

Molecular Genotyping of the Rhesus D Gene in Fetal DNA Extracted from Maternal Plasma

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Determining Fetal RhD Blood Group

- Prediction from parental phenotypes.
- Direct serological phenotyping can be performed from fetal red blood cells obtained from procedures such as Percutaneous Umbilical Blood sampling (PUBs).
- Fetal RhD phenotype can be predicted from the *RHD* genotype of the fetus.

Benefits of *RHD* Fetal Genotyping

- Enables non-invasive prediction of the RhD phenotype of the fetus. This is particularly important in RhD negative antenatal patients who have anti-D present at a level associated with HDN, as a result of sensitization during a previous pregnancy.
- Large scale screening of all RhD negative antenatal patients to enable appropriate use of Routine Antenatal Anti-D Prophylaxis (RADDP).
 - Human Blood Product
 - Cost (£23/500 IU)

Project Aims

- To develop a method of extracting fetal DNA from maternal plasma and amplifying the *RHD* gene.
- To establish the sensitivity and specificity of the method.
- To establish the stage during gestation when the method was most accurate.
- To determine the time of sample separation when the method was most accurate.

The *RHD* and *RHCE* genes

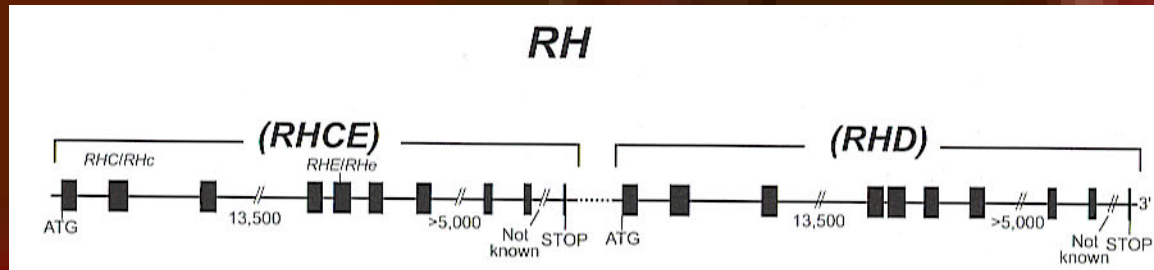


Diagram taken from Denomme, G.A. et al (2000)

- The Rh blood group antigens are derived from two genes, *RHD* and *RHCE*.
- *RHD* and *RHCE* are highly homologous with each other, both consisting of 10 exons which produce a polypeptide chain of 417 amino acids, with only 8.4% divergence from each other.
- Great care needs to be taken when designing the method that *RHD* is amplified not *RHCE*.

The *RHD* Gene continued

- In Caucasians approx 15% of the population are RhD negative, the vast majority of which are due to a complete deletion of *RHD*.
- However, *RHD* has other complexities. An important example of this is the *RHD* pseudogene (*RHD* ψ) = *RHD* positive genotype but an RhD negative phenotype.

