Current and Potential Applications for Hematologic Variables in Dairy Herd

Metabolic and Nutritional Monitoring

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The concept of blood analysis for herd-level interpretation, frequently referred to as metabolic profiling, in dairy herds appears to have taken hold about the time that automatic serum chemistry analyzers became available in the early 1970s. The hope was that large numbers of blood samples could be analyzed quickly and economically and that detailed information relative to metabolic and nutritional status of the cows could be practically available. The concept is appealing because metabolic disease remains a major health challenge in dairy herds. Interest in the technique, however, has waxed and waned over the years because of many questions about its diagnostic value relative to its cost. Nevertheless the concept remains popular with many veterinary clinicians because they see a need for additional diagnostic and metabolic monitoring techniques, beyond what are currently available.

The interest and potential value of monitoring metabolism in dairy cows can be quickly understood from looking at the distribution of cow losses from death and culling, relative to parturition, as illustrated in this slide. These early lactation losses are related primarily to metabolic disease. Thus, an increased ability to determine the metabolic status of cows, particularly around parturition would appear to be of great value.

Sampling of blood may seem advantageous in the investigation of metabolic disease because direct changes in metabolite concentrations can be measured. However, there are many disadvantages, including the expense of blood collection, shipment, and laboratory analysis. To increase the practicality of metabolic monitoring, alternatives to blood sampling should always be sought. Such alternatives could include analysis of other samples, such as milk, feces, or urine. Other kinds of monitoring, such as body condition scoring and rumination monitoring could also supply metabolic and nutritional information more practically and for some applications more accurately than blood analysis. Nevertheless, there may be some types of information that is best obtained from blood analysis. As herd sizes increase, the cost of blood analysis may become less, relative to the potential cost of errors in nutrition or management. In large herds such errors would be multiplied over many cows whereas the number of samples necessary for analysis may not increase proportionately to herd size.
**Is This a Nutritional Evaluation?**

There has been some discussion as to whether metabolic profiling is a form of nutritional evaluation, or is it something else. In my mind it clearly is part of nutritional evaluation or assessment. Any problems identified through changes in blood metabolite concentrations would need to be rectified by modifications in dietary management. Such changes may be more complex that just changing ration formulations and may involve such things as different cow grouping and feeding strategies, but they are clearly modifications in nutritional management.

In this discussion, I’d like to emphasize the concept of nutritional assessment, which I define as those observations about the animal which reflect on its nutritional status. Blood analysis clearly is not the only part of nutritional assessment, but in some cases it can be useful. Nutritional assessment forms a portion of the overall evaluation of nutrition, which must include evaluation of the diet and management as well.

**Why Isn’t Diet Evaluation Enough?**

Evaluation of the diet, including its nutrient profile and physical characteristics, should always be a first step in the investigation of metabolic disease. However, one must recognize that this may not be enough to identify the cause of a problem, even if the problem is related to dietary formulation. Ruminant digestion and nutrient supply is extremely complex. To address this complexity, increasingly intricate computer models for dairy cow diet formulation have been designed. These models involve considerable uncertainty and it may be necessary to modify diets formulated by such models, based on nutritional assessment. Furthermore, even the most well designed diets are subject to human error in formulation and delivery. Such errors are not always easy to identify, but may be suggested by nutritional assessment.

**Nutritional Assessment**

Just a few more comments on nutritional assessment, as I use the term. It’s essentially “listening to the cows” via whatever means they have to communicate. It encompasses a broad variety of animal responses. Blood variables can be among the animal responses monitored.

I think nutritional assessment can and should be an area of synergy and cooperation among veterinarians and nutritionists, not an area of confrontation. This synergy should be for the benefit of their mutual clients.
What are the Challenges in Interpreting Blood Variables?

The influence of diet and metabolic status on blood composition has been demonstrated in many ways over thousands of controlled experiments in animal research laboratories and institutions. The challenge comes in trying to interpret these findings in the uncontrolled world of commercial herds. The frequent comment relative to the interpretation of blood variables in commercial herds is: “The results are just too variable, no sense can be made of them.” Variation, however, can be both our friend and enemy in herd-level evaluation of blood variables. Ascribable variation is our friend. This is the variation that we can attribute to a definable condition, the kind of things we can make decisions about. Random variation is our enemy; it is the “background” variation that may obscure the ascribable variation for which we are looking. Statistical analysis is designed to assist us in differentiating the ascribable variation that we seek from the background of random variation.

Statistical Approaches to Metabolic Profile Testing – the “Clinical Pathology” Approach

Reference ranges for clinical chemistry variables, as typically used in the evaluation of individual patients, are based on the distribution of values within a reference population, presumably of healthy individuals. Such reference ranges are designed to identify diseased individuals. This approach is very difficult to apply to metabolic profiling in dairy herds for several reasons.

First, in metabolic profiling we are attempting to identify variation within the range of healthy individuals. That is to say, we are trying to identify the response to nutritional and metabolic stress that is still within the range of normal homeostatic control. Once normal homeostatic control has failed, the animal develops clinical disease, which usually contributes to biochemical abnormalities not directly related to the nutritional inputs in which we are interested. Thus reference ranges typically available from clinical chemistry laboratories are not appropriate for metabolic profile testing.

Furthermore, if we are to define appropriate reference ranges for dairy metabolic profiling they will need to be complex enough to account for expected changes across the lactation cycle. Such a table of reference ranges for several variables has been established for Italian dairy cattle (Bertoni and Trevisi 2013), but even this reference range is built on confidence intervals that makes classification of individual animals difficult.

