 2021). Among the different omic levels, the metabolome has been specially addressed as the closest indicator of changes in the biological function, integrating the external (e.g., diet) and internal (e.g., genotype) factors that influence metabolism (Goldansaz et al., 2017; Moore et al., 2017). Thus, using one-proton nuclear magnetic resonance (**1H$^+$NMR**), we and others identified and quantified low molecular weight metabolites in d 0 and d7 recipient plasma that correlate and predict the reproductive performance within the cognate estrus cycle (Phillips et al., 2018; Gómez et al., 2020b,c; Funeshima et al., 2021). In addition, the competence of fresh and cryopreserved [i.e., frozen-thawed (**F-T**) and vitrified-warmed (**V-W**)] IVP embryos to establish pregnancy and reach birth after transfer was noninvasively determined in embryo culture medium (**CM**) by UHPLC-MS/MS (Gimeno et al., 2021) and GC-MS/MS (Gómez et al., 2021). In this way, the combination of one biomarker identified in recipients with one biomarker from the embryonic side permits considerable improvement in pregnancy prediction to term (>90%; Gómez et al., 2021).

Given the strong metabolomic differences between recipient breeds (Gómez et al., 2020d), the control of recipients and certain laboratory factors is a must to identify reliable, pregnancy predictive metabolite biomarkers. We therefore should consider the range of metabolite concentrations in the scope of the recipient breed, to a certain extent the embryo CM, and the status of the embryo (fresh or cryopreserved), although specific metabolites are independent of such effects (e.g., ornithine; Gómez et al., 2020b,c). The analysis by 1H$^+$NMR of the plasma metabolome allows for accurate quantification of absolute metabolite concentrations, as wells as reliable results, allowing comparisons through different experiments (Goldansaz et al., 2017; Emwas et al., 2019).

Together with V-W embryos, IVP embryos cryopreserved by conventional slow freezing have recently become of interest because of the possibility of direct transfer and simplicity of use, without a need for laboratory equipment (Sanches et al., 2016; Jia et al., 2018; Zolini et al., 2019; Gómez et al., 2020a; Álvarez-Gallardo et al., 2021). We have recently identified metabolite biomarkers in the CM of such embryos before embryo transfer (**ET**; Gimeno et al., 2021), which are useful for identifying "true" competent recipients as previously shown with CM of V-W IVP embryos analyzed by GC-MS/MS (Gómez et al., 2021). However, the identification of pregnancy and birth metabolite biomarkers among recipients transferred with F-T embryos has not yet been investigated.

In this work we investigated the metabolite biomarkers in blood plasma on d 0 (estrus time) and d 7 (hours before ET time) which can predict predefined stages of pregnancy and birth in heifers transferred with F-T embryos. The biomarkers and metabolite signals that we previously identified in a CM subset from the cognate embryos (Gimeno et al., 2021) were used to obtain an accurate definition of the pregnancy potential of the recipient—and the tandem embryo and recipient—by detecting recipients acting as false negatives using a novel iterative in silico strategy. We hypothesized that, as shown with V-W and fresh embryos and their recipients, the predictive capacity of the recipient would increase once supported by accurate definition of the embryonic competence.

MATERIALS AND METHODS

Experimental procedures were conducted following the guidelines of the Declaration of Helsinki and approved by the Animal Research Ethics Committees of SERIDA (PROAE 26–2016; Resolución de 25 de Julio de 2016 de la Consejería de Medio Rural y Recursos Naturales), in accordance with the European Community Directive 86/609/EC. Reagents were purchased from SIGMA unless otherwise stated.

Embryo Production

Embryos were produced in vitro from slaughterhouse ovaries following detailed procedures previously described for oocyte collection, in vitro maturation, and in vitro fertilization, which was performed with n = 7 single different bulls [n = 4, Asturiana de los Valles (**AV**), and n = 3 Holstein bulls; Gómez et al., 2020c]. For in vitro culture (**IVC**), presumptive zygotes were cultured first in groups in modified synthetic oviduct fluid (**mSOF**) with AA [MEM nonessential amino acids solution (#M7145), 3.3 μL/mL; and BME amino acids solution (#B6766), 45 μL/mL], citrate (0.1 μg/mL), myo-inositol (0.5 μg/mL), and 6 mg/mL BSA (#A3311) with or without 0.1% (vol/vol) FCS (#F4135). In vitro culture (**IVC**) was carried out in droplets under mineral oil at 38.7°C, 5% CO_2, 5% O_2, 90% N_2, and saturated humidity. On d 6 (143 h PI), excellent and good quality (grade 1 and grade 2) morulae and early blastocysts were selected and cultured individually for 24 h in 12 μL mSOF with 0.5 mg/mL

polyvinyl-alcohol PVA (P8136) under mineral oil. Day 7 embryos showing very good to excellent morphological quality were either transferred as F-T or fresh. Embryonic stages transferred were expanded blastocysts (cryopreserved and fresh) and blastocysts (fresh). The CM of embryos (10 μL) was collected and stored at −150°C and analyzed by UHPLC-MS/MS to identify pregnancy and birth biomarkers, as described in a previous work (Gimeno et al., 2021).

Embryo Freezing and Thawing

Slow freezing procedures were described in detail (Gómez et al., 2020d). Briefly, expanded and fully expanded blastocysts were washed in PBS + 4 g/L BSA and loaded in a freezing medium containing PBS (P4417), 1.5 *M* ethylene-glycol and 20% CRYO3 (5617, Stem Alpha) for 10 min. Embryos were aspirated in a French straw, loaded between 2 columns with PBS + 0.75 M EG + 20% CRYO3, and 2 further columns PBS + 0.75 M EG + 20% CRYO3 separated by air. The straw was closed with a plug and loaded into a programmable freezer (Crysalis, Cryocontroller PTC-9500) at −6°C for 2 min and seeded with super-cooled forceps. Straws remained for 8 further min at −6°C and were subsequently cooled at −0.5°C/min up to −35°C. Finally, the straws were stored in LN_2 until use. For thawing, the straws were held for 10 s in air and 30 s in a water bath at 35°C and carefully dried with 70% ethanol. Each thawed straw with a single embryo was mounted in a 35°C ET catheter and directly transferred to d 7 synchronized recipients.

Procedures Involving Animals

Animals were housed in the experimental herd of the Centro de Biotecnología Animal, SERIDA, Deva (Spain). Recipients used in our study were healthy, tested free from the 7 most prevalent infectious diseases virtually affecting reproduction in the region, and underwent a gynecological examination.

Animal Feeding and Management

Animals were held in a body score condition 2.5 to 3.5 (scale 0–5) with the same basic diet throughout. From April to late October or early November, animals were fed on pasture, with individual concentrate supplementation (2–3 kg/d) given from August onward. Before estrus synchronization and up to pregnancy d 62, animals were fed indoors with concentrate (3–5 kg/d) from programmable, automated dispensers, and barley or oats and hay ad libitum. The indoor ration was also given from late October or early November until March or April to all animals and, at any time of year, from 10 to 15 d before birth. The concentrate contained 14.5% protein, >7% crude fiber, >3.5% fat, leading to >12% ME (MJ kg/DM), and an appropriate mixture of minerals and vitamins. The minimum amounts of raw foods in concentrate were 33% corn grain, 11% soybean, and 14% oats. Recipients were synchronized in estrus with an intravaginal progestagen device (PRID Alpha, CEVA) for 8 to 11 d, followed by a $PGF_{2\alpha}$ analog (Dynolitic, Pfizer) injected 48 h before progestagen removal. Recipients selected for transfer were those observed in standing estrus by experienced caretakers (2–3 times per day checks), or monitored in heat with an automated sensor system (Heatphone-Medria, Humeco, Huesca, Spain). In the absence of clear estrous signs, progesterone (**P4**) concentration was used to select recipients, with P4 fold change (**FCh**) d 7/d 0 > 8 and d 7 P4 values >3.5 ng/mL.

Blood Plasma Collection and Processing

Blood was collected in EDTA vacuum tubes from coccygeal vein puncture. Blood tubes were refrigerated at 4°C and centrifuged at 2,000 × *g*. Supernatant plasma was aliquoted and stored at −150°C until nuclear magnetic resonance (NMR) analysis.

Plasma P4 was measured on d 0 and 7 at fixed times (10 a.m. on d 0, expected estrus; and d 7, 4 to 6 h before ET); such times were informative of recipient fertility by metabolic fingerprint and metabolite biomarkers in earlier studies (Muñoz et al., 2014a,b; Gómez et al., 2020b,c). An ELISA test (EIA-1561, DRG Diagnostics) was used for blood P4 measurement. The test was sensitive beginning at 0.5 ng/mL, showing <1% cross-reactivity from steroids other than P4. Inter- and intra-assay variation coefficients were 7 and 6% respectively.

Embryo Transfer to Recipients

Embryos were transferred nonsurgically, under epidural anesthesia, at a fixed time 9 d + 4 to 6 h after progestagen removal. Before ET, all recipients were examined for detection of a healthy corpus luteum in one ovary by ultrasonography. Pregnancy was diagnosed by ultrasound scanning on d 40, 62, and monitored until birth.

Table 1 summarizes the numbers of ET performed, pregnancy rates per recipient breed, and embryo cryopreservation status in this study (N1 = 45 fresh+ 63 F-T embryos; total N1 = 108). From a previous study (Gimeno et al., 2021), we obtained the metabolome of the cognate culture medium of the embryo transferred, available in several cases (N2 = 70). Recipients were transferred up to 4 times if nonpregnant in previous

ET, as we found that metabolites associated with pregnancy prediction were independent of ET repeats (Gómez et al., 2020d). A description of each sample ET and covariate factors used in this study is shown in Supplemental Table S1 (https://doi.org/10.17632/sc2vnbfrw7.1; Gimeno et al., 2022a). Holstein heifers (1.64 yr old on average at the time of first ET) represented the most abundant ET data set, whereas AV breed and their crosses were 1.75 yr old on average at first ET. The assignment of type of embryo transferred to a recipient at each ET and fertilizing bull did not follow predefined patterns. The ET were repeated after 30 to 60 d from the former nonpregnant diagnosis (on d 40 or 62). The ET were preferentially performed in rounds of 3 to 8 recipients, with embryos sired by one (habitually) or 2 bulls per round and one or 2 embryo culture conditions.

Nuclear Magnetic Resonance ($1H^+$NMR) Analysis of Blood Plasma

Blood plasma samples were thawed at room temperature. A volume of 300 μL of plasma was mixed with 300 μL of chilled methanol and 300 μL of chilled chloroform. The mixture was vortexed and then kept at −20°C for 30 min. After centrifugation (15,000 × *g*, 10 min, 4°C) to separate the polar phase, the sample was evaporated in a Speedvac (ThermoScientific) at 35°C. Samples were stored at −20°C before analysis.

The $1H^+$NMR samples were prepared using extracted, dried blood plasma with the addition of 200 μL of 0.2 *M* potassium phosphate buffer in deuterium oxide (pH 7.4 ± 0.5) and 10 μL of 3.2 m*M* trimethylsilylpropanoic acid (**TSP**). Deuterium oxide provided a field frequency lock and TSP a chemical shift reference. The resulting solution was transferred to conventional 3-mm NMR tubes.

Briefly, $1H^+$NMR spectra were recorded at 298K on a Bruker Ascend 600 MHz spectrometer (Bruker, Sadis), equipped with a TCI cryoprobe. Standard $1H^+$NMR spectra were obtained using a NOESY pulse sequence with a 90° pulse, a relaxation delay of 20 s, and 64 scans in a time domain of 64K data points. Data were processed with 0.2 Hz of line broadening for the exponential decay function using TopSpin version 3.2 software (Bruker Daltonik).

Spectral assignments were performed using the free version of ChenomX 7.1 software (ChenomX), in-house database, and Livestock Metabolome Database (http://lmdb.ca/). Metabolite quantification was carried out using TSP signal with a known concentration, as reference (TSP = 152 μ*M*).

