Understanding subfertility at a molecular level in the female through the application of nuclear magnetic resonance (NMR) spectroscopy

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BACKGROUND: Understanding the aetiology of subfertility and female reproductive tract disorders at a molecular level may improve success rates in fertility treatment. Such understanding may be gained by the application of metabonomics technologies to tissues or biofluids. Metabonomics is concerned with the quantification of molecules in the metabolome and uses nuclear magnetic resonance (NMR) spectroscopy as one of the main technological platforms. This review concentrates on NMR studies of the female reproductive tract and discusses further possible applications. While full metabolic profiling is relatively recent, targeted NMR studies of biofluid and tissue has a longer history.

METHODS: Searches were carried out on MEDLINE®, PubMed, SciFinder® Scholar 2007 and ISI Web of Knowledge® for papers about NMR spectroscopy or metabonomics of the female reproductive tract and subfertility.

RESULTS: NMR spectroscopy has been employed for the compositional analysis of various elements of the female reproductive tract, including cervical mucus, follicular fluid (FF), ovarian tissue, fallopian tubes and uterine matter. NMR was used to document for the first time a change in FF lipoprotein concentration during follicular development. NMR analysis of granulosa cells from rats has revealed that follicle-stimulating hormone increases the activity of the pentose pathway, having crucial implications for ovarian stimulation regimens. In the uterine matter work, it has been shown by NMR that glycolysis is rapidly stimulated by estrogen, and in another study, citrate in uterine fluid was found as a potential biomarker for adenomyosis. NMR has also been used to show that chlamydiae are able to achieve higher energy reserves by stimulating glucose transport in host cells.

CONCLUSIONS: A range of NMR spectroscopic techniques have been applied to the analysis of the female reproductive tract, however great potential remains for further studies. Incorporation of metabonomics techniques into female fertility research may be valuable for understanding subfertility and predicting outcomes of assisted conception treatments.

Key words: fertility / female reproductive tract / nuclear magnetic resonance / metabonomics / metabolomics
Introduction

Background

Subfertility is a complex disorder with significant medical, psychosocial and economic aspects. According to the Human Fertilization and Embryology Authority (HFEA), subfertility affects one in seven couples in the UK (HFEA, 2008). This review has been written considering female causes for subfertility only; male factor subfertility, affecting ~30% of subfertile couples is beyond the scope of this review (Hull et al., 1985). A cause for subfertility is not identified in ~25% of couples (Hull et al., 1985; HFEA, 2008), and a combination of male and female factors have been reported to occur in ~39% of cases (Thonneau et al., 1991). Approximately 10–15% of couples will have more than one cause for their subfertility (Hull et al., 1985; Templeton et al., 1990; HFEA, 2008). Female aetiology for subfertility can be classified into ovulatory dysfunction, which in 2006 affected 8.4% of couples (HFEA, 2008), and can be subdivided into hypothalamic pituitary dysfunction and hypothalamic pituitary failure and incorporates polycystic ovarian syndrome (PCOS) and ovarian failure. A second major cause is pelvic and tubal disease. Tubal disease, including tubal obstruction and pelvic adhesions due to infection, contributes to 15% of subfertility cases and endometriosis contributes 3% (HFEA, 2008). Furthermore, Chlamydia trachomatis is present in 11% of the sexually active population aged 19 years or younger (Macmillan et al., 2000) and is a major cause of pelvic inflammatory disease (PID) (Westrom and Wolnerhanssen, 1993; Paavonen and Eggert-Kruse, 1999). Cervical mucus defects or dysfunction and uterine abnormalities, such as fibroids or abnormalities of shape and adenomyosis are among the rarer disorders associated with fertility problems (Hull et al., 1985; Devlieger et al., 2003).