Moreover, the objectives of metabolic profile testing are generally herd-level objectives. Some method is necessary to evaluate the herd performance based on a sampling of individual animals. Thus, specific herd-based reference ranges are necessary.

Statistical Approaches to Metabolic Profile Testing – the “Epidemiology” Approach

In epidemiology the objective is generally to define a diseased or “at risk for disease” population. Thus the statistical question is one of inclusion of the patient within an at risk population. This in contrast to the exclusion of a patient from a normal population, as was the objective in the clinical pathology approach. Use and application of this approach is rather new in metabolic profiling, but has been investigated and has created important insights.
Statistical Approaches Applied to Serum Non-esterified Fatty Acids (NEFA)

We are going to discuss the epidemiological approach to serum NEFA in some detail. We’ll use NEFA as an example in part because I believe NEFAs are the most useful blood variable currently available for the management of metabolic diseases of transition dairy cows, one of the largest disease challenges in the dairy industry. Moreover, there is considerable epidemiological research information available relative to NEFAs. Thus, we can discuss these epidemiological techniques in general, as they might apply to other blood variables that might be included in a metabolic profile.

What Are NEFA?

The upper molecule in the figure at left is a single fatty acid, in this case a non-esterified fatty acid. It is mostly composed of a long hydrocarbon chain, similar to diesel fuel; it is a high energy molecule. The molecule at the bottom is a triglyceride. It is composed of three fatty acids joined to glycerol by ester bonds. Thus, these fatty acids are referred to as being esterified. Splitting the triglyceride by breaking the ester bonds, as depicted by the dark line, results in the creation of non-esterified fatty acids. There are other forms of esterified fatty acids, besides triglycerides, but triglycerides are the form in which fat is stored in adipose tissue.

Non-esterified fatty acids are high energy molecules that can support the energy needs of animals when the dietary energy supply is insufficient to meet their needs. They are normal, indeed critically important metabolites. Non-esterified fatty acids found in the blood originate from adipose tissue, not directly from dietary fat. They serve a critical role in energy homeostasis. There can be a very broad range of serum NEFA concentrations in healthy animals, depending on their energy status.

1 Transition dairy cows = cows in the period between three weeks prior to three weeks after calving.
The serum NEFA concentration is regulated by the relative rate at which NEFA is released from adipose tissue. The NEFA removal rate from serum is relatively constant, thus making the release rate the primary determinant of serum concentration. The positive factors stimulating NEFA release are illustrated at left and are primarily driven by negative energy balance, although stress may contribute to the stimulatory effect. Inhibition of NEFA release is dramatically controlled by insulin.

Metabolic Factors Increasing Blood NEFA in Dairy Cows

Dairy cows in early lactation are expected to be in negative energy balance because the rate of increase in energy demand due to lactation exceeds the rate of increase in energy supply from dietary intake; appetite lags behind milk production. Thus, serum NEFA concentrations in early lactation are expected to exceed those at other times. In addition, dairy cows normally develop some degree of insulin resistance near parturition. This seems to be part of a natural process that helps direct energy from the dam to the calf. In cows that are obese, this insulin resistance may be exacerbated. Furthermore, a large adipose mass (obesity) may in itself lead to increased NEFA release. Moreover, stress, which may be due to environment or infectious disease, may even further contribute to the release of NEFA from adipose tissue.

Management factors influencing serum NEFA concentrations in transition cows are not the topic of this talk. However, they are complex and involve such things as cow grouping, dietary fermentability, and diet changes across the entire lactation cycle. Thus, blood profiling of serum NEFA during the transition period provides information relative to the adequacy of nutritional management across all phases of the mature dairy cow life cycle.

Why do we care about serum NEFA concentrations?

There is ample evidence of a positive association between serum NEFA concentrations during the transition period and “negative downstream events,” including displaced abomasum, clinical ketosis, retained placenta and metritis, reduced fertility, and reduced milk production. The exact cause and effect nature of this association is not completely understood, but the existence of this association is very well established.

Much of the recent epidemiological research involving serum NEFA concentrations, of which I’m aware, has been done at these institutions: Michigan State University, Ontario Veterinary College, and Cornell University. It’s primarily this research that we’ll discuss in the next several slides.
In the slide at left and the one that follows, the bars represent the incidence of disease occurring during the first month of lactation in 1556 dairy cows distributed across 95 Michigan dairy herds. Blood samples were taken during the last 4 weeks of gestation, when the cows were apparently healthy. Disease occurrence was recorded by farm personnel. The cows were retrospectively divided based on their serum NEFA concentrations during the dry period. The method by which they were classified is described below. There was a dramatic and significant \( P<0.01 \) increase in disease incidence with increasing serum NEFA concentration. This was true for all diseases monitored, except for milk fever.

Many investigators have found similar results, although study designs have differed. The cause-and-effect relationship underlying this association is not completely understood and is under intense investigation. It will not be a major topic of this talk, but appears to involve negative energy balance, hepatic lipidosis (fatty liver), and generalized inflammatory responses.

**How do we apply this association in practice?**

We’ve discussed the general challenges associated with the traditional clinical pathology approach to reference ranges as it could apply to dairy herd metabolic profile testing. All of those concerns, especially those related to time of sampling relative to calving are particularly important with respect to serum NEFA. There has been considerable research recently in applying epidemiological techniques to the interpretation of NEFA values, and that will be the topic of the next several slides, as will another approach known as **statistical process control.**
The epidemiological approach is to predict which animals are at risk of disease. In order to make that prediction we have to define disease. For the studies we will be discussing below the diseases considered were displaced abomasum, clinical ketosis, retained placenta, or a combination of the three.