Experimental Design and Statistical Analysis

This study analyzed the metabolite concentrations in d 0 and 7 plasma of recipients diagnosed as pregnant or open at specific time endpoints (d 40, d 62, and birth). The experimental sequence is shown in Figure 1. Analyses included separate studies with embryos transferred fresh and after freezing-thawing. Metabolites that differed in concentration were identified, and, among them, candidate metabolite biomarkers were obtained, as well as altered underlying metabolic

Table 1. Recipients [Asturiana de los Valles (AV); Asturiana de la Montaña (AM); and Holstein] used and pregnancy rates (%) obtained after transfer of fresh and frozen in vitro produced embryos at gestational endpoints d 40, d 62, and at birth

Embryo type	Recipient breed	N1[1]	N2[2]	Gestational endpoint[3] d 40	d 62	Birth
Fresh	AV	6	5			
	AM	5	1			
	Holstein	34	19			
Total		45		29/45 (64)	26/45 (58)	25/44[4] (57)
Total			25	18/25 (72)	17/25 (68)	15/25 (60)
Frozen	AV	15	13			
	Crossbred	5	2			
	Holstein	43	30			
Total		63		37/63 (59)	35/63 (55)	29/63 (46)
Total			45	28/45 (62)	27/45 (60)	21/45 (47)

[1]N1: All recipients used, which provided n = 108 and n = 107 plasma samples collected on d 0 (estrus) and d 7 (hours before the embryo transfer time), respectively.

[2]N2: Recipients that had available the cognate culture medium metabolome of the embryo transferred (a subset from N1), obtained in a previous study (Gimeno et al., 2021).

[3]Day: age of the cultured embryo counted from the onset of in vitro fertilization. Data presented as a proportion (%).

[4]One recipient deceased after pregnancy d 62.

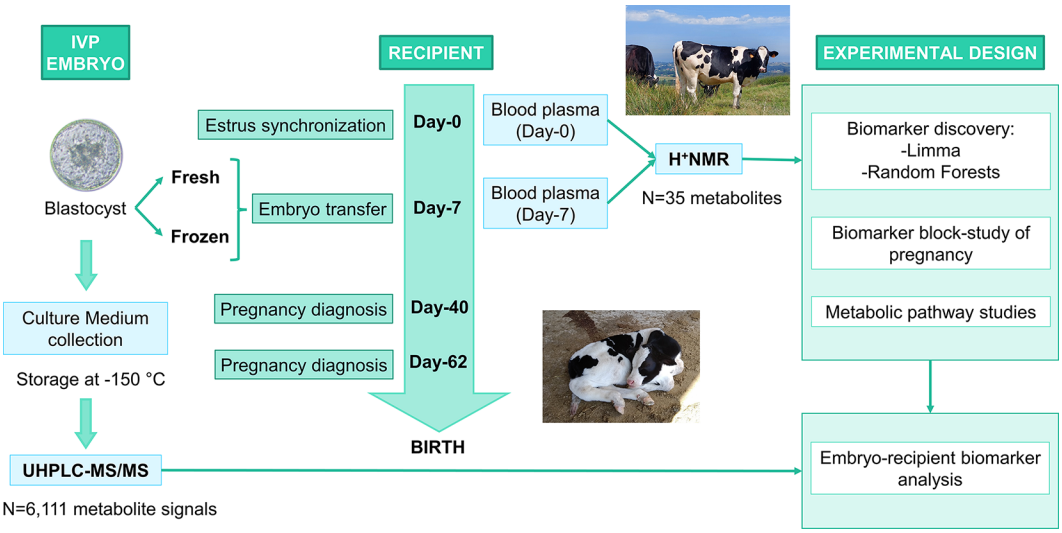


Figure 1. Workflow of experiments conducted in this study.

pathways where possible. For validation, metabolites in this study were compared with fresh controls made on purpose in this experiment, and with independent samples corresponding to other fresh and V-W embryos transferred to recipients in our experimental herd in previous studies (Gómez et al., 2020b,c).

Furthermore, because pregnancy is based on competent embryos and competent recipients, we performed a study to increase the predictive power of biomarkers in accordance with the pregnancy competence of the cognate embryo, which was obtained by metanalysis of data from a previous study (Gimeno et al., 2021). Ultimately, to overcome individual factors that could lead to distorted results, we introduced experimental randomness using high variability in recipients and in embryos. Thus, we ensured sample variability in recipient females from different batches over a 5-yr period (2016–2020) from Holsteins and AV cattle. From the embryonic perspective, since biomarker adjustment for each existing embryo production condition is unfeasible, we included 2 culture conditions (i.e., fetal calf serum or BSA within group culture from d 0 to 6), combined with fresh and F-T embryos, as it can be considered "randomized." Variation in oocyte origin included blind slaughterhouse ovary collection. Variation in fertilizing bulls included n = 7 individual bulls from 2 breeds used for IVF in the laboratory.

The NMR analysis in the recipient data set identified 46 metabolites in plasma, of which 35 were also quantified (detailed in Supplemental Table S2; https://doi.org/10.17632/sc2vnbfrw7.1; Gimeno et al., 2022b). Statistical analyses were performed with Metaboanalyst 5.0 (Chong et al., 2018) and Graphpad Prism software. Metabolite concentration data were scaled to adjust each variable/feature by a scaling factor based on the dispersion of the variable. Auto scaling consisted of mean-centering and division by the standard deviation of each variable, which was sufficient to reach a near-to-normal distribution (Figure 2).

Subsequently, metabolites identified and quantified were analyzed by univariate and multivariate statistics to detect confident biomarkers and pathways involved in pregnancy competence and their differences between recipients of F-T versus fresh embryos. The analytical procedures are described in the following sections.

Main Metabolites in the Entire Data Set

We analyzed metabolites identified in d 0 (n = 108) and d 7 (n = 107) plasma samples as a function of pregnancy diagnosis at d 40, d 62, and at birth. Covariates used in these calculations were recipient age (linear and quadratic) difference with the mean herd average, embryo cryopreservation, embryo culture, recipient, breed, NMR analysis, and individual bull.

Univariate Analysis: Linear Models with Covariate Adjustments (Limma). The overall data sets on d 0 and 7 were analyzed by Limma. This approach uses linear models to perform significance *t*-testing with covariate adjustments. The study included relevant metabolites as candidates with pregnancy predictive potential independent of cryopreservation.

Multivariate Analysis: Random Forests. A predictive model was built with metabolite features only as a function of the 3 pregnancy endpoints studied. The analysis was based on the machine learning classification algorithm Random Forests (**RF**). The mean decrease accuracy (**MDA**) tool from RF allowed obten-

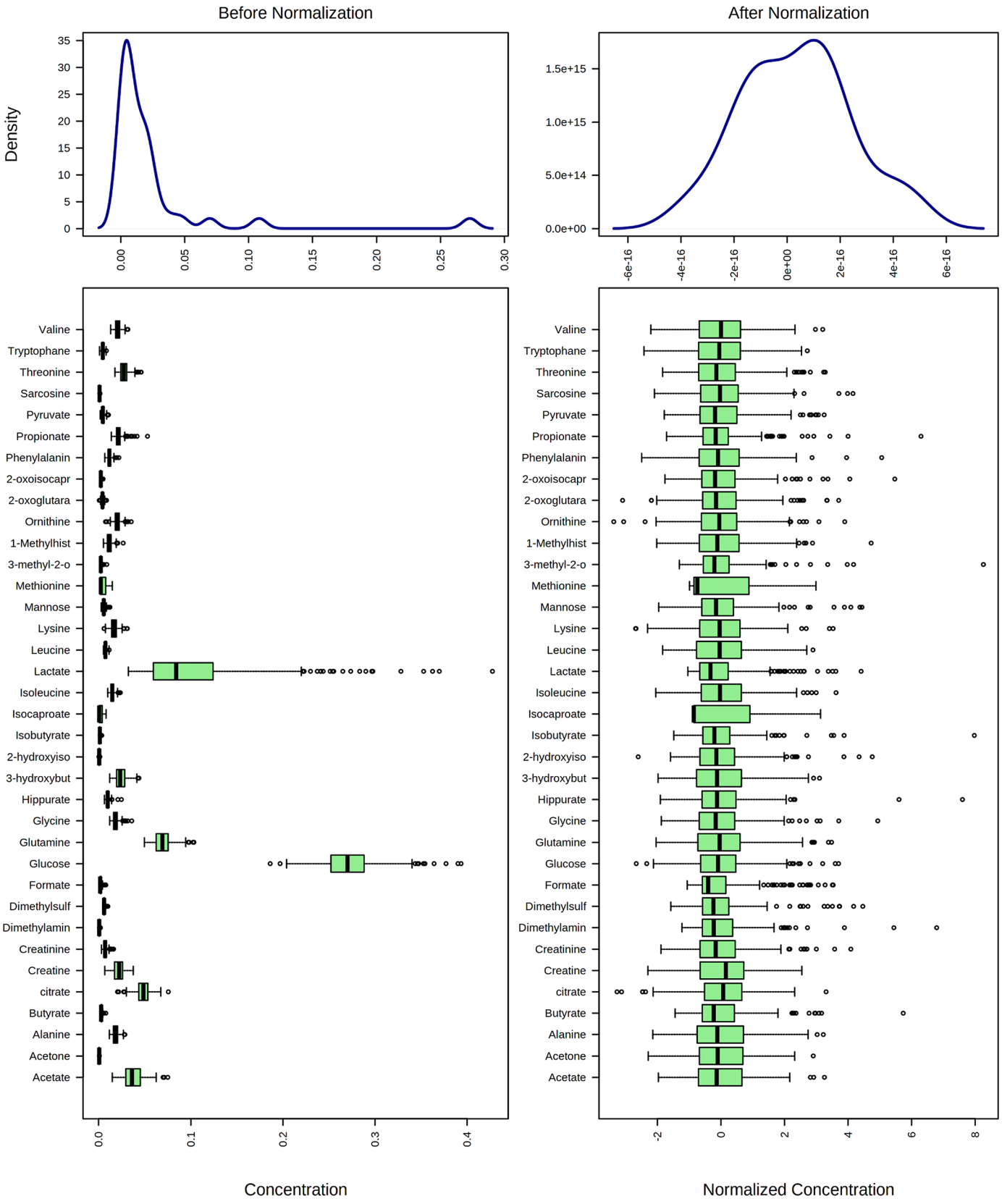


Figure 2. Normalization of metabolite concentration data.

tion of rankings based on feature contributions to the classification accuracy by permutation. The parameters used were n = 500 trees, n = 7 predictors (covariates), and positive randomness.

Main Metabolites: Biomarker Block Study of Pregnancy

Plasma samples (n = 108 from d 0 and n = 107 from d 7) were used in a block study in accordance with their controllable fixed factors (i.e., embryo cryopreservation, recipient breed, and day of blood collection) analyzed as a function of pregnancy endpoints. Blocks yielded more uniform data sets and increased the predictive power of metabolites (Gimeno et al., 2021). Once divided into blocks, metabolite concentrations were doubly analyzed with Wilcoxon test (univariate study) and *t*-test (for biomarker candidates with ROC-AUC >0.65). *P*-values were <0.05, and $0.05 > P < 0.10$ for tendencies. Some metabolites with nonsignificant ROC-AUC >0.65 were considered merely to illustrate specific blocks (marked in red in Supplemental Table S3; https://doi.org/10.17632/sc2vnbfrw7.1; Gimeno et al., 2022c), and only when the same metabolite was significant in other blocks or previously identified as a main metabolite in the general data set.

Metabolic Pathway Studies

In accordance with sufficiency of sample availability, 2 pathway studies were carried out in Metaboanalyst 5.0 tools with samples from animals diagnosed for pregnancy at d 40:

Day 40 Pregnant Versus D 40 Open Recipients Transferred with F-T Embryos: Metabolic Pathway Analysis. The largest breed-homogeneous block in samples (Holsteins; n = 23 pregnant and n = 20 open recipients) was selected and analyzed on d 0 and 7.