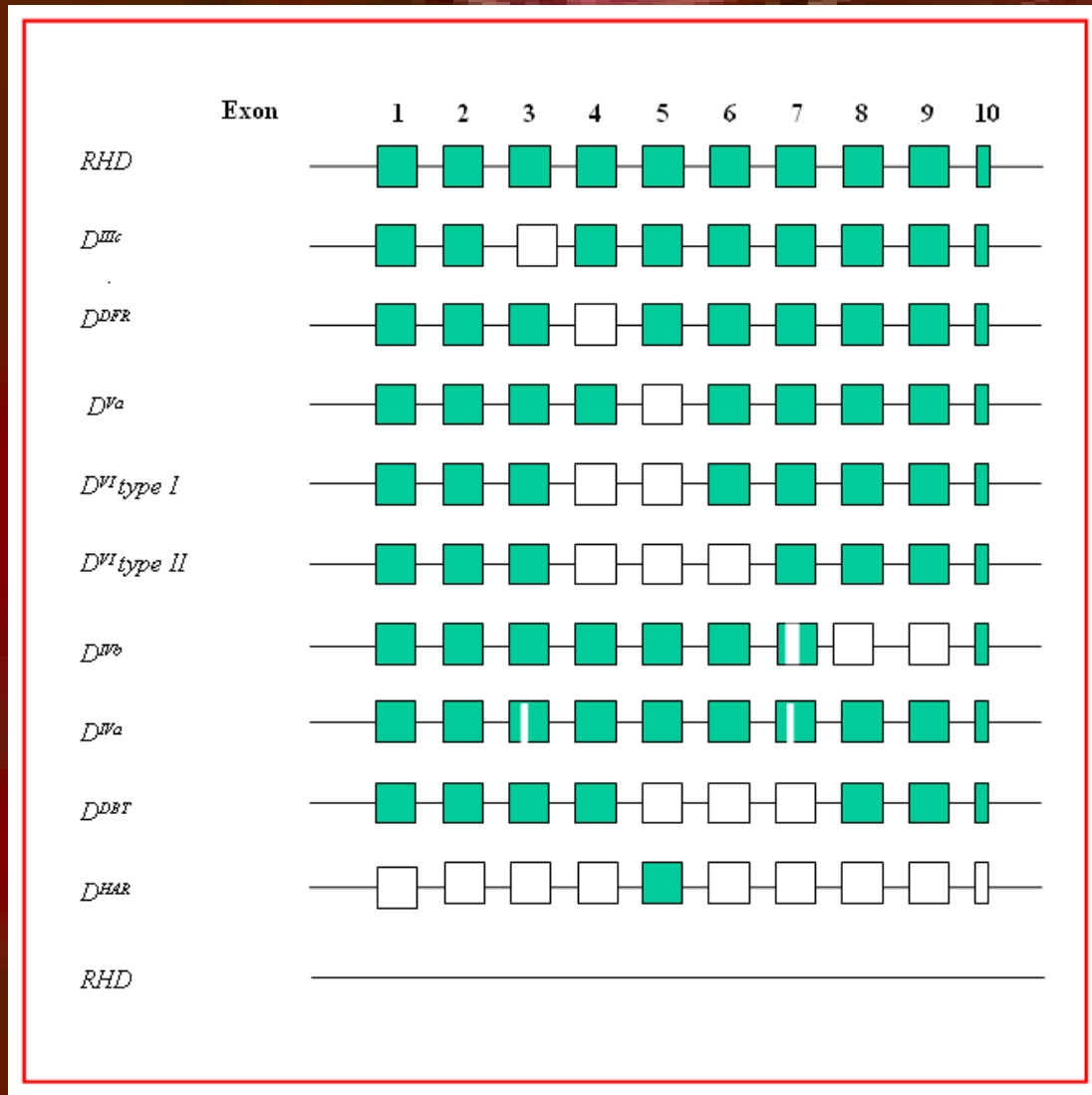
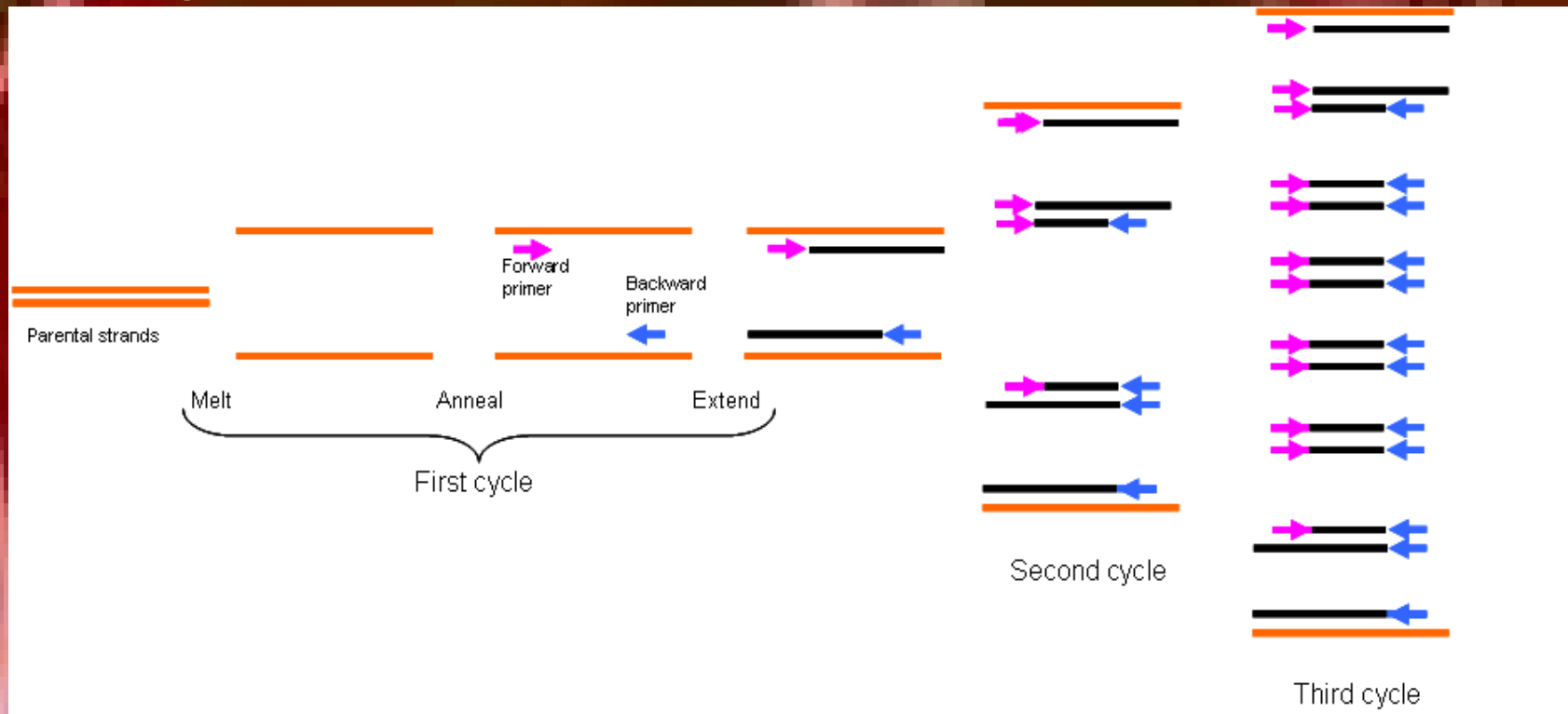