**Dichotomous and Continuous Variables**

Many of the outcome variables in which we are interested have dichotomous values; disease or not, pregnant or not, etc. In contrast, clinicopathological variables (usually blood concentrations) are typically continuous. For epidemiological evaluation there needs to be a means to compare them. This usually takes the form of a *cut point*, which is defined as a specific value of the continuous variable above which is designated *positive* and below which is designated *negative*. A *receiver operator characteristic curve* (ROC) is used to determine the optimum cut point. The optimum cut point maximizes both *sensitivity* and *specificity*.

<table>
<thead>
<tr>
<th>Sensitivity and Specificity Relative to Cut Points</th>
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<tbody>
<tr>
<td>• Sensitivity - the proportion of actual positives that are correctly identified</td>
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<tr>
<td>– The proportion of cows with high NEFA that developed DA</td>
</tr>
<tr>
<td>– High sensitivity - few false negatives</td>
</tr>
<tr>
<td>• Specificity - the proportion of negatives which are correctly identified as such</td>
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<tr>
<td>– The proportion of cows with low NEFA that did not get DA</td>
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<tr>
<td>– High specificity - few false positives</td>
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The slide above and on the right illustrates the tradeoff between sensitivity and specificity. The points on the distribution graph represent serum NEFA concentrations in cows measured after parturition and before disease occurrence. The solid points are cows that subsequently developed displaced abomasum while the open points are cows that did not. If were to choose the vertical line on the left as our cut point, it would have a high sensitivity; it would predict DA in nearly all the cows that did indeed develop DA, but it would also predict DA in many cows that did not develop the condition. If we were to choose the line on the right, it would have high specificity; we would predict DA in a relatively small number of cows that did not actually develop DA. The ROC curve is used to illustrate the overall accuracy of the test and to aid in choosing a point that maximizes both sensitivity and specificity.
The graph above plots sensitivity against 100-specificity, with both values measured in percent. For this graph the outcome is displaced abomasum and the cut point is a value of serum NEFA concentration. The dark solid line represents the relative change in sensitivity and specificity as the cut point is either increased or decreased. The dotted lines represent the 95% confidence interval of this value. The small square in the circle represents the optimum cut point, in this case 0.72 mM. This cut point achieves the highest combination of sensitivity and specificity. If a lower cut point is chosen, the square moves to the right along the solid line, representing greater sensitivity and reduced specificity. Likewise, if a higher cut point were chosen the square would move to the left along the line, representing lower sensitivity and increased specificity. If there were a perfect relationship between the prediction and outcome, the solid line in this graph would form a right angle along the upper and left boundaries of the graph. The area under the curve of such a relationship would be 1.0 (100%) as it would include all of the graph area. If the prediction were totally worthless, that is totally random with no predictive value, the solid line would form a diagonal, following the broken line in this graph. In that case the area under the curve would be 0.5, indicating a test of no predictive value.
The values in this table give us further information about the predictive value of serum NEFA, in this case when measured postpartum but before disease occurrence. In vet school we’re trained to look at the p values, which in this case are very small. This indicates we can have a high degree of confidence there is some associative relationship and that the association is not occurring due to chance. Beyond this let’s look at some other values. Remember we said that a perfect test would have an area under the curve of 1.0 and a totally worthless test would have an area under the curve of 0.5. Clearly the predictive value of serum NEFA is fairly mediocre, especially for diseases other than DA.

Moreover, let’s examine the likelihood ratio positive. This is sometimes called the risk ratio and indicates the relative risk of an animal positive for the test, in this case NEFA > 0.72 mM, of getting a DA compared to an animal negative for the test. In this case it indicates the risk of a DA in a positive cow (NEFA > 0.72 mEq/L) is three times the risk of cow with a lower DA. This seems like a lot, but we need to remember the actual probability of any cow getting a DA is generally low. If the probability any cow in the herd getting a DA is 5%, then the based on the risk ratio the probability of a cow with a serum NEFA > 0.72 mM getting a DA is 25% (McGee 2002). That’s better than playing the lottery, but hardly a perfect prediction. Thus we have to appreciate that the use of variables such as NEFAs has more potential in identifying trends and probabilities than in picking out individual animals for special treatment.

This table gives the same parameters as the previous table, except that it is based on blood samples taken prior to calving. Here the cut off value (critical threshold) is lower at 0.27 mEq/L. Note the p values are very small, but the strength of the association is even weaker than in the previous table based on values taken postpartum. Note the relatively small areas under the curve and likelihood ratios.

Adapted from PA Ospina et al., Journal of Dairy Science, 93(2): 546, 2010
This table shows the influence of serum NEFA, measured prepartum, on the likelihood of pregnancy at various times subsequent to the voluntary waiting period for the herd. On the average cows with prepartum NEFA > 0.27 mEq/L took ten days longer to reconceive, compared to cows with lower serum NEFA concentrations.

This table illustrates that serum NEFA has a relationship to milk production. In all animals high NEFA prepartum resulted in lower milk production, on average. In first parity animals high NEFA postpartum was related to higher milk production whereas in older cows it was related to a reduction in milk production.

What is the Application, or Relevance of this to Clinical Practice?

