

SLIDE NOTES FOR "CONTEMPORARY ISSUES IN FETAL LUNG MATURITY TESTING"

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Slide 4

The development of the human lung is a highly regulated and coordinated process that begins in the 3-week-old embryo with the creation of two lung buds during the embryonic phase. During the pseudoglandular phase these buds undergo successive rounds of branching morphogenesis to produce the lung lobes: three on the right and two on the left. Following branching morphogenesis, the canalicular phase is characterized by increased angiogenesis and the appearance of type I and II pneumocytes that will permit gas exchange. In the final stages of development, the fetal lung is prepared for its function as a gas exchange organ in an atmospheric environment. The most important of these saccular phase events is the production of surfactant that functions to decrease the surface tension at the alveolar air-liquid interface. Lung maturation is indicated by the appearance of fully mature alveoli during the final alveolar phase although new alveoli will continue to form for approximately three years.

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The production of pulmonary surfactant marks a critical point in lung development. Due to a hydrated inner layer, the surface tension of the alveoli is high which promotes their collapse. Pulmonary surfactants decrease this surface tension so that alveoli remain open upon exhalation and increases lung compliance.

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Surfactant is synthesized in the rough endoplasmic reticulum (RER) of the alveolar type II cell and is packaged into lamellar bodies, which are subsequently secreted by exocytosis. The secreted lamellar body unfolds to form tubular myelin (TM) that produces a phospholipid surface film at the air-liquid interface with the hydrophilic heads in the aqueous phase and the hydrophobic tails in the air phase. During the respiratory cycle as the film is compressed during exhalation, the film pressure rises, and a

compressed, closely packed monolayer of nearly pure DPPC is formed. Some phospholipids are excluded from the monolayer and form small aggregates that are ingested by macrophages or endocytosed and reprocessed by alveolar type II cells.

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Surfactant is primarily composed of phospholipids, the majority being phosphatidylcholine (lecithin), the critical component of surfactant and the one that contributes the most to the low surface tension. Because most of the PC has the saturated fatty acid palmitate in both acyl positions it is more accurately described as dipalmitoylphosphatidylcholine or DPPC. Additional components of surfactant include neutral lipids (cholesterol and fatty acids) and protein.

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As shown earlier, surfactant production begins at approximately 30 weeks. PC (lecithin) and PI appear at the same time but the concentration of PI peaks at 35 weeks while lecithin continues to increase to term. Notably, PG is the last surfactant to be synthesized which begins at approximately 35 weeks.

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Respiratory distress syndrome (RDS) is defined as respiratory distress that occurs in the newborn within the first few hours of life. It is also known as hyaline membrane disease and it is caused by a deficiency in pulmonary surfactant. It is the most common cause of respiratory failure in neonates and occurs with increasing frequency with decreased gestational age such that it is very common in infants born before 30 weeks and very rare in term infants. It's estimated that approximately 20,000 infants develop RDS each year in the United States.

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The pathophysiology of RDS is a vicious circle that begins with reduced pulmonary surfactant. This results in collapsed alveoli that are perfused but unventilated. This leads to hypoxia, hypercapnia, and a respiratory acidosis; conditions that produce vasoconstriction of pulmonary arteries and decreased pulmonary blood flow. The

resulting ischemic injury to the lung creates a secondary surfactant deficiency that exacerbates the disease. In addition, the pulmonary vasoconstriction causes epithelial cell damage. Plasma leaks into alveoli leading to the accumulation of fibrin which, together with necrotic cells, creates a hyaline membrane for which RDS was formerly named.

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Among risk factors for RDS, prematurity is the obviously the most important, especially in infants born at less than 37 completed weeks. There is a higher male-to-female ratio in incidence, morbidity, and mortality, which may be related to differences in sex steroids (testosterone decreases and estrogen increases phospholipid synthesis). Caucasian infants are at increased risk for unknown reasons. RDS is more common in C-sections as labor is known to increase fetal surfactant production. The second born infant of a twin gestation is at higher risk that is likely due to longer exposure to acute perinatal stress. And a history of RDS is a poorly understood risk factor and suggests a genetic predisposition.