Diagram adapted from Maaskant-van Wijk et al (1998)

The *RHD* Gene continued

- *RHD* gene is very complex and is associated with a large number of variations.
- To avoid errors in genotyping multiple areas must be amplified.
- We selected five areas of the gene to look at: exon 4, exon 5, two sections of exon 7 and exon 10 to cover as many different D variant types as possible.

Polymerase Chain Reaction (PCR)



Due to the very low concentration of fetal DNA in the maternal plasma, amplification of the fetal DNA is necessary before an accurate Rh genotype can be determined.

Control Issues....

- Include an ubiquitous internal positive control for the presence of DNA, the Human Growth Hormone (HGH). This indicates that DNA of any source is present and is NOT a specific control for fetal DNA.
- If the *RHD* gene is amplified, this acts as a positive control for fetal DNA as all antenatal patients were RhD negative.
- For male fetuses (regardless of Rh genotype) we amplified the Y chromosome specific gene *SRY*, acts as a positive control for fetal DNA in approx 50% of cases i.e. where the fetus is male.
- For female fetuses which are RhD negative..... Problem! but future resolution.

Sources of Fetal DNA

- Invasive Procedures
 - Amniocentesis
 - Percutaneous Umbilical Blood Sampling (PUBs)
- Non-Invasive Procedures
 - Nucleated Fetal Cells in Maternal Circulation
 - Cell-Free Fetal DNA in Maternal Plasma and Serum