All of the parameters discussed above were cow-level parameters. Should they be used for identification of individual cows to be identified for prophylactic treatment? The predictive value of the tests is probably not high enough for that. Even if the test were predictive enough, such an application would require testing every cow. This might occasionally be a short-term application, but disease prevention should always be the long-term objective.

Actions should be at the herd-level and involve such things as diet adjustments, change in cow grouping, body condition score management and similar things. So how do we apply these test values at herd level?
Herd-Level Approach

A herd-level evaluation implies that a number of cows are selected for testing and the results are used to predict responses; disease, fertility, etc., across the herd. To apply these tests at herd level, two decisions are necessary. First is the metabolite cut point, similar to the cow-level analyses. The second determining a critical proportion of tested animals above the cut point which will be called a “positive,” or undesirable result for the herd. This proportion is commonly referred to as the alarm level. The alarm level would be expressed as a percentage of the animals tested that were above the cut point. In the recent herd-level studies conducted (Ospina, Nydam et al. 2010, Chapinal, Leblanc et al. 2012) cut points were as established in cow-level studies and alarm levels were arrived at by maximizing the probability of classifying a herd in the upper or lower half of all herds, relative to the incidence of the outcome variable; disease, pregnancy, or milk production. Tested alarm levels were > 15%, > 25%, > 50%

The table at right compares the results of these two studies. The New York study observed that when 15% or more of tested animals were above the designated cut points, there was a significant (p<0.05) probability that disease (DA and clinical ketosis) would be higher and fertility and milk production lower than in herds with lower proportions of high NEFA values. The results of the Canadian study, at herd level, were somewhat different than those of the New York study in that no significant effect on herd disease incidence was observed, even at somewhat higher alarm levels.

The Canadian study did identify a negative herd-level effect on fertility when more than 30% of the cows had high NEFA, either before or after calving. A negative effect on milk production was determined related to high prepartum NEFA values. There were some important differences in the study populations between the two studies. Relative the Canadian study, the New York study population consisted of larger herds, higher disease prevalence, smaller sample sizes per herd, longer sampling time windows with respect to parturition, and a higher prevalence of high NEFA values.

<table>
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<tr>
<th>Comparison of Herd-Level NEFA Evaluations</th>
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<tr>
<td><strong>Prepartum Sampling</strong></td>
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<tr>
<td>Osapina et al. (New York)</td>
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<tr>
<td>Cut Point</td>
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<tr>
<td>&gt;0.25 mEq/L</td>
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<tr>
<td>Alarm Level</td>
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<tr>
<td>&gt; 15%</td>
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<td>Outcome</td>
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<tr>
<td>Disease</td>
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<tr>
<td>Osapina et al. (Canada)</td>
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<tr>
<td>Cut Point</td>
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<tr>
<td>&gt;0.5 mEq/L</td>
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<tr>
<td>Alarm Level</td>
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<tr>
<td>&gt; 30%</td>
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<tr>
<td>Outcome</td>
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<tr>
<td>Disease</td>
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<tr>
<td><strong>Postpartum Sampling</strong></td>
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<tr>
<td>Osapina et al. (New York)</td>
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<tr>
<td>Cut Point</td>
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<tr>
<td>&gt;0.7 mEq/L</td>
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<tr>
<td>Alarm Level</td>
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<td>&gt; 15%</td>
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<td>Outcome</td>
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<tr>
<td>Disease</td>
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<td>Osapina et al. (Canada)</td>
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<td>Disease</td>
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From PA Ospina et al., Journal of Dairy Science, 93(8): 3595, 2010
From N Chapinal et al., Journal of Dairy Science, 95(10): 5676, 2012
In the New York study there was a dramatic and linear increase in herd disease incidence as the proportion of cows with high postpartum NEFA values increased.

This and the following slide illustrate the very high proportion of the herds in the New York study that were above the determined alarm level. This indicates a very large proportion of herds have less than optimal nutritional management, relative to transition cow risk. The ramification is that there is a large opportunity in the US, and potentially Italian, dairy industries for improved profitability with improved metabolic and nutritional management.

Change in disease incidence at herd level based on the proportion of animals with postpartum NEFA = 0.7 mEq/L


Distribution of Herds with High NEFA Postpartum

Practical Herd-Level Application of Serum NEFA Testing

- **What we recommend at MSU DCPAH**
  - **For a herd “Snapshot”**
    - A one-time sample
  - **Prepartum**
    - Animals between 2 and 14 days of their due date
    - Seven or more animals
    - All available if less than seven
    - Cut point –
      - 14 to 7 days prepartum - > 0.27 mEq/L
      - 7 to 2 days prepartum - > 0.3 mEq/L
    - Alarm – > 25%
  - **Postpartum**
    - Animals between 2 and 14 days in milk
    - Seven or more animals
    - All available if less than seven
    - Cut point - > 0.7 mEq/L
    - Alarm – > 25%
### Sample Size for Estimation of Prevalence Above Cut Point

<table>
<thead>
<tr>
<th>Population at risk</th>
<th>Sample Size</th>
<th>Theoretical Herd Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3</td>
<td>72</td>
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<tr>
<td>4</td>
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<tr>
<td>12</td>
<td>8</td>
<td>288</td>
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<tr>
<th>Population at risk</th>
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<tbody>
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<td>13</td>
<td>9</td>
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<td>11</td>
<td>480</td>
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<tr>
<td>8</td>
<td>22</td>
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</tbody>
</table>

Based on an expected true prevalence of 20%. Theoretical herd size based on a 12 month calving interval and uniform calving. Results estimate prevalence within a 75% confidence interval and ± 10% precision.