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Signs of RDS usually appear within minutes of birth with characteristic tachypnea, prominent (often audible) grunting, nasal flaring, retractions, and cyanosis that is unresponsive to oxygen administration. The natural course of RDS is characterized by progressive worsening of cyanosis and shortness of breath. If inadequately treated apnea and irregular respirations occur as the infant tires.

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Preventing premature birth is the most effective way to prevent RDS. Alternatively, administration of steroids to the mother can be used to accelerate surfactant production. Treatment of neonates immediately after birth with exogenous surfactant delivered endotracheally can be effective if early delivery cannot be prevented. Surfactant replacement therapy has been credited with the largest drop of infant mortality in 25 years and they are approved for use for both prophylaxis in at-risk infants and as

treatment for RDS in effected newborns. Continuous positive airway pressure (CPAP) may also be used to deliver a stream of compressed air to the lungs.

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As shown on the left, RDS was responsible for 4.5% of all infant deaths in 1997 and was the 4th leading cause of infant death in the US. In 2005 that had decreased to 3% of all infant deaths so now RDS is the 7th leading cause of infant death. To give present a more global perspective, data compiled from the WHO is presented on the right.

The top figure shows the 10 countries with the highest infant mortality due to RDS and the bottom figures shows infant mortality due to RDS in the US, Canada, and the UK. The differences in RDS deaths in the US and the UK are curious because FLM tests are not often performed in the UK or throughout Europe. Instead of performing testing for fetal lung maturity, it is common practice to administer antenatal steroids and/or pulmonary surfactants to at-risk infants thereby making FLM testing unnecessary.

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Because fetal lung liquids contribute to amniotic fluid, the amount of surfactant in fetal lungs can be estimated by measuring the amount of surfactants in amniotic fluid. If they are to be performed then current recommendations call for evaluating lung maturity between 32 and 39 weeks of gestation. The purpose of testing is for appropriate decision making regarding delivery. To be clinically useful, tests for fetal lung maturity should have a high diagnostic sensitivity for immaturity and a high negative (mature) predictive value. As with all diagnostic tests, maximizing sensitivity is achieved by sacrificing specificity. As such, many infants with immature fetal lung maturity test results are born without RDS.

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This slide shows the history of FLM tests. Over a period of almost 20 years, several tests were developed. Only 7 are shown here because at one time or another, these were widely used in clinical laboratories. Some of them, like the lecithin/sphingomyelin (L/S) ratio and the surfactant/albumin (S/A) ratio are biochemical tests designed to quantify

the amount of surfactant in amniotic fluid while others, like the OD650 and foam stability tests, exploited the biophysical properties of surfactant. Of the 7 shown here, 6 appear to still be in use in the US.

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The figure on the left was compiled from recent CAP survey data. The most commonly used FLM test is the commercially available S/A ratio followed by detection of PG by agglutination, the L/S ratio, and then the detection of PG by TLC. A survey of laboratorians that I conducted earlier this year with Dr. Ann Gronowski at Washington University School of Medicine (St. Louis, MO) produced data consistent with the CAP survey data but it also shows that the lamellar body count is used by about 10% of laboratories and, interestingly, that the foam stability test is still in use in a small number of laboratories. These two tests aren't reflected in the CAP data as the CAP does not offer PT for these tests. Additional details about each of these tests follow.

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The S/A ratio is determined using fluorescence polarization. When added to amniotic fluid, a fluorophore partitions between surfactant and albumin. Polarized light is used to excite the fluorophore and the net decrease in polarization is measured. The change in polarization is a function of how rapidly the fluorophore is rotating and this is a function of the molecular masses of surfactant and albumin molecules.

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In a specimen with low amounts of surfactant relative to albumin, the net polarization remains high because rotation of the albumin molecules (with their greater molecular mass) is slower than the rotation of the surfactant molecules which have a much lower molecular mass and therefore rotate more rapidly.

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In contrast, in a specimen with high amounts of surfactant relative to albumin, the net polarization is low.

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The S/A ratio has many advantages. It has a high sensitivity for immaturity, it is rapid and precise, the result is quantitative, and it's essentially standardized because the test is commercially available from only one vendor. Commercial QC materials and PT challenges are also available. However, the test is affected by blood and meconium contamination and there is a wide, diagnostic grey zone for results that are between 40 and 54 mg/g which makes results in that range a challenge to interpret. Because the test is available from a single provider, laboratories that offer the test have limited options for alternative ways of determining the S/A ratio although ~9 US laboratories do perform a "home brew" version of this test.