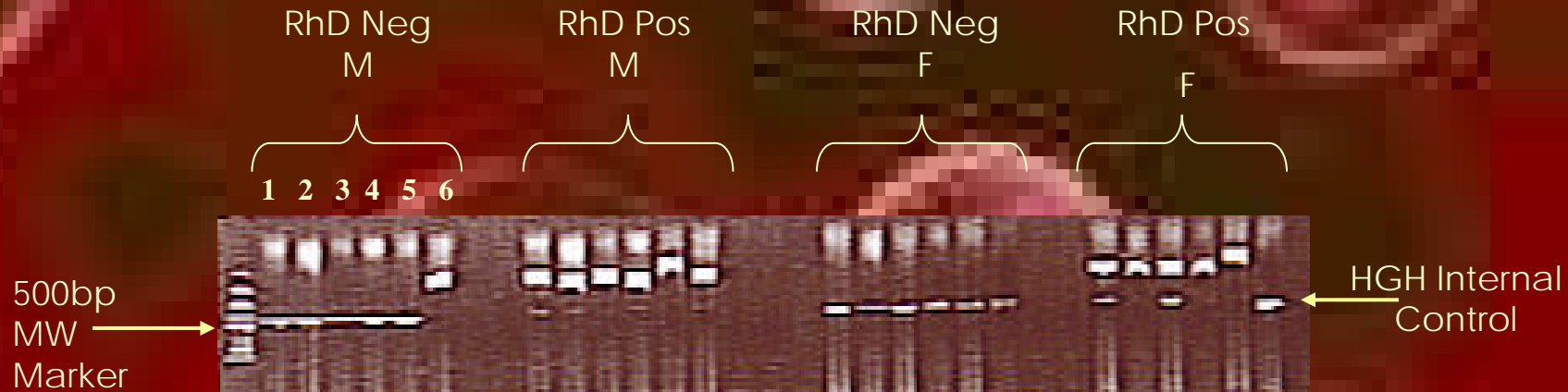
Sample Recruitment

- Patient information packs were sent out to RhD negative antenatal patients between 12-14 wks. All patients were vetted by head midwife to ensure there were no complications in the pregnancy.
- If the patient decided to take part they would bring the consent form along to their next antenatal appointment and consent would be taken by the midwife & an extra 5ml EDTA sample bled.
- Samples sent to Welsh Blood Service and plasma separated from buffy coat and frozen.

Results

- 154 Patient Information packs sent out by Welsh Blood Service to RhD negative antenatal patients.
- 34 RhD negative antenatal patients consented to take part in the study (22% uptake).
- Of the 34 participants 12 (35%) gave two separate samples at different stages during gestation.
- All samples were tested in duplicate by the PCR method described.
- On delivery of the baby the RhD phenotype and sex were requested from the hospital to compare with the genotyping results.

Results



- Lane 1 = *RHD* exon 4
- Lane 2 = *RHD* exon 5
- Lane 3 = *RHD* exon 7.1
- Lane 4 = *RHD* exon 7.2
- Lane 5 = *RHD* exon 10
- Lane 6 = *SRY*

What constitutes a RhD Positive genotype...

- Not all five exons were amplified on every occasion. It was therefore necessary to establish a minimum number of positive reactions required to report a Rh D positive genotype.
- False negatives are clinically more of a concern than false positives and with this in mind it was decided to set the minimum number of reactions required to 2.
i.e. samples having 2 positive reactions from the 5 exons tested are assigned a Rh D Positive genotype.
- This was applied to all the samples in the study:
 - 24/27 RhD positives genotyped correctly
 - 13/14 RhD negatives genotyped correctly