The treatment for subfertility includes ovulatory induction using either anti-estrogens or gonadotrophins (in the absence of tubal disease or male factor subfertility) and in vitro fertilization (IVF). Success rates for these treatments are variable and indeed with respect to IVF, live birth rates in the UK remain low (23.1% per cycle started in 2006; HFEA, 2008). Efforts also need to be made to improve the embryo selection and transfer of a single embryo (Cutting et al., 2008) in order to reduce the multiple pregnancy rate, which is a major cause of neonatal mortality and morbidity (RCOG, 2007). Additionally, there is a considerable cost to the individual and the National Health Service (NHS) (Ledger et al., 2006).

Causes of subfertility may lead to an imbalance of normal metabolism. It is postulated that a better understanding of the metabolic effects of various aetiologies of subfertility may help improve the likelihood of a pregnancy. Furthermore, it is hoped that identifying metabolic imbalances may contribute to the identification of non-invasive biomarkers for diagnostic and prognostic purposes.

The complete collection of low-molecular weight (LMW) compounds (≪1500 Daltons) in a cell, organ or organism is defined as the ‘metabolome’ (Wishart, 2007). These LMW molecules, referred to as ‘metabolites’, take part in, and are the end products of the metabolic reactions essential for normal cell function and growth. These include small peptides, lipids, carbohydrates, amino acids, vitamins and minerals (Wishart, 2007).

There has been much interest in the literature relating to metabolism within the reproductive tract incorporating the study of LMW compounds. In particular, within the ovary, folliculogenesis has been studied in depth (Nayudu et al., 2001; Smitz and Cortalvndt, 2002) and understanding is continually being developed, particularly in the context of in vitro growth and maturation of follicles (Picton et al., 2008). Folliculogenesis is the process by which the female germ cell develops within somatic germ cells of the ovary and matures into an oocyte with the potential for fertilization (Fortune et al., 2000; van den Hurk and Zhao, 2005). It is thought that early folliculogenesis is directed by cells within the ovary and endocrine signals from the pituitary are necessary for later development. The ovary in turn produces multiple feedback mechanisms that regulate pituitary physiology (Roche, 1996). Recent advances suggest that the oocyte itself provides signals which influence follicle metabolism and ultimate development (Albertini et al., 2001; Eppig, 2001; Hutt and Albertini, 2007; McLaughlin and McIver, 2009). Early studies in mice intimated that ovarian follicles produce large amounts of lactate during growth and maturation in vitro suggesting a metabolic preference for glycolysis (Boland et al., 1993). The same group (Boland et al., 1994) later demonstrated that growth and steroidogenesis were dependent on glucose concentrations and at low concentrations follicular development was significantly retarded. They conclude that follicles adopt a predominantly glycolytic mode of energy production to sustain growth and steroidogenesis, and that this is stimulated by gonadotrophins. It is thought that by utilizing the glycolytic pathway, oxygen resources can be preserved by the follicle (Godsen and Byatt-Smith, 1986), for use by the oocyte whose adenosine triphosphate (ATP) production is limited to oxidative metabolism via catabolism of nutrients such as pyruvate (Biggers et al., 1967). Harris et al. (2007) studied carbohydrate metabolism in murine pre-antral follicles by analysing spent follicle culture media and measuring metabolites using an automated analyser. They demonstrated increasing glucose consumption and lactate production during follicle development from the pre-antral to the antral stage. Furthermore, differences in nutrient turnover between follicles and oocytes with varying morphologies was found, suggesting thereby that metabolism and competence of gametes are related. Harris et al. (2005) also investigated the nutritional environments of the oocyte and later the mouse embryo as they pass along the reproductive tract, with a view to nutritionally and physiologically optimizing the in vitro culture medium for oocyte maturation. Utilizing ultramicrofluorometric methods for glucose, lactate and pyruvate and high performance liquid chromatography (HPLC) for protein analysis, significant differences were identified within follicular fluids (FFs) and fluids within the fallopian tube and within the uterus (Harris et al., 2005). There is also evidence for a fluctuation in the nutritional status of FF throughout the bovine oestrous cycle (Orsi et al., 2005). Studies of metabolism elsewhere within the human female reproductive tract have also been performed. In the 1990s, human fallopian tube was used as a ‘co-culture’ for preimplantation embryos (Bongso et al., 1990) in IVF. Studies have been performed to investigate the metabolic requirements for the human endosalpinx and Brewis et al. (1992) demonstrated that glucose is the major metabolic fuel of this organ, by performing an assay of four key metabolic enzymes using an ultra violet (UV) spectrophotometer. More recently the metabolism of arachidonic acid by the fallopian tubes has been studied, using reverse-phase HPLC, and the findings may be important in understanding gamete function, embryo transport and development (Huang et al., 2002). Metabolic components of hydrosalpinges (Chen et al., 2002) have also been measured using a blood analyser, demonstrating that potassium, calcium, glucose...
and lactate have lower concentrations in hydrosalpinges when compared with the fluid within normal fallopian tubes (Chen et al., 2002). This finding may explain subfertility in women with chronically inflamed but unobstructed fallopian tubes as these metabolites provide the direct environment for the early developing embryo.