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The S/A ratio has high sensitivity for predicting immature lungs and, therefore, has a high predictive value for maturity but it is not very specific and does a poor job at predicting immaturity.

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Because the risk of RDS decreases with increasing gestational age, the use of a single cutoff for maturity has limited utility. A few studies have confirmed that incorporating gestational age with the S/A ratio result improves the performance of the test.

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This table is from a report that combined data from the 3 largest outcome-based studies of the S/A ratio. The researchers used logistic regression analysis to model the odds of RDS as a function of the two variables and generated this table of relative risk. The risks are shown relative to that of a 37 week gestation (considered term) and the median S/A ratio at that age (70 mg/g).

You can appreciate the value of the table by looking at the relative risk of RDS for a 34 week old fetus with an S/A ratio result of 70 mg/g and see that, despite the result being mature, the infant would have a 3-fold greater risk of developing RDS. Conversely, a fetus at 34 weeks with an S/A result of 90 mg/g would be at decreased risk.

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The qualitative detection of PG can be accomplished using a commercially available test and is the second most widely used test after the S/A ratio. Equal volumes of amniotic fluid and a lecithin/cholesterol reagent are mixed together and combined with anti-PG antibody reagent. Agglutination is determined by visible inspection and indicates the presence of PG. Results for this test are reported as either "negative" (immature) or "low" or "high" positive (mature). Note that this nomenclature is opposite that used for other tests of fetal lung maturity (with positive meaning immature). A low positive result is one that produces small agglutinates with definite background clearing while a high positive result consists of obvious agglutinates of varying size with a distinctly clear background. Interpretations are facilitated by the inclusion of a negative, a low positive (0.5 µg/mL of PG) and a high positive (2.0 µg/mL of PG) control specimen for comparison with the test sample.

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This test has a high sensitivity for maturity and it's quick and simple to perform. It's unique among FLM tests in that it is unaffected by the presence of blood or meconium contamination and commercial QC and PT are available. However, PG is a late marker of pulmonary maturity which limits its utility as a lung maturity test and the interpretation of the qualitative result can be subjective. It too is available from a single vendor and supply issues can be problematic.

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Like the S/A ratio, the detection of PG is sensitive and has a high mature predictive value but it is not specific and has a low immature predictive value.

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The L/S ratio describes the relative change in the concentration of lecithin to that of sphingomyelin in amniotic fluid as determined by thin-layer chromatography (TLC). Although low, the concentration of amniotic fluid sphingomyelin remains largely constant throughout the last trimester of pregnancy and therefore serves as an internal standard

against which the concentration of lecithin can be compared. Because it was the first test of fetal lung maturity, it became the de facto gold standard and everything that came after it was compared to it. However, some consider it to be an undeserving gold standard and, when based on outcome, other FLM tests perform as well or better than the L/S ratio. Despite this, the L/S ratio maintains its status in the minds of many physicians as the “best test” for assessing lung maturity.

Among all the FLM tests, the L/S ratio is the most difficult to perform and requires considerable skill and expertise. The surfactants in the specimen are first extracted using methanol and chloroform which are then concentrated by dehydration and then spotted onto a silica gel TLC plate. After a 90 minute separation, the phospholipids are visualized with cupric acetate and charring and then quantified by scanning densitometry.

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Like the other FLM tests, the L/S ratio has high sensitivity for immaturity and commercial sources of QC and PT are available. As indicated in the previous slide, the test is time consuming, is technically difficult to perform, and is notoriously imprecise. It does require a larger volume of specimen than the other FLM tests and it, too, is affected by the presence of blood and meconium. Like the S/A ratio, there is a wide diagnostic grey zone that can be difficult to interpret.

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As with all FLM tests, the L/S ratio is sensitive but not specific and has a high mature predictive value and a low immature predictive value. Interestingly, despite it being considered the gold standard test, its performance is actually less robust than many of the other FLM tests.