http://epitools.ausvet.com.au/content.php?page=1Proportion&Proportion=0.25&Conf=0.75&Precision=0.1&Population=

### Statistical Process Control Charts

Statistical Process Control Charts refer to a technique developed for the manufacturing industry to aid in reducing variation in process output. For example, controlling the uniformity of parts coming of an assembly line. The technique incorporates change over time into the analysis. When applied to blood metabolite testing the time element not only is useful from a management prospective, but also aids in producing useful and practical information, even in relatively small herds.
Statistical process control (SPC) charts are reasonably easy to construct using Microsoft Excel and a freely available \textit{add in}\textsuperscript{2}. Making the SPC charts requires some applied knowledge of Excel, but in all probability if you can construct formulas and charts in Excel, you will be able to this \textit{add in} to create process control charts. The add in is available at no charge from this Web address.

\url{http://www.jstatsoft.org/v30/i13}

At this address you will find the add in which can be attached to your Excel program, examples illustrating its use, and a detailed paper describing the process.

There are various types of control charts, based on different parameters. All have this general structure. There are individual points connected by a line. Each individual point is plotted at a time point, which is on the X axis. The variable value is on the Y axis. Each point represents a value generated by a sample of several animals. In this chart, which is an X-bar, or average chart, the points are the average of several animals sampled on a single day. The central line is the grand average of the points. The other horizontal lines are the \textit{upper} and \textit{lower control limits}. Points falling outside of the control limits indicate an added component of variance. In other words, there has been a significant change.

\textsuperscript{2} An add in refers to a special application that can be inserted into the Excel program that gives Excel additional capabilities.
This chart in this slide is referred to as an attribute chart. It is based on the fraction of animals above the cut point. It represents samples taken at two-week intervals, 8 cows at each sampling. In this case the herd was having excellent success with high production and few sick cows. The average proportion of cows above the > 0.3 mEq/L cut point was less than 25%. Notice on this chart there is a wide range between the upper and lower control limits. At 8 cows per sample there needs to be a very large change before a single point would be classified as out of control. However, on the average this herd is below the 25% alarm level and except for one point has not varied significantly from that for over a year.

This is another attribute chart. This one from a herd with a high incidence of displaced abomasum and metritis. Samples were taken weekly with 16 animals tested at each sampling. Note that even with 16 animals per sample, the range between control limits is large. The average proportion of cows above the > 0.3 mEq/L cut point is 35%, well above the alarm level designated at 25%.

### Parametric Statistics and Control Charts

The previous charts were attribute charts and based on the proportions of animals above or below a cut point. Cut points are used to dichotomize data for epidemiological evaluation. Dichotomizing continuous data with a cut point has advantages in some applications. However, dichotomizing continuous data results in the loss information that may be gained from parameters such as means, ranges, and standard deviations. Inferences based on these statistical parameters are based on the assumption that the underlying distribution of the data follows a normal, or Gaussian, pattern.
The histogram (bars) portion of the figure on the right shows the frequency distribution of prepartum serum NEFA values from a large number of Michigan dairy cows. This distribution is typical of such NEFA distributions, whether in large or small populations of peripartum cows. The curve overlying the histogram is a normal distribution curve. Note that it does not fit the distribution of NEFA values very well.

This figure shows the distribution of the natural logarithms of the same NEFA values as in the previous graph. Note that they fit a normal distribution much better than did the direct NEFA concentration values. We'll not discuss here the reason for this change in the shape of the distribution pattern, other than to say that by making this transformation we can appropriately make use the statistical inferences inherent in parametric statistics. These inferences are critical to the interpretation of X-bar and range applications of the statistical process control charts.

This is average chart of log transformed NEFA values from a herd with excellent performance. There are two above the upper control limit, indicating a significant increase in mean NEFA concentrations at those points.

Distribution of Blood NEFA Concentrations in a population of 4700 peripartum cows in Michigan

Distribution of Natural Logarithms of Blood NEFA Concentrations in a population of 4700 peripartum cows in Michigan

X-Bar, or Average Chart

Prepartum NEFA concentration from an excellent herd, few sick cows
sample size per point - 8
This is an R, or range chart. Range values are calculated by subtracting the lowest value in each sample group from the highest value. This chart is based on the same data as used to create the Average chart above. Note the range exceeded the upper control limit at the same time point that the average chart value exceeded the control limit. This could possibly be due to the inclusion of a cow carrying twins, a lame cow, or some other specific animal that would suggest that re-evaluation of the mean data was appropriate.

Statistical Process Control Charts

- Historical record of data
- Easy to construct with free add in for Excel
- Easy to interpret
- Easy to present to clients
- Based on sound statistical principles
  — But in themselves do not represent epidemiology

The charts provide a sound statistical interpretation of the data, allowing you to interpret apparent changes as being significant or more likely to be due to random variation. The charts do not provide epidemiological information. We must rely on epidemiological studies to determine the effects of change on disease incidence or other important herd variables.
Considerations for Sample Collection for NEFA Testing

In this experiment cows in negative energy balance (upper line) or positive energy balance (lower line) were fed a TMR diet delivered once per day as indicated by the vertical line. Feed remained in front of the cows continuously. Blood samples were taken hourly. In the negative-energy-balance cows, there was a significant drop in NEFA concentration following the offering of fresh feed. On the average there was still substantial difference between the positive- and negative-energy-balance cows. Serum NEFA concentrations are probably best used as indicators of negative energy balance when sampling is done just before fresh feed is offered.