Day 40, Only Pregnant Recipients from Fresh Versus F/T Embryos: Metabolite Set Enrichment Analysis. This study responded to the hypothesis that pregnant recipients of fresh (n = 29) and F-T (n = 37) embryos would have different metabolic requirements for pregnancy in response to different embryos. Metabolite set enrichment analysis directly investigated a set of functionally related metabolites (SMPDB; Human) without the need to preselect compounds based on an arbitrary cut-off threshold.

Improving the Pregnancy Predictive Power of the Recipient Biomarkers by ROC Curve-based Models Supported by Embryonic Spectral Metabolite Features Obtained by UHPLC

Pregnancy depends on viable embryos and competent recipients. Thus, a viable embryo transferred to a noncompetent recipient, and vice versa, leads to nonpregnancy and false negative samples. On the contrary, if the pregnancy capability is defined in the embryo and the recipient, a more exact pregnancy prediction can be estimated (Gómez et al., 2021).

Among the recipients used in this work, a group of n = 70 recipient plasma samples had available complementary analysis of the embryo CM obtained in our previous study (Gimeno et al., 2021; see Table 1). Such analysis, performed by UHPLC/MS-MS metabolomics, led to obtain 6,111 confident spectral metabolite features with which we calculated a confident embryonic competence value as shown below (see Model Development section). The matched groups were recipients with fresh embryos (n = 25) and recipients with frozen embryos (n = 45).

Model Development

To refine birth expectations of recipient plasma in this study, we used a strategy to identify false negatives within paired embryos and recipients, as modified from Gómez et al. (2021). For sample imputation, recipients were divided into 4 predictive groups by breed (Holsteins, including 2 Holstein crossbred; and AV) in a 2 × 2 factorial design with the status of the embryo transferred (fresh or F-T). The optimal results for analyses in these groups of recipients combined 3 to 5 metabolites per group. Embryos were divided into 4 predictive groups by fertilizing bull breed (Holstein and AV) in a 2 × 2 factorial design with the cryopreservation of the embryo (fresh or F-T). The analyses within embryos combined up to 20 feature signals.

Combinations of recipients (metabolites) and embryos (feature signals) in each pregnancy predictive group were independently analyzed with the algorithm support vector machine (**SVM**), PLS-DA and RF to create pregnancy predictive biomarker models (ROC-AUC empirical *P*-value <0.01 by permutation). For training and testing, ROC-AUC curves generated by Monte-Carlo cross-validation were used with balanced subsampling to evaluate the feature importance. Classification models were performed with selected top features and validated on one-third of the samples that were left out. To avoid overfitting, K-means clustering was used to detect and avoid features with similar behavior to minimize the redundancy in biomarkers (i.e.,

features in the same cluster that behave more similarly) (Tester tool; Metaboanalyst 5.0). Thus, metabolite features from >3 clusters were selected in the recipient group, and 5 to 10 clusters in the embryo group. Among the tested algorithms SVM gave the highest ROC curve classification. Confusion matrices were obtained with actual and predicted values for birth and no birth both for embryos and recipients.

Predictions and Iterations

Confusion matrices between matched samples from recipients and embryos were compared. Samples were judged as misclassified when the predicted pregnancy status was not coincident with the actual pregnancy status. Criteria for corrections through iterations were based on the following 4 possible cases:

1) Actual "No birth" with recipient judged as "birth" and embryo judged as "no birth": reclassification of the recipient as "birth" for testing in a subsequent iteration.
2) Actual "no birth" with recipient judged as "no birth" and embryo judged as "birth": reclassification of the embryo as "birth" for testing in a subsequent iteration.
3) Actual "no birth" with recipient and embryo judged as "no birth": no reclassification for testing in a subsequent iteration.
4) Actual "birth" with recipient and embryo judged as "no birth": based on the evidence, recalculation as "birth" of both embryo and recipient in a subsequent iteration.

Through successive iterations with the Tester tool, samples that became matched in accordance with the actual pregnancy status were considered true, whereas samples that do not fit with their reclassification were considered false (error).

Recalculation of Birth Biomarker Values in Recipients

Iterations based on multivariate SVM analysis combining the embryo and recipient data led to a more accurate recipient classification for birth potential. Therefore, we hypothesized that the original ROC-AUC value of certain predictive metabolites would equally increase after iterations. Metabolite ROC-AUC classification and *t*-test of recipients were recalculated after iterative identification of recipients identified as "no birth" with predicted "birth" potential supported by embryo confusion matrix.

RESULTS

Main Metabolites in the Entire Data Set

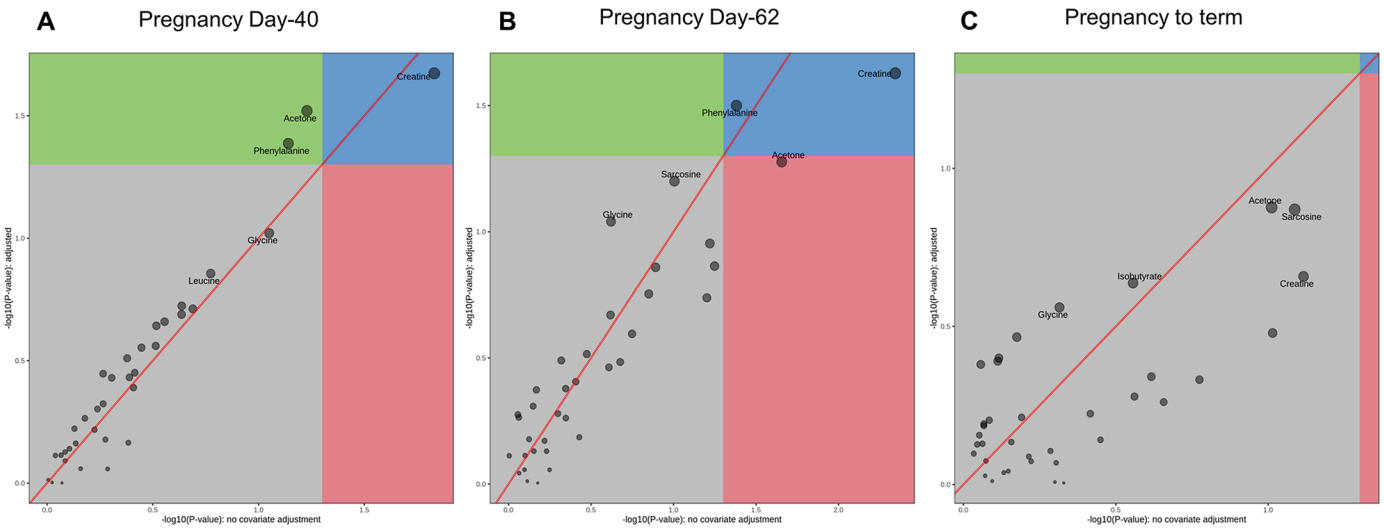
Limma D 0 Analysis (n = 108 Samples). Creatine was the most relevant metabolite on d 0, appearing at higher concentration in pregnant recipients when analyzed for d 40 and 62 endpoints, both as adjusted and nonadjusted by covariates (**A/A**; Figure 3). Acetone and L-phenylalanine were also A/A metabolites on d 40 and 62, respectively. However, L-phenylalanine at d 40 was significant only when adjusted, whereas acetone on d 62 was significant only if nonadjusted. No metabolite changing significantly on d 0 was detected as a function of birth.

Limma D 7 Analysis (n = 107 Samples). Diagnosis of pregnancy at d 40 led to significantly different FCh between 12 metabolites (i.e., L-glutamine, L-lysine, ornithine, 2-oxoglutarate, 2-oxoisocaproate, L-phenylalanine, 3-methyl-2-oxovalerate, butyrate, L-methionine, propionate, L-alanine and L-isoleucine; Figure 4). Of these, all were A/A but propionate and L-isoleucine, which were significant only when adjusted. Four of the above A/A metabolites also diagnosed pregnancy on d 62 (L-glutamine, L-lysine, ornithine and 2-oxoglutarate), and at birth, with L-glutamine, L-lysine and ornithine being A/A and 2-oxoglutarate being significant only when adjusted. Furthermore, birth revealed a d 40 A/A metabolite (L-phenylalanine) and a new A/A metabolite not recorded at d 40 and 62 (dimethyl sulfone).

Multivariate Analysis: Random Forests

Results of the RF analysis are presented in Figure 5, showing similar MDA predictive values between d 40 and 62 for d 0 and 7, but lower MDA values on d 0 at birth. Interestingly, on d 0 the consistency of the top MDA values creatine, acetone and L-phenylalanine at d 40 (Figure 5A) and d 62 (Figure 5C) with the univariate study was total, while at birth (Figure 5E) acetone and creatine were also in the top, but with a poorer MDA value than at the earliest endpoints.

On d 7, the highest consistency with the univariate study was shown by metabolites classified at the pregnancy d 40 (Figure 5B), with L-glutamine, L-lysine, ornithine, 2-oxoisocaproate, L-phenylalanine, 3-methyl-2-oxovalerate being included. In contrast, glycine ranked at the top by MDA but not in the univariate study. On d 62 (Figure 5D), the top MDA metabolites were 2-oxoisocaproate, tryptophan, glycine, 3-methyl-2-oxovalerate and L-isoleucine, while L-glutamine was also consistent with the univariate study, together with L-lysine, although this last showed a low MDA. At birth



	Pregnancy Day-40				Pregnancy Day-62				Pregnancy to term			
	Adjusted		Not adjusted		Adjusted		Not adjusted		Adjusted		Not adjusted	
Metabolite	Log FCh	P value	Log FCh	P value	Log FCh	P value	Log FCh	P value	Log FCh	P value	Log FCh	P value
Creatine	0.4067	0.0212	0.4750	0.0118	0.3932	0.0235	0.5503	0.0045				
Acetone	0.4117	0.0302	0.3640	0.0487			0.4536	0.0221				
L-Phenylalanine	0.3894	0.0410			0.4297	0.0316	0.3949	0.0416				

Figure 3. Covariate boxplot showing *P*-values for metabolites quantified in d 0 plasma of recipients transferred with fresh and frozen embryos that differ as a function of pregnancy on d 40 (A), d 62 (B), with and without covariate adjustment [covariates adjusted for each pregnancy stage were recipient age (linear and quadratic difference with the mean herd average), embryo cryopreservation, embryo culture, recipient breed, nuclear magnetic resonance analysis, and individual bull]. No metabolites fulfilling the required conditions were identified to term (C). The plot compares *P*-values for each metabolite both before (x-axis) and after (y-axis) covariate adjustment. Green section: features significant ($P < 0.05$) only after adjustment; red section: features significant only before adjustment; blue section: features significant in both cases; gray section: nonsignificant features. Log FCh: logarithm of fold change pregnant/nonpregnant.