The content of cervical mucus has long been recognized as having specific relevance to sperm migration and penetration (Sujan et al., 1963; Morales et al., 1993) and ultimately subfertility (Ulstein, 1972). In the 1950s, metabolic processes and content of the cervical mucus employing fractionation procedures was studied. This identified fructose, galactose, glucosamine, galactosamine, sialic acid, 13 amino acids (the major three of which were threonine, serine and proline) and glucose-containing material thought to be glycogen in the bovine cervical mucus (Gibbons, 1959). A consistent change in cervical mucus glucose concentration throughout the normal menstrual cycle was found. The pre-ovulatory and luteal phase fructose concentrations differ significantly (Van Der Linden et al., 1992).

In the above experiments, the researchers ascertained the presence and quantified concentrations of metabolites with the use of automated analysers, blood analysers, assays, UV spectrophotometry and reverse-phase HPLC. While some understanding has been gained using these techniques, more insight is required into the metabolic processes involved in subfertility. To this end, the focus of this review is to evaluate the role of nuclear magnetic resonance (NMR) spectroscopy for identifying metabolites within the female reproductive tract. In particular, this review will examine the past and present use of metabolite analysis through NMR spectroscopy, and will discuss the potential of such analyses for enhancing our understanding of subfertility. To this end, the focus of this review is to evaluate the role of NMR spectroscopy for identifying metabolites within the female reproductive tract. In particular, this review will examine the past and present use of metabolite analysis through NMR spectroscopy, and will discuss the potential of such analyses for enhancing our understanding of subfertility. Metabolite analysis can either be targeted, where a selection of specific molecules are identified and quantified, or global, which is more like a ‘fishing’ exercise where all the components of a medium are identified and quantified at once, and any trends within their concentrations are identified. The latter approach is known as ‘metabonomics’.

**Metabonomics**

Metabonomics (also referred to as metabolomics) is the non-targeted identification and quantification of all the metabolites in the metabolome, with the aim of measuring the response of an organism to a pathophysiological insult (such as disease, drug exposure and environmental changes) or genetic modification. This is the latest ‘omic’ technology, stemming from genomics, transcriptomics and proteomics, which measure the responses of living systems to stimuli at the genetic and cellular protein expression levels, respectively (Nicholson et al., 1999). Together, these technologies form the omics cascade (Fig. 1), which describes the flow of biological information, from the genome to the transcriptome, then the proteome and ending in the metabolome. Transcriptomics and proteomics have been useful in the observation of the effect of drugs on gene expression; however they offer little information regarding the global response to disease and desirable or undesirable drug effects, since they both ignore the dynamic metabolic status of the organism (Nicholson et al., 1999). Conversely, metabonomics allows the understanding of biological processes to be completed as it is capable of providing biological end-point markers of the cellular processes that occur as a result of the disease, drug exposure or altered gene function (Lindon et al., 2004). Pathophysiological insults cause abnormal cellular processes (Nicholson et al., 1999; Lindon et al., 2004). The response of an organism’s cells is to alter the concentrations of myriad metabolites in an attempt to maintain homeostasis (Lindon et al., 2003; Goldsmith et al., 2010). The metabolites inside the cells of an organism are in dynamic equilibrium with the metabolites inside the biofluids that perfuse, or are secreted by, the cells (Nicholson et al., 1999). Therefore, the effect of the insult on the organism will be translated into the change in composition of its biofluids (Nicholson et al., 1999; Lindon et al., 2004). By analysing biofluids (such as blood plasma/serum, urine and seminal fluid), metabolic profiles can be obtained which act as fingerprints for biochemical perturbations characteristic of a physiological state (Lindon et al., 2003, 2004).