In this experiment cows were in positive (lower line) or negative (upper line) energy balance. Initially blood samples were taken from the tail vessels when the animals were completely calm and relaxed (time 1). They were then moved from their stalls and worked through a restraint chute. Samples were taken immediately after return to their stalls and for 30-min intervals thereafter. In both the positive- and negative-energy-balance cows NEFA concentrations increased about 0.1 mEq/L (p < 0.05) at thirty minutes after restraint. Excitement should be held to a minimum when collecting samples for NEFA evaluation.

Ketone Bodies

The ketone bodies consist of acetoacetic acid, acetone, and beta-hydroxybutyric acid (BHB). Metabolically they are derived from NEFA, but are also derived from butyric acid as it is absorbed from the rumen. Serum concentrations of BHB in healthy animals in positive energy balance are probably derived primarily from ruminal butyrate. Ketone bodies are normal metabolites that serve a critical purpose in energy homeostasis.
This slide illustrates the metabolic association between adipose mobilization, serum NEFA concentrations and serum BHB concentrations. A large proportion of serum NEFAs are extracted by the liver. In the liver, NEFA may be converted to ketone bodies, but this conversion requires entry into the mitochondria. Non-esterified fatty acids that do not enter the mitochondria may be converted to triglycerides for storage or export from the liver. Entry into the mitochondria is a crucial branch point determining the proportion of NEFA that are converted to ketone bodies.

Glucose availability is a major determinant regulating the entry of NEFA into the mitochondria and thus the distribution of NEFA between ketone body and triglyceride synthesis.

The glucose needs of the cow are supplied primarily from hepatic synthesis. Substrates include propionic acid from rumen fermentation and lactic acid resulting from intestinal absorption of starch bypassing the rumen. The demand for glucose increases dramatically with calving, as can be seen from the figures in the slide at left. The large demand for glucose at the initiation of lactation stresses the cow’s glucose supply mechanism. Prior to calving, serum BHB concentrations are seldom elevated because there is much less stress on the glucose supply mechanism at that time.

- Fetal, placental and maternal requirements in late gestation
  - 1 kg/d
- Requirements for early lactation
  - 2.5 kg/d
    - At approximately 40 kg milk/day
- This is the reason BHB concentrations are seldom elevated prior to calving

This slide illustrates the complex and indirect relationship between energy balance and serum BHB concentrations. Dietary starch increases glucose supply by providing both propionic acid and lactate to the liver. The diagonal lined bars represent serum BHB concentrations while the stippled bars represent calculated energy balance. The cows on the high starch (60:40 concentrate to forage ratio) diet produced more milk and were in greater negative energy balance than the cows on the low starch (40:60 concentrate to forage ratio) diet. Despite the more severe negative energy balance in the cows on the high starch diet, serum BHB was significantly lower than in the low-starch-diet cows, illustrating that the effect of negative energy balance on serum BHB is moderated by glucose availability.

This slide illustrates the relationship of serum NEFA to serum BHB across a large number of postpartum cows. Note that high BHB concentrations are always associated with high NEFA concentrations, but that high NEFA concentrations are not always associated with high BHB. In all likelihood this represents variation in glucose precursor supply; cows with larger supplies of glucose precursors can sustain higher NEFA concentrations without becoming hyperketonemic, relative to cows with lower glucose precursor supplies.

**Conditions Necessary to Cause High βHB**

- Negative energy balance
  - Needed to cause NEFA mobilization
- Low glucose availability
  - Needed to promote NEFA entry into hepatic mitochondria
- Effect on value of pre- versus postpartum βHB samples
  - In general prepartum serum βHB values will be low because there is substantially less glucose demand prepartum.
This table shows the cow-level predictive efficiency of serum BHB relative to postpartum disease. The results are remarkably similar to the predictive efficiency of NEFA, when sampled postpartum. The sensitivity values are slightly higher for NEFA, but the likelihood ratios and areas under the curve are very similar.

Similar to the NEFA results, the effect of serum BHB concentrations on milk production varied by parity with first-lactation animals showing a positive association between serum BHB and milk production while in older animals the opposite association was observed.

Relative to the herd-level application of BHB results, there are differences similar to those observed for NEFA between the New York and Canadian studies. In the New York study postpartum BHB had a much stronger relationship to disease, fertility, and production than in the Canadian study. Prepartum BHB concentrations were not measured in the New York study. A very interesting observation is the negative relationship between prepartum serum BHB and milk production in the Canadian study.
In the New York study, the distribution of herds relative to high BHB concentrations was substantially different, relative to the distribution of high NEFA herds. There were substantially more high NEFA herds compared to high BHB herds.

In my estimation, NEFA generally provides more information than BHB relative to evaluation of transition cow risk, both pre- and postpartum. However, compared to NEFA, BHB has many practical advantages relative to sampling strategy (less effect of excitement, feeding time), the cost of the assay, and the availability of a cow-side test.

This is an extremely practical device that provides immediate results for BHB testing. Tests are done on whole blood, so there is no need for a centrifuge or any other equipment.

- Tests β hydroxybutyric acid in blood
- ~ $75 for meter
- ~ $2 to $4/test strip
- Well documented in cattle
- Very easy to operate
The size of the sampling window relative to parturition is an important consideration in BHB testing. As this slide shows, there is a dramatic drop in the incidence of subclinical ketosis over the first three weeks of lactation. If the sampling window is expanded in order to increase cow numbers in smaller herds, the alarm level should probably be adjusted downward. Perhaps more importantly, if the sampling window is narrowed because of the availability of more cows in large herds, the alarm level should be adjusted upwards.