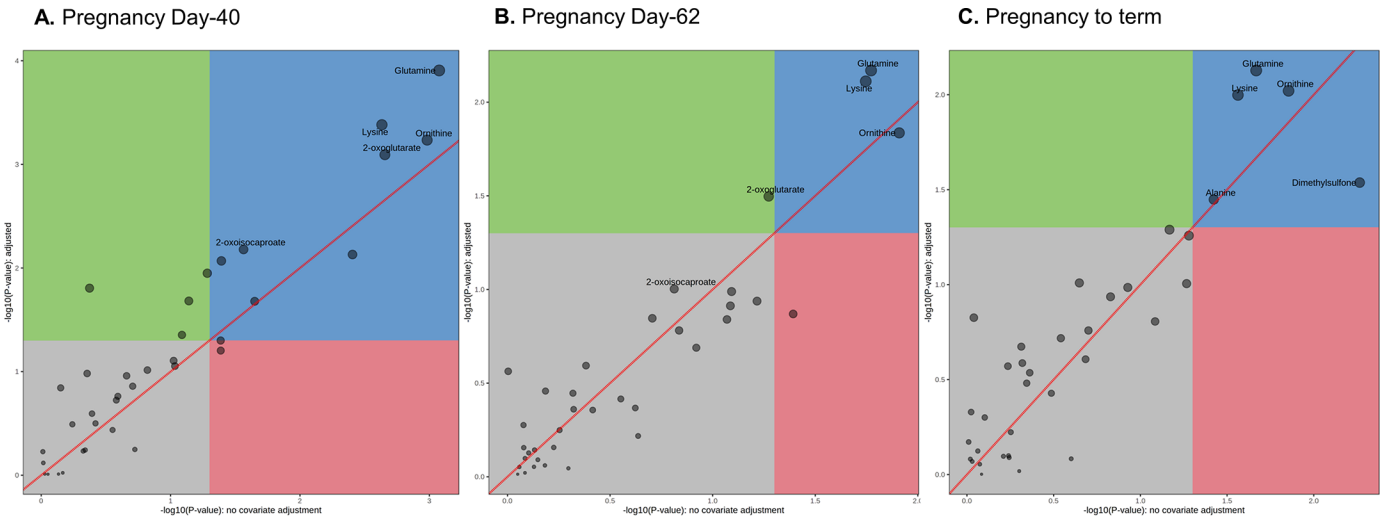
(Figure 5F), again L-lysine and L-phenylalanine shared relevance with the univariate study, together with L-glutamine and dimethyl sulfone, although these latter with much lower MDA scores. Valine and L-leucine were new metabolites ranked at birth.

Main Metabolites: Biomarker Block study of Pregnancy

A total of 190 pregnancy predictive blocks were obtained (Supplemental Table S3), distributed as 65 for d 0 and 125 for d 7. The numbers of blocks accounting for each pregnancy endpoint were similar (65, 64, and 61 for d 40, d 62, and birth, respectively), although they differed with cryopreservation (i.e., 33 for frozen embryos, 86 for fresh embryos, and 71 cryopreservation independent blocks). Among them, 71 blocks were independent from breed, 67 from Holsteins, 20 from non-Holsteins, and 32 from AV. The numbers of blocks were associated with the most represented breed (Holstein), but not with the most represented embryos (i.e., frozen). Thus, representative blocks for frozen embryos were fewer on d 0 plasma (9 in total, 3 at each endpoint) than in d 7 plasma (24 in total, 13 at d 40, 7 at d 62, and 4 at birth). The number of blocks at each gestational endpoint decreased as the pregnancy proceeded both within frozen embryos and independent of cryopreservation (16 and 26 blocks for d 40, 10 and 26 for d 62, and 7 and 19 at birth, respectively). In contrast, within fresh embryos, the number of blocks increased at each endpoint until birth (23, 28, and 35 blocks for d 40, d 62, and at birth; Figure 6). Blocks with upregulated metabolites in pregnant samples were 63, while 126 blocks contained downregulated metabolites.

Among 35 metabolites analyzed, 28 were considered biomarker candidates acting as pregnancy predictive in specific sample blocks. Metabolites not represented

were acetate, formate, 3-hydroxybutyrate, isocaproate, lactate, mannose, L-methionine, and pyruvate. Metabolites were ranked by the number of blocks in which they participated according to pregnancy endpoints, recipient breed and transferred embryo status (Figure 6). Creatine was an outstanding metabolite (20 blocks), showing the widest and most uniform distribution over pregnancy endpoints (7, 6, and 7 blocks for d 40, d 62, and at birth, respectively), and cryopreservation (8 frozen, 6 fresh, and 6 noncryopreservation dependent). Creatine was a pregnancy biomarker associated with Holsteins (6 blocks), cattle groups with Holstein representation (i.e., non-Holsteins, through crossbred, 6 blocks) and breed independent (7 blocks; higher proportion of Holsteins). In contrast, the pure AV group with the double muscled AV cattle showed just 1 block for creatine. The other metabolites that completed the top-10 ranking, bearing 115/190 blocks, were mainly AA and one tri-carboxylic acid: L-lysine, L-valine, L-phenylalanine, L-glutamine, L-leucine, L-ornithine, 2-oxoglutarate, L-



	Pregnancy Day-40				Pregnancy Day-62				Pregnancy to term			
	Adjusted		Not adjusted		Adjusted		Not adjusted		Adjusted		Not adjusted	
Metabolite	Log FCh	P value	Log FCh	P value	Log FCh	P value	Log FCh	P value	Log FCh	P value	Log FCh	P value
L-glutamine	-0.7792	0.0001	-0.6632	0.0006	-0.5609	0.0068	-0.4742	0.0141	-0.4742	0.0141	-0.4563	0.0175
L-lysine	-0.7156	0.0004	-0.5957	0.0019	-0.5480	0.0077	-0.4454	0.0190	-0.4459	0.0190	-0.4050	0.0325
Ornithine	-0.6971	0.0005	-0.6423	0.0007	-0.5032	0.0146	-0.4728	0.0128	-0.4728	0.0128	-0.4542	0.0162
Dimethylsulfone									-0.4181	0.0290	-0.5589	0.0032
2-oxoglutarate	-0.6697	0.0008	-0.6098	0.0016	-0.4369	0.0318	-0.3891	0.0440	-0.3891	0.0440		
2-oxoisocaproate	-0.5152	0.0066	-0.4498	0.0139								
L-phenylalanine	-0.5392	0.0074	-0.5815	0.0017					-0.3655	0.0480	-0.3947	0.0318
3-methyl-2-oxovalerate	-0.5078	0.0085	-0.4176	0.0241								
Butyrate	-0.4746	0.0112	-0.3962	0.0333								
L-methionine	-0.2191	0.0156	-0.1907	0.0234								
Propionate	-0.4398	0.0208										
L-alanine	-0.4688	0.0210	-0.4457	0.0217								
L-isoleucine	-0.4197	0.0442										

Figure 4. Covariate boxplot showing *P*-values for metabolites quantified in d 7 plasma of recipients transferred with fresh and frozen embryos that differ as a function of pregnancy on d 40 (A), d 62 (B) and to term (C) with and without covariate adjustment [covariates adjusted for each pregnancy stage were recipient age (linear and quadratic difference with the mean herd average), embryo cryopreservation, embryo culture, recipient breed, nuclear magnetic resonance analysis, and individual bull]. The plot compares *P*-values for each metabolite both before (x-axis) and after (y-axis) covariate adjustment. Green section: features significant ($P < 0.05$) only after adjustment; red section: features significant only before adjustment; blue section: features significant in both cases; gray section: nonsignificant features. Log FCh: logarithm of fold change pregnant/nonpregnant.

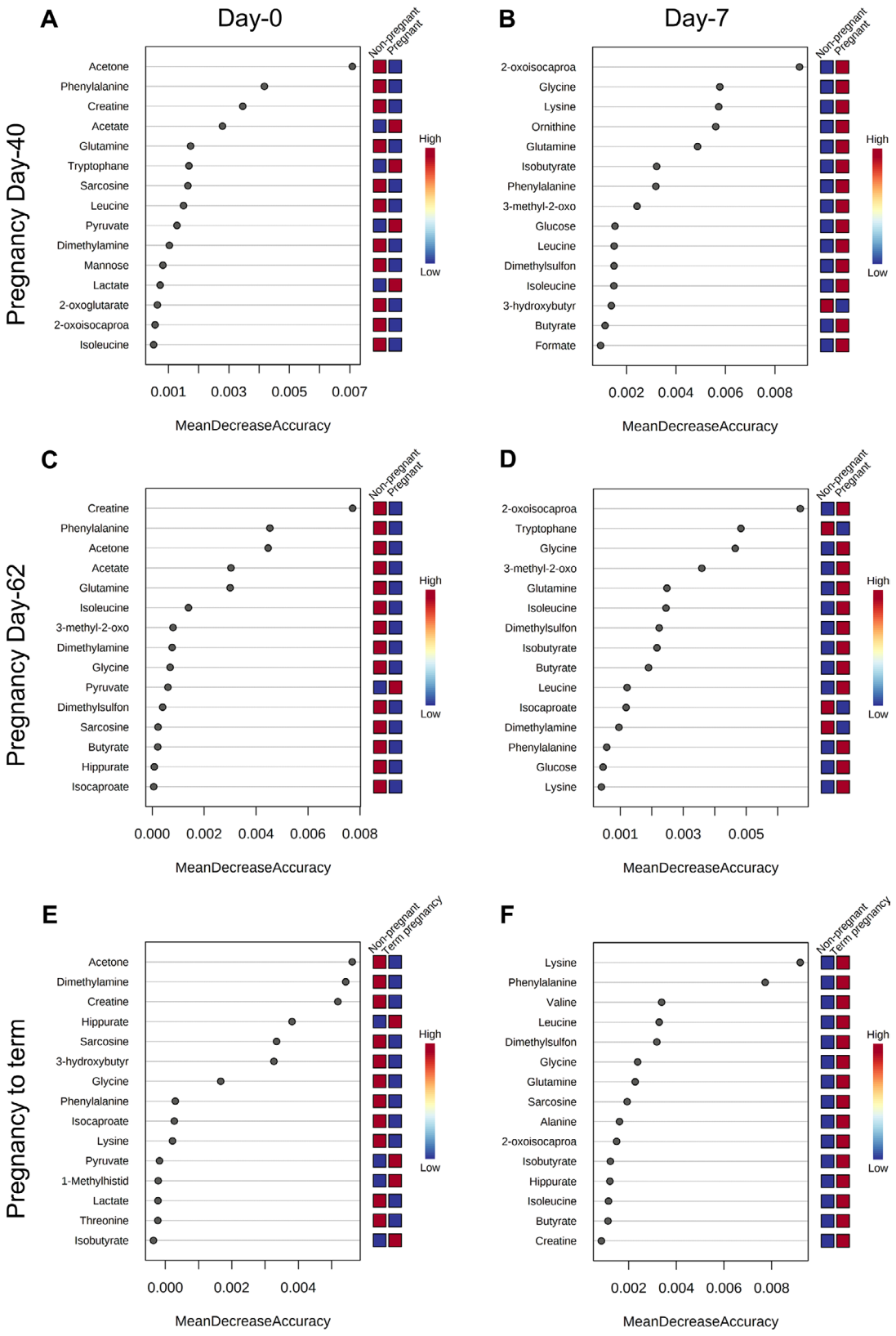


Figure 5. Metabolites identified in d 0 (A, C, E) and d 7 (B, D, F) plasma from recipients transferred with frozen and fresh embryos ranked by their contributions to classification accuracy to predict pregnancy (mean decrease accuracy; random forest classification algorithm) on d 40 (A, B), d 62 (C, D), and birth (E, F). Red: higher concentration. Blue: lower concentration.