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In 1979, 8 years after the introduction of the L/S ratio, the qualitative detection of PG by TLC (as part of the L/S ratio) was described using a modification of the L/S ratio from a 1-dimensional procedure to a 2-dimensional one. However, of the labs that still perform this test, 80% utilize the 1-dimensional method of the L/S ratio and report the presence

or absence of the PG band. The test was developed in an effort to improve the poor specificity of the L/S ratio by decreasing the number of "false immature" results with a mature result being one that had an L/S ratio greater than the established maturity cutoff and a PG that was >2% of the total phospholipid content.

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The most recent FLM test developed was the lamellar body count (LBC) in 1989 when it was noted that lamellar bodies were similar in size to blood platelets and so could be enumerated using an automated cell counter.

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Numerous outcome-based studies have been published investigating the clinical utility of this method using the Coulter brand of cell counters and some of the larger studies using these types of cell counters are shown here. Studies are separated into those that did not centrifuge specimens and those that did. Centrifugation of specimens before testing has been shown to be unnecessary and is no longer recommended. The LBC is a sensitive test for immaturity but is not very specific and so the predictive value of mature results is high but not those of immature results.

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The LBC is sensitive for immaturity and is quick and easy to perform on instruments (automated cell counters) that are widely available and requires only a small volume of specimen. Unfortunately, the test is affected by blood and meconium and it's considered a lab developed test which some may consider a disadvantage. There are no commercial sources of QC or PT materials for the LBC although the CAP will introduce a PT challenge for the LBC in 2010. Importantly, due to differences in counting methods, the same maturity cutoff cannot be used across all brands of automated cell counters.

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As mentioned previously, consensus guidelines for the LBC were published several years ago. Some of the key points are shown here. The consensus was that centrifugation of specimens should not be performed and, furthermore, cutoff counts were

recommended. However, these cutoffs did not consider possible differences in counts obtained from cell counters other than those manufactured by Coulter.

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The influence of cell counter on the LBC has been investigated by Dr. Gronowski and colleagues. They compared the Sysmex XE-2100, Advia 120 and Cell-dyn 3500 hematology counters to the Coulter GenS counter. In the figure, the panels on the left show a concordance plot with results from Coulter on the x-axis and the comparative method on the y-axis. The two vertical and horizontal lines represent the consensus cutoffs of 15,000 and 50,000/uL. The panels on the right are Bland-Altman plots of the concordance data.

The Sysmex counter compares very favorably to the Coulter method with an overall concordance of 86% and, similarly, the Advia also compares fairly well to the Coulter with a concordance of 78%. However, counts from the ADVIA appear to plateau at ~50,000/uL and so further comparisons are needed to know if different cutoffs are needed for that instrument. The Cell-dyn was only 66% concordant with the Coulter counter with clear differences as the LBC increases suggesting that a higher cutoff for maturity (possibly 80,000/uL) would be needed for LBCs determined on this instrument.

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Multiple gestations account for 1-2% of all US deliveries but account for 10% of NICU admissions, largely because of premature delivery. However, data regarding FLM testing in twin pregnancies is sparse and mixed. Studies have reported that S/A ratio values in twin pregnancies are higher compared with those in singleton pregnancies at similar gestations whereas other have reported no difference. Discordant values appear to occur more frequently at earlier gestational ages but many of these reports used the L/S ratio, which, because of its high imprecision, may have contributed to the varying results in concordance. Still, it is suggested that amniocentesis of both twins be performed when the gestation is <32 weeks of gestation and FLM testing is required.

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In 1996, the ACOG recommended a sequential approach to FLM testing. Because a mature result from any test was strongly predictive of lung maturity, little additional information was to be gained by the performance of multiple assays.

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Last year, the ACOG updated its practice bulletin on FLM.

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While the new guideline acknowledges the high mature predictive value of all FLM tests, it no longer specifically mentioned the sequential testing approach and instead suggests that an FLM test be utilized on the basis of specimen quality. That is, the test(s) performed should be selected based on the presence or absence of contaminants such as blood and meconium. This doesn't mean a sequential testing approach isn't valuable and it remains a sensible approach given that many labs offer a variety of FLM tests and that there is an increasing need for appropriate utilization of laboratory resources.