**Feeding time consideration for BHB sampling**

In animals in positive energy balance and not in early lactation, serum BHB concentrations peak at 2 to 4 hours post feeding. This is apparently due to the absorption of butyric acid that forms rapidly in the rumen after feeding. How this relates to the evaluation of negative energy balance and glucose supply is not clear. It seems reasonable that samples taken just before feeding probably represent the greatest portion of metabolic effect on BHB production. Therefore, when possible, I think BHB samples should be taken just prior to feeding, just as with samples for NEFA testing.

### Prevalence of Subclinical Ketosis in a Normal Population

![Graph showing prevalence of subclinical ketosis](image)

*Prevalence of Subclinical Ketosis in a Normal Population*


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**Practical Herd-Level Application of Serum BHB Testing**

- **What we recommend at MSU DCPAH**
  - For a herd “Snapshot”
    - A one-time sample
  - **Postpartum**
    - Animals between 2 and 21 days in milk
    - Seven or more animals
      - All available if less than seven
    - Cut point - > 1.0 mM
    - Alarm – > 15 %
      - Many well managed herds will have 0% prevalence
Other potential variables for metabolic profile testing

In very early lactation, as illustrated by this graph, dairy cows are generally challenged by negative protein balance that is of similar magnitude to their negative energy balance. Their status relative to labile protein reserves prior to calving may determine how well they can adapt to this negative protein balance, and how it affects their disease resistance and production capacity. Thus some assessment of protein status might be useful in metabolic profile design.

Protein metabolism involves the continual destruction of all body proteins in order to provide a pool of amino acids for protein resynthesis. The relative balance of proteins lost to this amino acid pool compared to those synthesized may affect the body reserves of critical proteins, such as several visceral proteins. This may be particularly critical when amino acids are lost to milk production and potentially to gluconeogenesis.

Serum albumin has been used frequently in dairy herd metabolic profiles as an indicator of protein status. While it is less than ideal for this purpose due to its relatively long half-life and large body pool size, there are currently few alternatives in bovine medicine. Creating tests for bovine prealbumin or retinol binding protein may offer better means of assessing protein status. One alternative is to measure serum vitamin A concentrations, which are a very close reflection of retinol binding protein concentrations.

**Serum Proteins as Indicators of Protein Status**

- **Albumin**
  - Half life ~ 20 days
  - Large body pool
- **Prealbumin (aka transthyretin)**
  - Half life ~ 2 – 3 days
  - Small body pool
  - Best hematological protein status indicator (human)
  - No bovine assay currently
- **Retinol binding protein**
  - Half life ~ 12 hr
  - Very small body pool
  - Concentration closely tied to retinol concentration
  - Dynamics similar to prealbumin
The dark solid line in this graph illustrates the typical dynamics of serum albumin with respect to the lactation cycle in a group of health cows. The depression near calving has been generally observed in numerous experiments.

Serum albumin has not been critically evaluated relative to predicting dairy cow disease. However, it is my clinical impression, as well as of others (R Van Saun, personal communication) that serum concentrations less than 29 to 30 g/L in dry dairy cows are associated with increased disease risk.

I think we need better means of evaluating protein status. This may involve the use of serum proteins other than albumin, or the use of other techniques such as ultrasonic measure of loin eye diameter as a measure of skeletal muscle depletion. The situation is complicated by the role that inflammation plays in modifying the concentrations and relationships of serum proteins.

The role of inflammation in modifying the serum concentrations of numerous proteins, including albumin, is well known. The serum concentrations of positive acute phase proteins increase while those of negative acute phase proteins decrease under the influence inflammatory mediators such as TNFα and IL6. What is more recently understood is the role that metabolism and metabolic disease may play in release of inflammatory mediators.

Role of Inflammation in Modifying Serum Protein Concentrations
- Inflammatory cytokines influence hepatic production of serum proteins
  - Positive acute phase reactants
    - Haptoglobin, ceruloplasmin, Alpha 2-macroglobulin
  - Negative acute phase reactants
    - Transferrin, retinol binding protein, albumin
- Thus, inflammatory signals may influence serum protein concentrations, complicating their implications in predicting protein status.
There appears to be a complex interrelationship between adipose tissue and inflammatory mediators of the body. This relationship is such that inflammatory events of any kind may enhance lipid mobilization. In addition, it is coming to light that adipose mobilization and large adipose mass (obesity) may in themselves enhance and augment the inflammatory response. This makes the evaluation of serum proteins as metabolic profile variables challenging. Multivariable parameters may aid in sorting out this complexity.

Adipose tissue contains a substantial number of inflammatory cells, mostly macrophages. The numbers of macrophages increase with increasing adipose tissue mass and with stimulation of adipose mobilization. This is established in rodents and work is currently in progress to investigate these phenomena in cattle. Furthermore, the macrophages in adipose, under stimulation of adipose mobilization, change in phenotype from the more quiescent M2 cells to the more inflammatory M1 cells. These cells release inflammatory cytokines that can enhance the damaging effects of infectious disease, and may promote the mobilization of adipose fat.
The dark solid line in this slide shows in a group of healthy dairy cows the pattern of serum haptoglobin, a positive acute phase protein, over the period of late gestation and early lactation. The spike in serum haptoglobin at calving is expected, but its degree appears related to disease risk. Cattle with both metabolic as well as infectious diseases will have exacerbated peripartum haptoglobin responses. Haptoglobin is a marker of inflammatory response and the extent of its increase in the serum of peripartum cows is related to the degree of inflammatory stimulation, which may be from infectious or metabolic events.