	Blocks	Plasma		Day-40			Day-62			Birth			By breed					
Metabolite	Total	0	7	Fresh	Frozen	Both	Fresh	Frozen	Both	Fresh	Frozen	Both	All	H	No H	AV	avAUC	Block-AUC
Creatine	20	7	13	2	4	1	2	2	2	2	2	3	7	6	6	1	0.7182	14.3640
L-Lysine	12	3	9	2	0	3	2	0	2	2	0	1	4	6	0	2	0.7409	8.8908
L-Valine	12	3	9	2	0	1	2	0	1	3	0	3	4	3	1	4	0.7137	8.5644
L-Phenylalanine	12	4	8	0	3	2	1	2	1	1	2	0	4	8	0	0	0.6765	8.1180
L-Glutamine	11	2	9	2	1	2	2	0	1	2	0	1	4	7	0	0	0.7035	7.7385
L-Leucine	10	4	6	2	1	1	2	1	0	2	0	1	3	6	0	1	0.7681	7.6812
L-Ornithine	10	3	7	2	0	2	2	0	1	2	0	1	4	6	0	0	0.7479	7.4790
2-oxoglutarate	10	1	9	2	0	2	2	0	1	2	1	0	4	5	1	0	0.6966	6.9660
L-alanine	9	3	6	1	0	2	2	0	1	2	0	1	4	1	1	3	0.7173	6.4557
L-Glycine	9	0	9	1	0	0	2	0	2	2	0	2	4	4	1	0	0.6930	6.2370
Dimethylsulfone	8	2	6	2	0	1	2	0	1	2	0	0	3	3	0	2	0.6998	5.5984
1-Methylhistidine	7	1	6	0	0	1	1	0	2	1	0	2	3	0	3	1	0.7006	4.9042
Acetone	6	4	2	0	2	1	0	1	2	0	0	0	1	0	3	2	0.7438	4.4628
Creatinine	5	2	3	0	0	1	0	0	1	2	1	0	2	0	1	2	0.7504	3.7520
2-hydroxyisobutyrate	5	4	1	0	1	0	0	1	0	2	1	0	2	0	3	0	0.7119	3.5595
L-Isoleucine	5	3	2	0	0	1	1	0	1	1	0	1	2	0	0	3	0.6964	3.4820
Butyrate	5	4	1	1	0	1	1	0	1	1	0	0	3	0	0	2	0.6936	3.4680
Propionate	5	3	2	1	0	1	1	0	1	1	0	0	3	0	0	2	0.6877	3.4385
Sarcosine	4	4	0	1	0	1	0	0	1	0	0	1	1	0	0	3	0.6963	2.7852
L-Threonine	4	3	1	2	1	0	1	0	0	0	0	0	2	2	0	0	0.6858	2.7432
2-oxoisocaproate	4	0	4	0	1	1	0	1	1	0	0	0	0	4	0	0	0.6747	2.6988
L-Tryptophane	3	2	1	0	0	0	1	0	0	0	0	2	1	0	0	2	0.6951	2.0853
Glucose	3	1	2	0	0	0	0	0	2	1	0	0	2	1	0	0	0.6800	2.0400
3-methyl-2-oxovalerate	3	0	3	0	1	0	0	1	0	1	0	0	1	2	0	0	0.6726	2.0178
Citrate	2	0	2	0	0	1	0	0	1	0	0	0	0	0	0	2	0.8133	1.6266
Dimethylamine	2	1	1	0	0	0	0	0	0	2	0	0	1	1	0	0	0.7146	1.4292
Hippurate	2	1	1	0	0	0	1	0	0	1	0	0	2	0	0	0	0.7076	1.4152
Isobutyrate	2	0	2	0	1	0	0	1	0	0	0	0	0	2	0	0	0.6747	1.3494
TOTAL	**190**	**65**	**125**	**23**	**16**	**26**	**28**	**10**	**26**	**35**	**7**	**19**	**71**	**67**	**20**	**32**		

Figure 6. Heatmap representative of abundance of blocks within metabolites according to the day of the sample (i.e., d 0 or d 7 plasma), pregnancy endpoints (i.e., d 40, d 62, and birth) and transferred embryo status (i.e., frozen, fresh, or both), and recipient breed (all, H: Holstein, No H: No Holstein, or AV: Asturiana de los Valles). The ROC-AUC average (avAUC) of each metabolite is also shown. Block*avAUC value considers the number of conditions into which the metabolite is discriminant for pregnancy and their avAUC values and represents the importance of each metabolite.

alanine, and L-glycine. The 10 metabolites ranked by their average ROC-AUC (**avAUC**) (0.8133 to 0.7137) were citrate, L-leucine, creatinine, L-ornithine, acetone, L-lysine, creatine, L-alanine, dimethylamine, and L-valine. We estimated the importance of each metabolite with the Block*avAUC value ranking, which considers the number of conditions in which the metabolite is discriminant for pregnancy and their avAUC values. Once again, creatine (Block*avAUC = 14.3640) ranked first followed by L-lysine, L-valine, L-phenylalanine, L-glutamine, L-leucine, L-ornithine, 2-oxoglutarate, L-alanine, L-glycine (Block*avAUC = 6.2370).

For practical purposes and identification of the best metabolites that can be used as biomarkers in each condition, Table 2 shows the top 4 ROC-AUC candidate biomarkers identified in recipient plasma per block. Abundance of these top biomarkers was more prominent on d 7 (53) than d 0 (20), and biomarkers were more predictive for d 40 and 62 than at birth. Lower abundance of biomarkers that predicted birth is observed mainly in recipients transferred with frozen embryos, which had just one predictive metabolite to term pregnancy. In a lesser extent, such a reduction of birth biomarkers was also seen in blocks independent on cryopreservation, with the exception of Holsteins at birth, with 4 biomarkers in Table 2. In contrast, biomarkers of recipients that received fresh embryos did not show pronounced losses of their predictive ability to term.

Metabolic Pathway Studies

Day 40 Pregnant Versus D 40 Open Recipients Transferred with F/T Embryos: Metabolic Pathway Analysis. In correspondence with the higher number of regulated metabolites identified, d 7 pathway analysis led to the discovery of pathways that significantly differed between pregnant and open Holstein recipients as diagnosed on d 40 (Figure 7). The following pathways (*Bos taurus*) stood out due to their impact:

Table 2. The top 4 ROC-AUC candidate biomarkers identified in recipient d 7 and 0 (highlighted in blue) plasma for each block determined by breed, cryopreservation (Cryo; recipients of frozen embryos (Fz); recipients of fresh embryos (Fh); dash indicates independent from embryo cryopreservation), and pregnancy endpoint

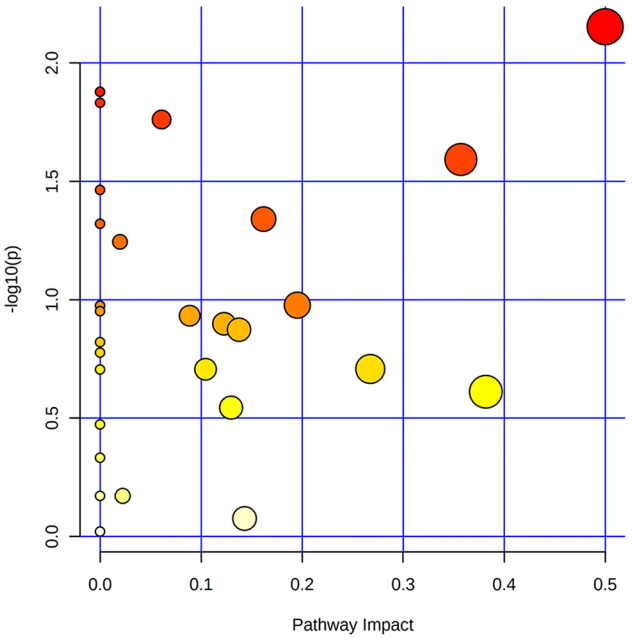
			Biomarker 1		Biomarker 2		Biomarker 3		Biomarker 4	
Breed[1]	Cryo[2]	Endpoint	Metabolite-Day	AUC	Metabolite-Day	AUC	Metabolite-Day	AUC	Metabolite-Day	AUC
—	—	d 40	Glutamine-7	0.677	Lysine-7	0.676	2-oxoglutarate-7	0.663	Phenylalanine-0	0.661
—	—	d 62	1-Methylhistidine-7	**0.718**	Glycine-7	**0.703**	Creatine-0	0.668		
—	—	Birth	Glycine-7	**0.669**						
—	Fz	d 40	Creatine-7	0.729	Phenylalanine-7	0.698				
—	Fz	d 62	Creatine-7	0.732	Acetone-7	0.656				
—	Fz	Birth	Creatine-7	0.707						
—	Fh	d 40	Lysine-0	0.804	Ornithine-0	0.804	Glutamine-0	0.774	2-oxoglutarate-7	0.763
—	Fh	d 62	Valine-7	0.771	Lysine-7	0.769	Ornithine-7	0.751	Leucine-7	0.747
—	Fh	Birth	Ornithine-0	0.781	Lysine-7	0.768	Dimethylsulfone-7	0.764	Creatine-0	0.737
Holstein	—	d 40	Ornithine-7	0.710	Glutamine-0	0.708	2-oxoglutarate-7	0.701	Lysine-7	0.701
Holstein	—	d 62	2-oxoglutarate-7	0.671	Ornithine-0	0.671	Lysine-7	0.668	Glutamine-7	0.650
Holstein	—	Birth	Lysine-7	0.682	Ornithine-7	0.659	Creatine-7	0.655	Glutamine-7	0.653
Holstein	Fz	d 40	Phenylalanine-7	0.744	Leucine-7	0.717	2-oxoisocaproate-7	0.700	Creatine-7	**0.698**
Holstein	Fz	d 62	Creatine-7	**0.691**	Phenylalanine-7	**0.669**				
Holstein	Fz	Birth	**Phenylalanine-7**	**0.662**						
Holstein	Fh	d 40	Ornithine-7	0.830	Lysine-7	0.826	Creatine-0	0.758	Leucine-0	0.758
Holstein	Fh	d 62	Lysine-0	0.806	Ornithine-7	0.791	Leucine-0	**0.747**	Valine-7	0.747
Holstein	Fh	Birth	Lysine-0	0.831	Ornithine-7	0.801	Creatine-7	0.733	Glutamine-7	0.707
AV	—	d 40	Citrate-7	0.813	Acetone-0	0.800	Alanine-7	0.787		
AV	—	d 62	Citrate-7	0.813	Acetone-0	0.800	Alanine-7	0.787		
AV	—	Birth	Glycine-7	0.669						
No-H	—	d 40	Creatine-7	**0.695**						
No-H	—	d 62	Creatine-0	0.776	Acetone-0	0.754	1-Methylhistidine-7	**0.718**	Glycine-7	**0.703**
No-H	—	Birth	1-Methylhistidine	0.751	Creatine-7	0.692				
No-H	Fz	d 40	Creatine-0	**0.750**	Creatine-7	**0.750**				
No-H	Fz	d 62	**2-hydroxyisobutyrate-0**	**0.743**						
No-H	Fz	Birth	Creatine-7	0.780						

[1]AV = Asturiana de los Valles; No-H = non-Holstein recipients consisting of AV; AM = Asturiana de la Montaña; dash indicates recipient from all breeds Blocks not represented due to low numbers of recipients: AV-Frozen; AV-Fresh; crossbred, AM, pooled as "No Holstein" recipients (No-H).

[2]Cryo = cryopreservation; FZ = recipients of frozen embryos; Fh = recipients of fresh embryos; dash indicates independent from embryo cryopreservation.

[3]**Bold AUC**: tendencies in either Wilcoxon test or *t*-test or both ($P < 0.10$).

[4]**Bold in metabolite and AUC**: AUC >0.65 but nonsignificant.



Pathway Name	P value	FDR	Impact
Phenylalanine, tyrosine and tryptophan biosynthesis	0.007	0.089	0.5
Purine metabolism	0.013	0.089	0
Pyrimidine metabolism	0.013	0.089	0
Nitrogen metabolism	0.013	0.089	0
D-glutamine and D-glutamate metabolism	0.015	0.089	0
Arginine biosynthesis	0.017	0.089	0.061
Phenylalanine metabolism	0.026	0.113	0.357
Aminoacyl-tRNA biosynthesis	0.034	0.113	0
Alanine, aspartate and glutamate metabolism	0.046	0.148	0.162
Valine, leucine and isoleucine biosynthesis	0.048	0.148	0

Figure 7. Metabolome view showing all matched pathways (*Bos taurus*) differentially regulated between d 40 pregnant and open Holstein recipients according to the P-values (color gradient) from the pathway enrichment analysis and pathway impact values (dot sizes) from the pathway topology analysis. *P*-values, false discovery rate (FDR) and impact value of the top regulated pathways (pregnant/nonpregnant ratio) are detailed.

phenylalanine, tyrosine, and tryptophan biosynthesis; phenylalanine metabolism; alanine, aspartate, and glutamate metabolism; and arginine biosynthesis.