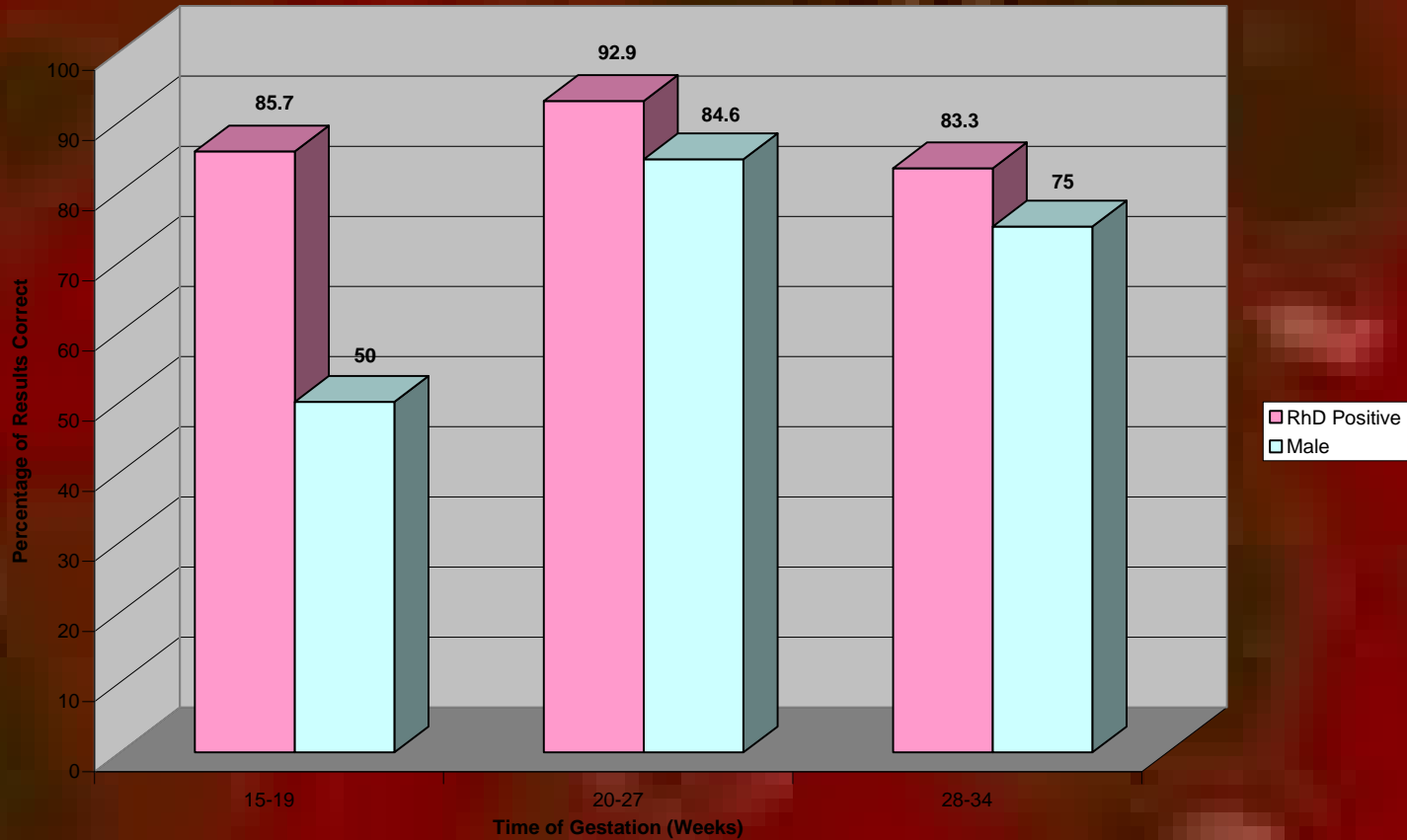
Accuracy of determining the Sex of the Baby

- Out of the 46 samples tested:
 - 19 delivered male fetuses
 - 25 delivered female fetuses
 - Unable to obtain a result for one
- Out of these, 15/19 male samples genotyped correctly and 24/25 female samples genotyped correctly.