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In January of this year, the *New England Journal of Medicine* published an article about the timing of elective repeat C-sections and neonatal outcome (NEJM 2009;360:111-20). The results clearly demonstrated that delivery at <39 weeks of gestation was associated with adverse respiratory outcomes and they concluded that elective delivery before 39 weeks be discouraged unless FLM was demonstrated. This is consistent with the current ACOG guidelines that a scheduled delivery at <39 weeks not be performed unless FLM is confirmed.

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Two years ago, investigators at the University of Mississippi Medical Center published a paper (J Perinatology 2007;28:20-3) that noted a decrease in the number of FLM tests that were being performed in their laboratory. In response to this, they surveyed members of the Society for Maternal Fetal Medicine regarding their use of FLM tests and 60% of them commented that their use had declined. When asked why they were

ordering fewer tests, the most common response was that the tests were not needed for patient care.

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Intrigued by that report, Dr. Gronowski and I surveyed laboratorians to find out if other labs were seeing similar trends. More than 6,000 individuals were invited to take the survey and 4.1% responded. Of these 4%, 2% provided their FLM test volume data.

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These data did indicate a slight decrease in the number of FLM tests being performed each year in US laboratories which was significant when adjusted for the number of FLM tests performed per site.

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We also decided to survey Fellows of the Society for Maternal Fetal Medicine to get additional information about their use and opinions of FLM tests. 3.2% of individuals invited to take the survey responded.

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When asked about the frequency with which they ordered FLM tests, only 26% indicated their use of FLM tests was decreasing with most indicating that there had been no change in overall use. For those who did indicate decreased utilization, 82% cited that the results were not needed for patient care.

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However, when asked if they could provide their current level of care without the benefit of ANY FLM test results, only 9% responded that they could.

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Consistent with ACOG guidelines, most physicians do not order FLM tests before 32 or after 39 weeks of gestation and FLM tests are either always or sometimes ordered more than 50% of the time between 34 and 39 weeks.

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When asked their impressions of the clinical performance of the various FLM tests, all but the foam stability test were considered excellent or good by more than 50% of respondents.

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Last year, Abbott Diagnostics announced that it was going to retire its legacy systems, including the TDx instrument that performs the S/A ratio. While many of the assays performed on these instruments have been moved to other Abbott platforms, there has been no formal announcement by Abbott regarding the fate of the S/A ratio, however they have committed to providing its customers a minimum of 12 months notice before retiring the test. Labs that currently offer the S/A ratio will need to consider how this change will impact their FLM test menu.

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If the S/A ratio is the only FLM test currently offered, then labs may opt to send FLM tests to a referral lab. However, results from our survey of SMFM Fellows indicated that most physicians prefer to have FLM test results within 12 hours of ordering the test and this turn-around time may be difficult to achieve if using a referral laboratory.

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Labs may opt to offer an alternate FLM test, particularly if the S/A ratio is currently the only FLM test they provide. In that case, it would be a good idea to include physician users of FLM tests in the discussions of possible replacement tests. Fellows of the SMFM indicated more frequently that they would prefer either the L/S ratio or the LBC over the other test options if the S/A were not available.

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There are some notable caveats to consider when choosing possible replacement tests. Due to the technical expertise required to perform the test well, laboratories should not offer the L/S ratio unless 15 or more specimens per week are tested. As a replacement

test, the detection of PG isn't as robust as the S/A ratio because it is a late marker of maturity and it's a qualitative result. There has also been a history of reagent supply issues for the rapid agglutination test. The foam stability test is not commercially available and needs to be developed by the laboratory. Furthermore, the test requires the use of absolute ethanol that absorbs moisture easily which can compromise test performance and the results are not quantitative. The LBC is also a lab developed test but the instrumentation to perform it is readily available and requires minimal technical expertise to perform correctly.

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In summary, RDS is a serious problem in premature infants but its incidence is decreasing steadily. All currently available FLM tests are excellent predictors of lung maturity but not of lung immaturity. While the overall frequency of FLM testing appears to be on the decline, FLM tests are not likely to become obsolete any time soon. The potential loss of the widely used S/A ratio means that many laboratories will need to offer a replacement FLM test. Of the possible replacement tests, the LBC is an easy and rapid test to perform but, as a lab developed test, it requires a thorough validation with special attention paid to cutoff values for maturity.