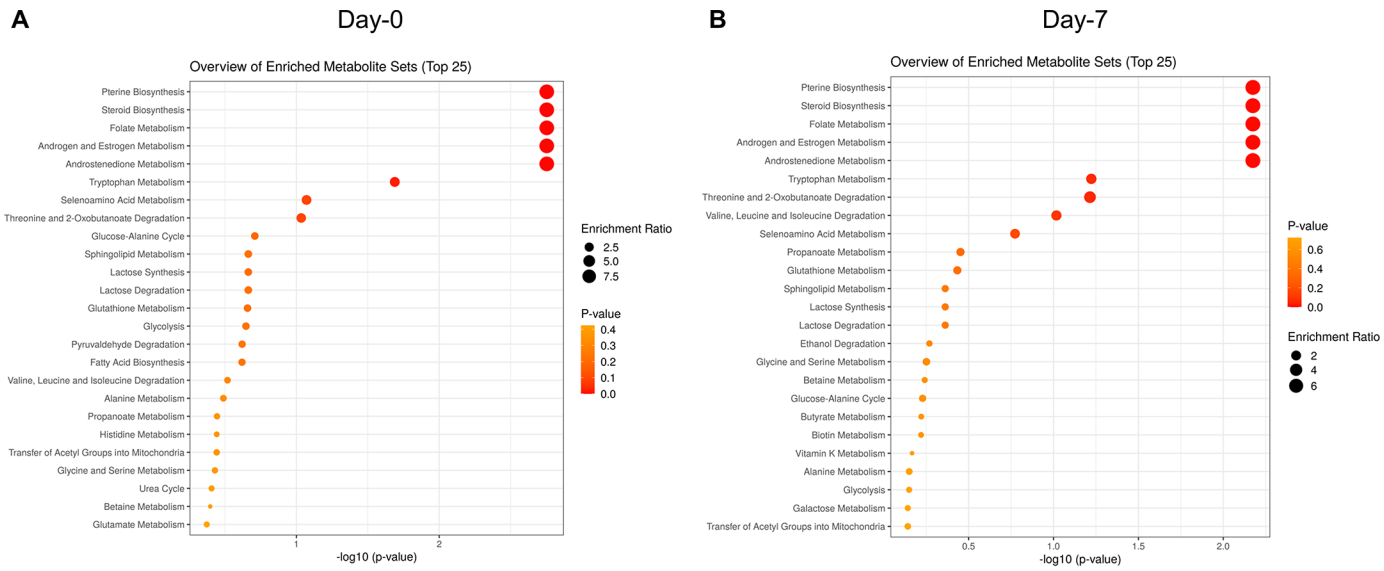
Day 40, Only Pregnant Recipients from Fresh Versus F-T Embryos: Metabolite Set Enrichment Analysis (MSEA). All pregnant recipients were used with separate analysis for d 0 and 7 (Figure 8). Interestingly, the same top 6 metabolically enriched sets were identified on d 0 (Figure 8A) and d 7 (Figure 8B): pterine biosynthesis, steroid biosynthesis, folate metabolism, androgen and estrogen metabolism, androstenedione metabolism, and tryptophan metabolism. Enrichment and significance were more affected on d 0 than on d 7.

Improving the Pregnancy Predictive Power of the Recipient Biomarkers by ROC Curve-based Models Supported by Embryonic Spectral Features

Predictions and Iterations. Table 3 shows the results of the combined pregnancy prediction. Embryos showed the highest ROC-AUC values in the sample classification models [i.e., ranging between 0.948 to 1.000 (iteration 1) and 0.948 to 0.981 (iteration 2)], with iteration 2 being not necessary for fresh embryo samples and no misclassification (i.e., 25/25 correctly classified samples). Unsolvable, erroneous classification of some samples occurred in the frozen embryo group with 2 "birth" misclassified as "no birth," and 2 "no birth" classified as "birth" coinciding with the same classification error in the recipient, which precluded the identification of which—the embryo or the recipient—was a candidate to correct for the next iteration; such misclassifications remained after 3 iterations, leading to 41/45 correctly identified samples in frozen embryos.

Recipients of fresh embryos were correct for 24/25 samples. Holsteins needed 2 iterations, and only one "no birth" sample remained misclassified as "birth." Recipients from AV were correctly classified with no iteration. Within frozen embryos, the Holstein recipients (the largest embryo-recipient matched sample data set with 32 ET, including 2 crossbred), interestingly, had 9 "no birth" cases initially classified as "birth"; of them, after 3 iteration rounds, the ROC-AUC value grew from 0.737 to 0.855, and 8 of these recipients were definitely classified as "birth" as matched with noncompetent embryos. The AV recipients of frozen embryos showed one case of unsolvable classification from "birth" to "no birth," with only one iteration needed.

Recalculation of Birth Biomarkers in Recipients. The original Holstein data set containing 49 samples was fed with the new predicted status of "birth" in 9 "no birth" recipients (see Table 3) and a new ROC-AUC for single biomarkers was calculated (Table 4). Interestingly, together with L-phenylalanine, 12 new biomarkers predicted birth with significant



Pathway Name	P value	FDR
Pterine biosynthesis	0.002	0.020
Steroid biosynthesis	0.002	0.020
Folate metabolism	0.002	0.020
Androgen and estrogen metabolism	0.002	0.020
Androstenedione metabolism	0.002	0.020
Tryptophan metabolism	0.020	0.194
Selenoamino acid metabolism	0.085	0.659

Pathway Name	P value	FDR
Pterine biosynthesis	0.007	0.076
Steroid biosynthesis	0.007	0.076
Folate metabolism	0.007	0.076
Androgen and estrogen metabolism	0.007	0.076
Androstenedione metabolism	0.007	0.076
Tryptophan metabolism	0.060	0.496
Threonine and 2-oxobutanoate degradation	0.061	0.496
Valine, leucine and isoleucine degradation	0.096	0.684

Figure 8. Enrichment of metabolite set pathways in pregnant fresh versus pregnant frozen on d 0 (A) and d 7 (B). The size of the circles per metabolite set represents the enrichment ratio and the color represents the *P*-value. Tables down each plot describe the top metabolic pathway enriched, *P*-value and false discovery rate (FDR).

ROC-AUC >0.65 regarding the original recipient data set, with a particular reinforcement of the predictive role for creatine (ROC-AUC = 0.851; Figure 9 shows the ROC-AUC and boxplot for creatine). Biomarkers predictive of earlier pregnancy stages (d 40/d 62) in the original data set appeared again at birth after iteration (i.e., phenylalanine, L-leucine, 2-oxoisocaproate and creatine), while 5 new biomarkers with ROC-AUC >0.700 were identified (butyrate, 1-methylhistidine, L-glutamine, D-glucose, L-glycine).

DISCUSSION

In this work, for the first time, we identified biomarkers and metabolic pathways representative and predictive of pregnancy and birth in bovine recipients of IVP F-T embryos. In previous works we used Fourier transform infrared spectroscopy to predict pregnancy and birth in IVP (Muñoz et al., 2014a) and in vivo cultured (Muñoz et al., 2014b) embryos, as well as in the plasma of their recipients. However, the identification of molecules involved in pregnancy was not possible until more recent work with NMR with recipients of V/W and fresh embryos (Gómez et al., 2020b,d). Variability in fertilizing bulls, oocyte origins, culture systems, and cryopreservation techniques increase the value of identified biomarkers in our studies. Likewise, the large number of samples and their use in different animal cohorts and breeds represents a proof of cross-validation often absent in livestock metabolomics studies (Goldansaz et al., 2017). Showing the same relationship between a studied phenotype and a biomarker in independent populations increases the value of the biomarker even if no obvious mechanistic relationship can be observed (Mayeux, 2004; Agakov et al., 2011; te Pas, et al.; 2017).

Thus, the pregnancy and birth biomarkers identified here were both specific of F-T embryos and shared with V/W embryos and fresh embryos (Gómez et al., 2020a,b, 2021). The presence of different metabolite

Table 3. Transferred embryos (fresh, Fh; or frozen, Fz) matched with their recipients showing the actual gestational status at birth and the ROC-AUC value (ROC) predicted for birth after iterations (ITER-) calculated with the algorithm support vector machine (SVM) within specific groups of embryos and recipients[1]

Embryo			Recipient			Actual	Predicted status in embryos				Predicted status in recipients					
sample	Group	Bull	Sample	Group	Breed	status	ITER-1	ROC	ITER-2	ROC	ITER-1	ROC	ITER-2	ROC	ITER-3	ROC
1487	AV H Fh	AV	5111	H Fh	H	Birth	Birth	1.000			Birth	0.887	Birth	0.993		
1509	AV H Fh	AV	7278	H Fh	H	Birth	Birth	1.000			Birth	0.887	Birth	0.993		
1572	AV H Fh	AV	7277	H Fh	H	Birth	Birth	1.000			**No birth**	0.887	Birth	0.993		
2504	AV H Fh	AV	4290	H Fh	H	Birth	Birth	1.000			Birth	0.887	Birth	0.993		
2384	AV H Fh	H	7531	H Fh	H	Birth	Birth	0.983			Birth	0.887	Birth	0.993		
2437	AV H Fh	H	8447	H Fh	H	Birth	Birth	0.983			Birth	0.887	Birth	0.993		
2438	AV H Fh	H	2363	H Fh	H	Birth	Birth	0.983			Birth	0.887	Birth	0.993		
2439	AV H Fh	H	3613	H Fh	H	Birth	Birth	0.983			Birth	0.887	Birth	0.993		
2454	AV H Fh	H	6094	H Fh	H	Birth	Birth	0.983			Birth	0.887	Birth	0.993		
2575	AV H Fh	H	4557	H Fh	H	Birth	Birth	0.983			**No birth**	0.887	Birth	0.993		
2576	AV H Fh	H	7550	H Fh	H	Birth	Birth	0.983			Birth	0.887	Birth	0.993		
1757	AV H Fh	H	3955	H Fh	H	Birth	Birth	0.983			Birth	0.887	Birth	0.993		
1758	AV H Fh	H	1959	H Fh	H	Birth	Birth	0.983			Birth	0.887	Birth	0.993		
1571	AV Fh	AV	5140	H Fh	H	No birth	No birth	1.000			**Birth**	0.887	**Birth**	0.993		
2142	AV H Fh	H	4290	H Fh	H	No birth	No birth	0.983			**Birth**	0.887	No birth	0.993		
1488	AV Fh	AV	3844	H Fh	H	No birth	No birth	1.000			No birth	0.887	No birth	0.993		
2308	AV Fh	AV	4290	H Fh	H	No birth	No birth	1.000			No birth	0.887	No birth	0.993		
2309	AV Fh	AV	7530	H Fh	H	No birth	No birth	1.000			No birth	0.887	No birth	0.993		
1993	AV Fh	AV	3613	H Fh	H	No birth	No birth	1.000			No birth	0.887	No birth	0.993		
2505	AV Fh	AV	7235	AV H Fh	AV	Birth	Birth	1.000			Birth	0.925				
2143	H Fh	H	4943	AV H Fh	AV	Birth	Birth	0.997			Birth	0.925				
2385	AV H Fh	H	1381	AV H Fh	AV	No birth	No birth	0.983			No birth	0.925				
2386	AV H Fh	H	7237	AV H Fh	AV	No birth	No birth	0.983			No birth	0.925				
2456	AV H Fh	H	1344	AV H Fh	AV	No birth	No birth	0.983			No birth	0.925				
1473	AV Fh	AV	6022	AV H Fh	AM	No birth	No birth	1.000			No birth	0.925				
1520	AV Fz	AV	6887	H Fz	H	Birth	Birth	0.948	Birth	0.948	Birth	0.737	Birth	0.840	Birth	0.855
1521	AV Fz	AV	6886	H Fz	H	Birth	Birth	0.948	Birth	0.948	Birth	0.737	Birth	0.840	Birth	0.855
2175	AV Fz	AV	7922	H Fz	H	Birth	**No birth**	0.948	**No birth**	0.948	Birth	0.737	Birth	0.840	Birth	0.855
1782	AV Fz	AV	6810	H Fz	H	Birth	Birth	0.948	Birth	0.948	Birth	0.737	Birth	0.840	Birth	0.855
1846	AV Fz	AV	7540	H Fz	H	Birth	Birth	0.948	Birth	0.948	Birth	0.737	Birth	0.840	Birth	0.855
1848	AV Fz	AV	7541	H Fz	H	Birth	Birth	0.948	Birth	0.948	Birth	0.737	Birth	0.840	Birth	0.855
1680	H Fz	H	4710	H Fz	H	Birth	Birth	0.973	Birth	0.981	Birth	0.737	Birth	0.840	Birth	0.855
2242	H Fz	H	7530	H Fz	H	Birth	Birth	0.973	Birth	0.981	**No birth**	0.737	Birth	0.840	Birth	0.855
2319	H Fz	H	1356	H Fz	H	Birth	Birth	0.973	Birth	0.981	Birth	0.737	Birth	0.840	Birth	0.855
1900	H Fz	H	5988	H Fz	H	Birth	Birth	0.973	Birth	0.981	Birth	0.737	**No birth**	0.840	Birth	0.855
1522	AV Fz	AV	6480	H^{+}Cb Fz	Cb	Birth	Birth	0.948	Birth	0.948	**No birth**	0.740	Birth	0.840	Birth	0.855
1523	AV Fz	AV	6885	H^{+}Cb Fz	Cb	Birth	Birth	0.948	Birth	0.948	Birth	0.740	Birth	0.840	Birth	0.855
2489	AV Fz	AV	7530	H Fz	H	No birth	No birth	0.948	No birth	0.948	**Birth**	0.737	**Birth**	0.840	**Birth**	0.855
1842	AV Fz	AV	7530	H Fz	H	No birth	No birth	0.948	No birth	0.948	**Birth**	0.737	**Birth**	0.840	**Birth**	0.855
1675	H Fz	H	2398	H Fz	H	No birth	No birth	0.973	No birth	0.981	**Birth**	0.737	**Birth**	0.840	**Birth**	0.855
1678	H Fz	H	4787	H Fz	H	No birth	No birth	0.973	No birth	0.981	**Birth**	0.737	**Birth**	0.840	**Birth**	0.855
2178	AV Fz	AV	7531	H Fz	H	No birth	No birth	0.948	No birth	0.948	**Birth**	0.737	**Birth**	0.840	**Birth**	0.855
2285	H Fz	H	713755	H Fz	H	No birth	No birth	0.973	No birth	0.981	No birth	0.737	**Birth**	0.840	**Birth**	0.855
2041	H Fz	H	4557	H Fz	H	No birth	No birth	0.973	No birth	0.981	**Birth**	0.737	**Birth**	0.840	**Birth**	0.855
1901	H Fz	H	2398	H Fz	H	No birth	No birth	0.973	No birth	0.981	**Birth**	0.737	No birth	0.840	No birth	0.855
2516	AV Fz	AV	880	H Fz	H	No birth	**Birth**	0.948	**Birth**	0.948	**Birth**	0.737	No birth	0.840	**Birth**	0.855
1613	H Fz	H	5140	H Fz	H	No birth	**Birth**	0.973	**Birth**	0.981	**Birth**	0.737	No birth	0.840	**Birth**	0.855
2177	AV Fz	AV	713755	H Fz	H	No birth	No birth	0.948	No birth	0.948	No birth	0.737	No birth	0.840	No birth	0.855
1679	H Fz	H	7136	H Fz	H	No birth	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855