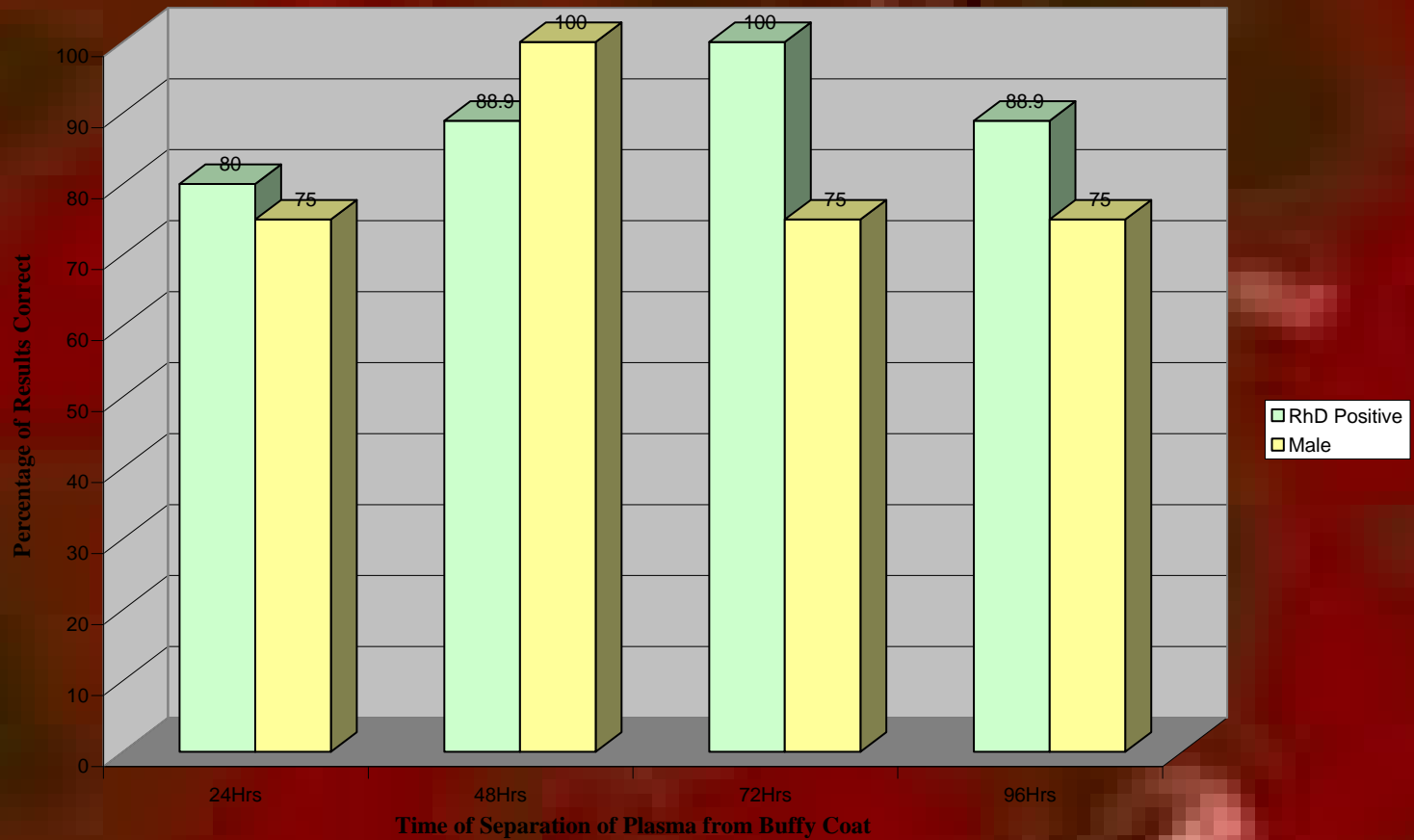
The Effect of Gestational Age & Time of Sample Separation on the Accuracy of Fetal Genotyping

- All RhD positive samples and Male baby samples in the study were split into three groups for gestational age:
 - 15-19 weeks gestation
 - 20-27 weeks gestation
 - 28-34 weeks gestationand five groups for time of separation: 24Hrs, 48Hrs, 72 Hrs, 96Hrs and 120Hrs.
- It was expected that there would be an increase in the accuracy the later in the pregnancy the sample was taken and the earlier the plasma was separated from the buffy coat, But!