**Multivariate Parameters in Blood Profiling**

The complex association of metabolic and inflammatory events in early lactation dairy cattle creates a challenge in the design and interpretation of metabolic profiles. Multivariate parameters that combine several blood components into a single variable may be developed to aid in interpreting this complexity. For example, some function based on the combination of values from both positive and negative acute phase proteins may aid describing the extent of protein depletion relative to the extent of inflammatory stimulation.

**Urea**

- Serum urea
  - Measured as urea nitrogen (US)
  - Measured as urea (Europe)
- Reflection of rumen ammonia concentration
  - Which is intern a reflection of the relative concentrations of rumen available energy and rumen available nitrogen
This chart shows the variation in serum urea nitrogen concentrations in four groups of cattle fed extremely divergent rations (Herdt and Stevens 1981). Diet 1 was high protein (17% CP)-low energy (1.3 Mcal NEI/kg), diet 2 was high protein-high energy (1.8 Mcal NEI/kg), diet 3 was low protein (10% CP)-low energy, and diet 4 low protein-high starch. The animals were fed twice per day at the time points indicated by the vertical lines. Blood samples were taken at hourly intervals over the day. The effect of high protein is dramatic and the effect of rumen available energy with in the high protein diets is dramatic. Feeding time variation is minimal except in the high protein-low energy diet.

### Urea

- Probably underutilized relative to adjusting dietary protein characteristics
- No reason to think serum values are preferable to milk
  - Dry cow values, however, could be important
- There has been perhaps too much emphasis on the reproductive aspects of serum urea concentrations and not enough on the nutritional aspects

### Serum Calcium

In the US there is considerable interest currently in monitoring serum calcium concentrations in a profiling approach. Serum concentrations measured within 24 hr after calving indicate the cow’s capacity to mobilize calcium reserves in the support of lactation and thus, her metabolic resistance to milk fever and subclinical hypocalcemia. Concentrations measured outside of that time window are difficult to interpret.
In one study (Chapinal, Leblanc et al. 2012) serum calcium concentrations were evaluated relative to herd-level outcomes. These investigators did find a significant herd-level effect of serum calcium concentration on health, reproductive, and production responses. This was true for calcium concentrations measured either in the week before calving or the week afterwards. These are important observations that warrant further observation. However, I think it important to consider that the cut point used in this study may need to be adjusted within the week-long sampling windows of this study.

### Serum Calcium

<table>
<thead>
<tr>
<th>Time Window</th>
<th>Cut point</th>
<th>Alarm level</th>
<th>Effect</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week -1</td>
<td>&lt; 2.1 mM</td>
<td>&gt;5%</td>
<td>Reproduction</td>
<td>0.04</td>
</tr>
<tr>
<td>Week +1</td>
<td>&lt; 2.1 mM</td>
<td>&gt;35%</td>
<td>DA</td>
<td>0.003</td>
</tr>
<tr>
<td>Week +1</td>
<td>&lt; 2.1 mM</td>
<td>&gt;15%</td>
<td>Milk Production</td>
<td>0.01</td>
</tr>
</tbody>
</table>

From N Chapinal et al., Journal of Dairy Science, 95(10): 5676, 2012

**Distribution within time window difficult to determine**

### Serum Calcium – Appropriate Cut Point

- Seems to keep getting revised upward in the US
  - 1.9 mM, 2.0 mM, 2.1 mM
- I think 2.1 mM may be unrealistic
- Needs at the least to be adjusted for parity

This chart shows herd-adjusted parity effects on serum calcium concentrations within 24 hr of calving. The results are from approximately 80 US dairy herds tested at DCPAH. Note the apparent linear decrease in calcium over parity, especially over the first three parities. I think the implication is that, at least through parity 4, the hypocalcemia cut point may need to be adjusted for parity.
The Future of Nutritional Assessment by Technological Means

Before discussing the future of blood variables and metabolic profiling as components of nutritional assessment, I’d like to comment on nutritional assessment in general. I think the future in this area is bright. As herd sizes increase and more animal husbandry is provided by a labor force less experienced than in years past, the need to monitor cows by technological means will increase. Furthermore, increasing herd sizes will make management and nutritional errors more costly as they are multiplied by increasing numbers of animals. Thus, making increased monitoring more critical for avoiding errors, or catching them before they have clinical consequences.

Variables for tracking could include such things as manure analysis, milk analysis, rumination monitoring, and many other possibilities. Collection of data will need to be easily automated and interpretations will need to be clear.

Nutritionists, as well as veterinarians, will need to be willing to modify diets and management strategies in accordance with assessment findings. We have to be willing to listen to the cows more than the computers. This said, there must be a means to track the outcomes of decisions made based on nutritional assessment techniques.

What is the future of blood analysis in the framework of nutritional assessment?

Blood analysis is potentially valuable in some situations, but probably not as a “first line,” routine technique. Blood sampling will always be costly and difficult, if not impossible to automate. Inexpensive tests, and especially rapid cow-side tests that can be performed by personnel with minimal training will be important. For those tests that must be sent to an off-farm laboratory, prices must be in line with value. For those assays amenable to automated laboratory assays there may be excess instrument capacity that could be creatively applied to economically serve agriculture as well as increase the profitability of the laboratories themselves.

Most of all, blood tests will need to provide clearly interpretable information that is not obtainable by other means.

References