Continued

Table 3 (Continued). Transferred embryos (fresh, Fh; or frozen, Fz) matched with their recipients showing the actual gestational status at birth and the ROC-AUC value (ROC) predicted for birth after iterations (ITER-) calculated with the algorithm support vector machine (SVM) within specific groups of embryos and recipients[1]

Embryo			Recipient			Actual	Predicted status in embryos				Predicted status in recipients					
sample	Group	Bull	Sample	Group	Breed	status	ITER-1	ROC	ITER-2	ROC	ITER-1	ROC	ITER-2	ROC	ITER-3	ROC
1725	H Fz	H	2476	H Fz	H	No birth	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
1799	H Fz	H	7922	H Fz	H	No birth	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
2284	H Fz	H	1487	H Fz	H	No birth	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
2297	H Fz	H	9925	H Fz	H	No birth	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
2298	H Fz	H	9925	H Fz	H	No birth	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
2557	H Fz	H	9925	H Fz	H	No birth	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
1732	H Fz	H	2032	H Fz	H	No birth	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
1790	H Fz	H	2032	H Fz	H	No birth	No birth	0.973	No birth	0.981	No birth	0.737	No birth	0.840	No birth	0.855
1519	AV Fz	AV	6203	AV Fz	AV	Birth	Birth	0.948	Birth	0.948	**No birth**	0.911				
2517	AV Fz	AV	7234	AV Fz	AV	Birth	Birth	0.948	Birth	0.948	Birth	0.911				
2518	AV Fz	AV	1382	AV Fz	AV	Birth	**No birth**	0.948	**No birth**	0.948	Birth	0.911				
1847	AV Fz	AV	7230	AV Fz	AV	Birth	Birth	0.948	Birth	0.948	Birth	0.911				
1614	H Fz	H	4758	AV Fz	AV	Birth	Birth	0.973	Birth	0.981	Birth	0.911				
1615	H Fz	H	3598	AV Fz	AV	Birth	Birth	0.973	Birth	0.981	Birth	0.911				
2294	H Fz	H	7239	AV Fz	AV	Birth	Birth	0.973	Birth	0.981	Birth	0.911				
2296	H Fz	H	7237	AV Fz	AV	Birth	Birth	0.973	Birth	0.981	Birth	0.911				
1783	AV Fz	AV	7235	AV Fz	AV	No birth	No birth	0.948	No birth	0.948	No birth	0.911				
1905	AV Fz	AV	4943	AV Fz	AV	No birth	No birth	0.948	No birth	0.981	No birth	0.911				
2283	H Fz	H	1382	AV Fz	AV	No birth	No birth	0.973	No birth	0.981	No birth	0.911				
2295	H Fz	H	7237	AV Fz	AV	No birth	No birth	0.973	No birth	0.981	No birth	0.911				
2551	H Fz	H	1275	AV Fz	AV	No birth	No birth	0.973	No birth	0.981	No birth	0.911				

Table 4. Biomarkers predictive of birth identified by H^+NMR in d 7 plasma of Holstein recipients of frozen embryos after recalculation of metabolite concentration values by iterative identification of actual "no birth" recipients with predicted "birth" potential supported by embryo confusion matrix;[1] many of these biomarkers did not predict birth before iteration

Metabolite	ROC-AUC	T-Test	Log FCh
Creatine	0.85117	0.00000588	0.46652
Butyrate	0.75251	0.0030875	0.218
1-Methylhistidine	0.72575	0.035673	0.27461
L-Glutamine	0.71572	0.013677	0.12309
D-Glucose	0.70736	0.0079813	−0.11701
L-Glycine	0.70736	0.022528	0.17702
L-Isoleucine	0.68896	0.014258	0.14392
L-Leucine	0.67726	0.038238	0.14411
L-Phenylalanine	0.67559	0.02963	0.1214
Propionate	0.67391	**0.052078**	0.13442
Hippurate	0.66890	0.033148	0.12401
L-Alanine	0.66555	0.025607	0.14095
2-oxoisocaproate	0.66388	0.022209	0.12641

[1]Predictive values of recipients calculated by multivariate support vector machine calculations (empirical value $P < 0.01$ by permutation). Tendencies $0.05 > P < 0.10$ shown in **bold**.

biomarkers led to identification of regulatory pathways that differed not only between open and pregnant recipients, but also between recipients of cryopreserved and fresh embryos (Gómez et al., 2020b,c).

Profiling Embryonic Competence Improves Evaluation of Recipient Competence

The biomarkers we discovered in recipients of F-T embryos were not observed in recipients of V/W embryos in a previous report (Gómez et al., 2020c). However, after iterative pregnancy prediction combining information from recipient plasma and embryo CM, we here identified L-glutamine and L-glycine as early pregnancy biomarkers (i.e., d 40 and 62) for Holstein recipients, as shown before within V/W embryos (Gómez et al., 2020c). The iteration procedure detected misidentified recipients with a high ROC-AUC probability value. Previously we hypothesized and showed that absence of reciprocal information from embryos and recipients holds up the identification of pregnancy biomarkers (Muñoz et al., 2014a,b; Gómez et al., 2021). The rationale can be explained from the information of the embryonic side, which is long-term predictive because pregnancy rates to term greatly change with embryos produced with or undergoing different technologies, such as cloning, cryopreservation and certain culture conditions (e.g., serum; Liu et al., 2013; Carrillo-González and Maldonado-Estrada, 2020; Gómez et al., 2020a; Valente et al., 2022). These technologies share similar early pregnancy rates, followed by a cascade of pregnancy losses, more pronounced with extremely altered embryos (i.e., clones). The similar early pregnancy rates between IVP embryos and more competent embryos (e.g., fresh in vivo), endorse our findings. Thus, the embryo, as responsible for most miscarriages, hides the true recipient competence to term as shown in a pioneer work modeling pregnancy failure after d 60 (McMillan, 1998). In the present work we observed misclassification due to the pregnancy losses experienced by frozen embryos, interestingly, whose competence was accurately identified in the large data set once supported by embryonic metabolite signals.

In our study, more embryos than recipients were identified as nonviable for pregnancy; thus, independent recipient classification led to nonpredictive results (i.e., more recipients were judged as viable than effectively pregnant). In contrast, recipient classification weighed with viability of embryos, half of which were qualified as nonviable, improved pregnancy prediction of recipients. Furthermore, the specificity of biomarkers is consistent with the type of embryo and its cryopreservation status, which could also imply, in a certain extent, a special capacity of the recipient to carry one or another embryo type to term. At the same time, complementary information from the embryo and the recipient predicts better to term and makes it possible to mate viable embryos with competent recipients, thus optimizing biomarker identification, leading to improved birth rates. We also emphasize that, in healthy recipients, the embryo, as responsible for most of miscarriages, shows an early metabolic fingerprint of such pregnancy losses that translate into measurable changes in culture medium.

Metabolic Differences Between Recipients of Fresh and F-T Embryos

Recipients of fresh and cryopreserved (i.e., V/W) embryos show distinct metabolic profiles in d 0 and 7 plasmas (Gómez et al., 2020b,c, 2021), in agreement with the present study with frozen embryos. The rationale of metabolic differences between recipients of F-T and fresh embryos must be explored in the consequences of embryo freezing. Thus, F-T and fresh embryos that lead to pregnancy and birth exhibit distinct metabolite concentrations in their surrounding CM; such differences are particularly relevant for AA and lipids (Gimeno et al., 2021).