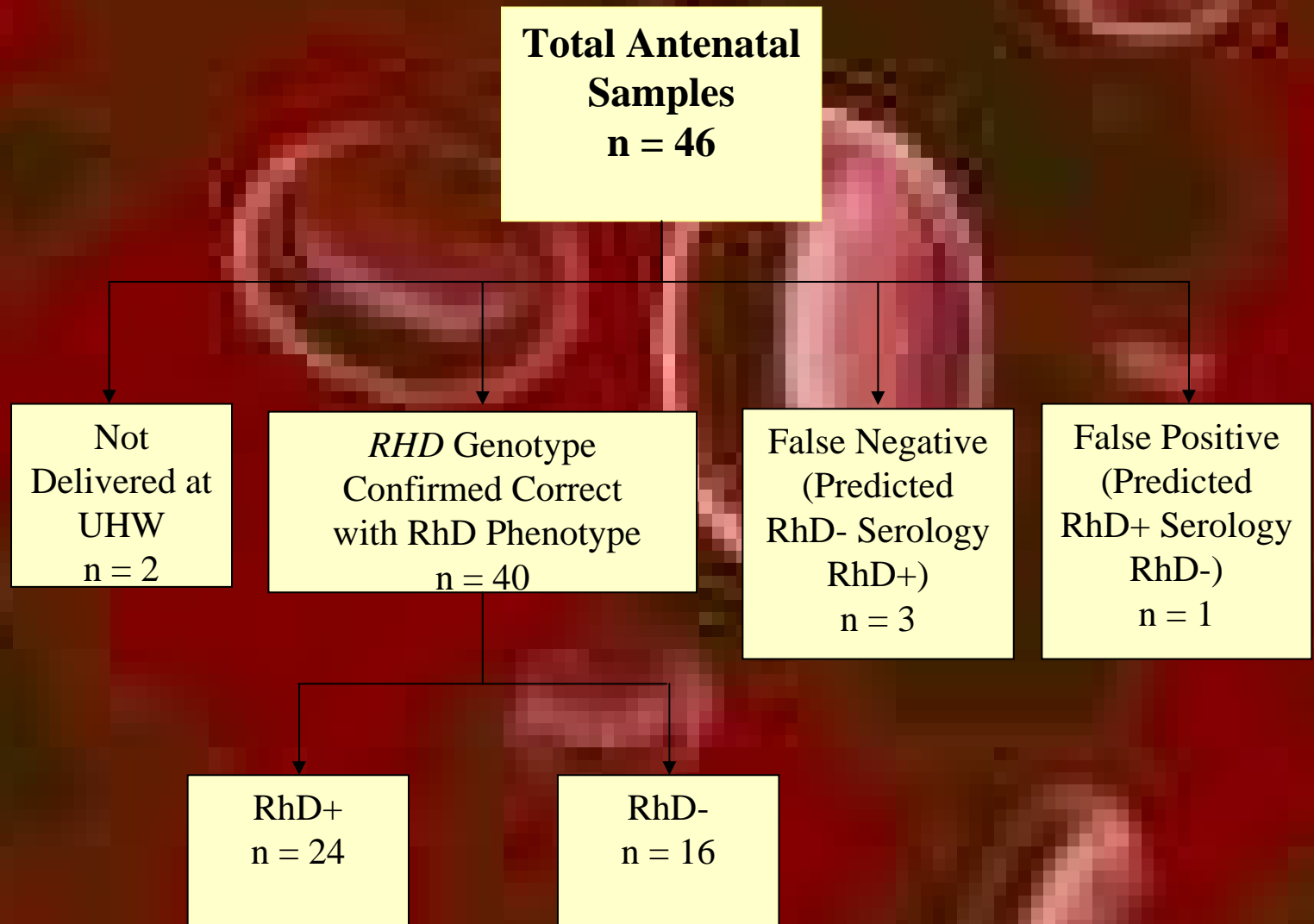
Effect of gestation age..



The effect of time to sample separation.....



Summary of Results



Conclusions

- The number of samples examined was not high enough to establish the earliest time during gestation and the time of separation when genotyping is most accurate.
- The technique is currently not sensitive enough for diagnostic application in a clinical setting, but the results of the study are encouraging with 40/44 samples where the phenotype was known being genotyped correctly.
- The lack of a positive control for RhD negative female fetus' needs to be overcome.
- In the long run an automated real-quantitative PCR method may need to be established.

Future Perspectives ?

- ? Routine genotyping of mums with antibodies with a potential to cause HDN. Current IBRGL service
- ? Routine mass screening of all Rh D Negative antenatal patients for appropriate administration of antenatal anti-D prophylaxis. IBGRL and UCL trial.

Acknowledgements

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