In previous studies, lipid metabolism was related to pregnancy success both in IVP embryos (Gimeno et al., 2021) and recipients (Gómez et al., 2020c, 2021). The involvement of lipid metabolism has also been reported in live embryos collected from Jersey cows, which have higher, different lipid content than Holsteins (Baldoceda et al., 2015). Such embryos show poorer survival to cryopreservation (Gilbert et al., 2022), and lower

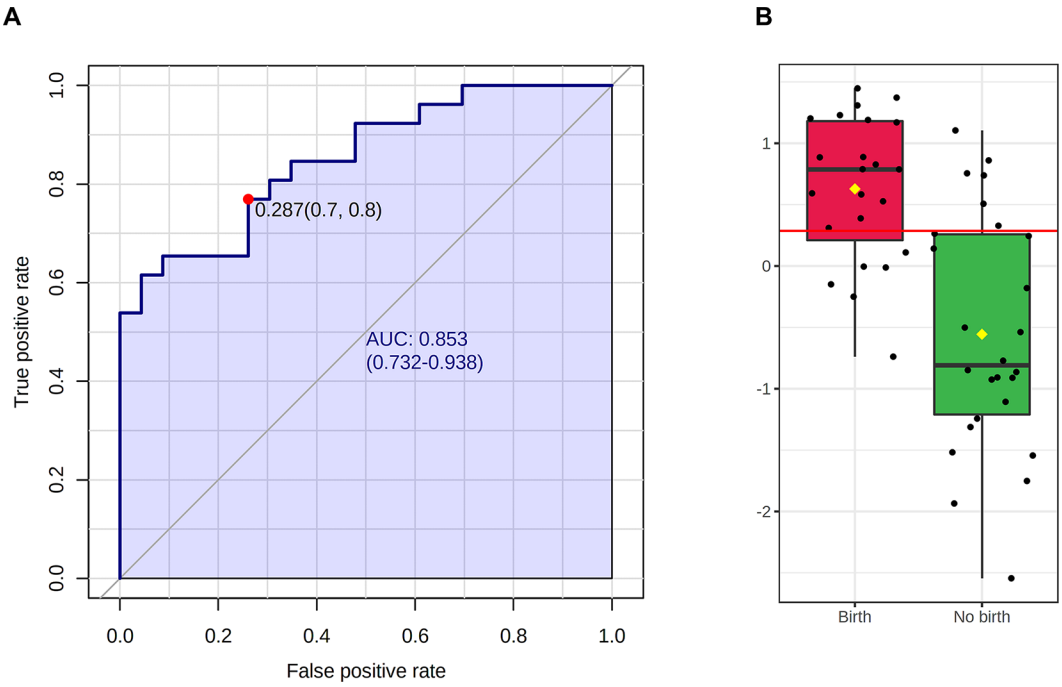


Figure 9. ROC-AUC (A) and boxplot (B) obtained after iterations for creatine concentrations that differ between recipients that reached or not birth. Black dots represent the concentrations of all samples of each metabolite scaled as log fold change values. The notch indicates the 95% confidence interval around the median. The mean concentration of each group is shown by a red diamond. The optimal cut-off is indicated as a horizontal red line (i.e., the closest to the left-hand corner in the corresponding ROC-AUC).R

pregnancy rates after transfer once F-T (Steel and Hasler, 2004). Not only the total lipid contents, but certain lipids are responsible for decreased survival to cryopreservation (de Andrade Melo-Sterza and Poehland, 2021). Thus, in the embryonic cell, the freezing damage reduces membrane contents in lipids such as lysophosphatidylcholine (Janati Idrissi et al., 2021), while the best freezing survivors, in vivo embryos, have membranes enriched in phospholipids such as phosphatidylcholines (Sudano et al., 2012; Janati Idrissi et al., 2021). Phosphatidylcholines are in turn methylated phosphatidylethanolamines, a lipid family we detected as acting as pregnancy biomarkers in F-T embryos (Gimeno et al., 2021). In accordance with a previous work with V/W and fresh embryos (Gómez et al., 2020c), we propose that recipients of cryopreserved embryos that become pregnant should have the intrinsic uterine ability to counteract at least the cryopreservation damage in the embryo, facilitating immediate embryo recomposition in the uterus upon transfer (e.g., membrane damage). Such a short-term effect would allow implantation of long-term damaged embryos, likely carrying serious epigenetic damage from cryopreservation, including cytogenetic alteration (Maldonado et al., 2015; Inaba et al., 2016; Hayashi et al., 2019), which would be prone to undergo late miscarriage, as explained by some specific biomarkers that differed between birth and earlier pregnancy stages (i.e., d 40) identified in the embryo CM (Gimeno et al., 2021; Gómez et al., 2021). As an example, the miscarriage biomarker dimethyl adipate also acts as a robust pregnancy biomarker mainly in F-T embryos, but at early pregnancy stages (Gimeno et al., 2021). In contrast, fresh embryos would not show such specific damages, and therefore the restorative ability exerted by the histotroph over the embryo could be a reason for differences shown in pregnancy biomarkers between recipients of fresh and cryopreserved embryos, provided that a proportion of plasma metabolites is reflected in the uterine fluid (**UF**; Hugentobler et al., 2007). Thus, we identified 6 differentially enriched pathways in d 40 pregnant recipients from fresh and F-T embryos, both within plasma samples on d 0 and 7, which were pterine biosynthesis, steroid biosynthesis, folate metabolism, androgen and estrogen metabolism, androstenedione metabolism, and tryptophan metabolism. Furthermore, the fact that up to 60% losses of IVP embryos take place between d 8 and 17 (Mamo et al., 2011; Hue et al., 2019) suggests that the environment that the embryo challenges just after ET greatly conditions its fate. Furthermore, the uterus responds to embryos bearing distinct competence with changes in the AA concentrations in the UF (Groebner et al., 2011). Differences in histotroph AA between cows (less

fertile) and heifers (more fertile) remain at least until gestational d 19 (Forde et al., 2017).

Further evidence of differences between recipients of F-T and fresh embryos are defined by creatine. In our case, creatine was a consistent pregnancy and birth biomarker, mainly with F-T but also with fresh embryos; and mainly on d 7, but also on d 0. Creatine showed positive FCh in recipients of F-T embryos (8 blocks; higher in pregnant), whereas the recipients of fresh embryos showed negative FCh (6 blocks; lower in pregnant). Interestingly, proteins of creatine metabolism were upregulated in UF from cows yielding poor quality in vivo embryos (Aranciaga et al., 2021), which is consistent with our results for fresh embryos. To a lesser extent, the same reversed FChs were observed for creatinine (contrary to creatine, 1 block negative in frozen, positive in fresh) and 2-hydroxyisobutyrate (3 frozen blocks and 2 fresh blocks higher and lower in pregnant recipients, respectively). The upregulation of general and AA metabolism in cows carrying poor quality embryos described by Aranciaga et al. (2021) is also consistent with our distribution of blocks and with the multiple AA pathway activation we recorded in recipients of F-T embryos. In this way, the number of blocks that had lower metabolite concentrations in pregnant recipients accounted for double the quantity than nonpregnant ones.

Relevant Metabolite Biomarkers of Pregnancy

Collectively, embryonic biomarkers would predict better birth than earlier stages, while recipients, in general, show more predictive power in the beginning of pregnancy (Muñoz et al. 2014a; Gómez et al., 2020b,c). In contrast, in vivo collected embryos, the highest viability standard, transferred fresh after a 24-h culture step to analyze CM by Fourier transform infrared spectroscopy in a simultaneous trial performed in 2 independent laboratories (France and Spain), showed higher predictive ability on d 60 than at birth (Muñoz et al., 2014b), while recipients of such embryos showed similar predictive capacity through gestational endpoints.

Ornithine, L-lysine and L-glutamine were relevant biomarkers in our study. Such metabolite concentrations increase on d 0 in females later diagnosed as pregnant by AI (Phillips et al., 2018). Ornithine was a consistent biomarker for pregnancy at d 40, mainly among fresh embryos produced under a variety of culture conditions and in all different breeds examined in previous studies (Gómez et al., 2020b,c) and in the present study, suggesting robustness as a confident biomarker. Recently, ornithine, was identified as one of 2 important metabolite hubs in d 0 plasma of AI pregnant and nonpregnant recipients, as well as glutamine in nonpregnant recipients (Banerjee et al., 2022). Ornithine is involved in the arginine biosynthesis pathway, which was upregulated in open versus pregnant F-T Holsteins diagnosed on d 40, together with phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, alanine, aspartate, and glutamate metabolism pathways. Moreover, in the nonpregnant group, the ART3 gene (ADP-ribosyltransferase-3, an arginine-specific ADP-ribosyltransferase) was downregulated and positively correlated with ornithine (Banerjee et al., 2022). Ornithine and arginine are precursors for polyamine synthesis. Ornithine decarboxylase 1 converts arginine into ornithine which in turn forms putrescine (Lenis et al., 2017). Polyamines are essential for early embryonic development and successful pregnancy establishment (Fozard et al., 1980; Lenis et al., 2017; Pendeville et al., 2001). Interestingly, expression of ornithine decarboxylase 1 was found to be significantly lower in biopsies from blastocysts resulting in resorption compared with those resulting in calf delivery (El-Sayed et al., 2006). In porcine parthenotes, addition of polyamines to the culture medium increased the blastocyst rate, total cell number, and decreased apoptosis (Cui and Kim, 2005). L-Arginine and ornithine are regulated throughout pregnancy and, interestingly, hold their concentrations in serum, uterine fluid and fetal-annex fluids under nutritional restriction (Crouse et al., 2019), which suggest they can be suitable biomarkers. Whatever the reproductive technique used to reach pregnancy, there is a widely supported association between ornithine and fertility.

The pregnancy predictive ability of biomarkers in recipients was lower with frozen embryos, particularly at birth, in contrast with both fresh and early pregnancy stages of F-T (summarized in Table 2). We reported similar findings with recipients of V/W embryos (Gómez et al., 2020c), with one single biomarker being predictive both at birth (L-glycine) and at earlier pregnancy stages. In the present study, L-glycine also predicted at earlier stages and, with a lesser capability, at birth, but independent of breed. However, combining the information provided by embryos improves the capacity to predict birth in recipients, as shown with L-glycine herein. Another interesting biomarker, acetone, was altered in all groups except in pure Holstein, and mainly in recipients transferred with F-T embryos. In a previous work we identified acetone as a birth biomarker in AV recipients transferred with V/W embryos, but not with fresh embryos (Gómez et al., 2020b). Therefore, acetone seems to be a biomarker associated with cryopreservation. Similar to the present work with F-T embryos in AV and non-Holstein breeds, 2-hydroxyisobutyrate predicted birth in d 7 plasma of

AV recipients transferred with V/W embryos (i.e., AV, AM, and crossbred; Gómez et al., 2020b).

Supplementation to CM with specific metabolites identified in this study could improve the metabolic status of the embryo. However, as the study was carried out in an experimental herd, results can be constrained by specific nutrition, environmental conditions, and embryo culture systems; therefore, the extent of our results to other scenarios need caution to be made. Although the combined methodology to detect and mate competent embryos and recipients led to upgraded biomarkers in recipients and embryos consistent in part with other studies, further proofs of validation are necessary yet.

CONCLUSIONS

Generally, embryos were more predictive of birth than of earlier pregnancy stages; on the contrary, recipients of frozen embryos showed more predictive power at the beginning of pregnancy, while recipients of fresh embryos were more predictive at birth. However, used together, such complementary information leads to better pregnancy prediction to term. Cryopreservation, although showing not significantly more miscarriage rates in our sample set, led to obtaining some specific biomarkers which were different from recipients of fresh embryos. Furthermore, more biomarkers differed between initial (i.e., d 40 and 62) pregnancy endpoints and birth among recipients of F-T embryos, confirming the drop of biomarker abundance observed at birth in previous studies with V/W embryos. We also observed that pregnancy competence is lower in a high proportion of our IVP embryos than in recipients, making embryo studies more reliable than investigating recipient competence, which we can estimate over 80 to 85% of ET cycles, in agreement with previous findings. Therefore, the expected gain of pregnancy prediction is much greater with embryos (45–55%) than with recipients. Predicting on d 0, although less efficient because more biomarkers appear on d 7, entails advantages in terms of decision making (one week before ET). The use of recipient biomarkers combined with embryo biomarkers (obtained in a noninvasive way) provides a method both to improve the reliability and accuracy of single biomarkers, and used together, to highly increase the pregnancy expectation with F/T embryos in cattle.

[1]AV = Asturiana de los Valles, includes one recipient Asturiana de la Montaña (AM); H = Holstein; Cb = crossbred. Divergencies between the actual status and the predicted status in an iteration are marked in **bold**. Significant ROC-AUC values calculated with *P*-value <0.01 by permutation.

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