![Figure 1](image-url)  
**Figure 1** The ‘omics’ cascade describes the flow of biological information in an organism (Di Leo et al., 2007).
this way metabonomics has potential for the identification of bio-
markert(s) for diseases and other stimuli.

**Metabonomics procedure**
The process of a metabonomics investigation involves sampling bio-
fluids or tissues from control and test subjects, paying close attention
to the inclusion and exclusion criteria for the cohort and to the hand-
ing and storage of the samples. Immediately upon collecting the
samples, metabolic activity needs to be ceased until the time of analy-
sis. This can be achieved by snap freezing the tissue or biofluid samples
in liquid nitrogen before storing at \( -80 \)°C. Once samples have been
collected, they are analysed (qualitatively and if possible, quantitatively)
and the data are compiled (Fig. 2). Next, the data are processed using
a suitable technique capable of identifying regions of interest among
the multitude of data points that result from sample analysis (Fig. 2).
This is generally achieved using a multivariate analytical technique,
which is often preceded by a process of data-reduction.

NMR spectroscopy is ideal for the analysis of biological samples since
it is the only technique that can be used to analyse intact tissues or bio-
fluids with little or no sample preparation, such as pre-separation prior
to analysis. Furthermore, the analysis is non-destructive to the sample
and requires only small sample volumes, which is ideal when studying
fluids that are not readily available in large volumes. One of the main
limitations of NMR spectroscopy is its low sensitivity relative to tech-
niques such as UV and infra-red (IR) spectroscopy and mass spec-
trometry (MS) with the consequence that longer experimental times
are required to get the good signal to noise ratio required for reliable
subsequent quantification and statistical analysis procedures (Elipe,
2003). UV and IR spectroscopy do not provide the resolution required
to differentiate between the various components of complex mixtures
such as biofluids. Thus, MS is the only other platform widely used in this
area. MS is significantly more sensitive than NMR (under favourable con-
ditions sub-nanomolar species may be detected) and generally requires
less material than that utilized in an NMR experiment. It is a truly ‘high-
throughput’ platform. However, MS generally requires chemical deriva-
tization and pre-separation steps in order to select individual metab-
lites for analysis, since different types of metabolites cannot be
analysed simultaneously. Pre-separation is usually achieved by coupling
MS to liquid or gas chromatography. Other disadvantages of MS com-
pared with NMR spectroscopy are that it suffers from variable detection

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**Figure 2** A typical metabonomics procedure requires sample collection, followed by analysis using a suitable technique, data reduction and finally
multivariate analysis to interrogate the sample data. This example is for analysing FF using NMR spectroscopy. Part 1 shows ultrasound directed fol-
licular aspiration to obtain FF samples for analysis. The three-dimensional ultrasound image is of a human stimulated ovary containing several follicles
from which FF is aspirated. Following NMR analysis in Part 2, the spectral data are reduced. A common data reduction method, ‘binning’, involves
dividing the spectrum into regions of equal width and calculating the area under the signals within each region, this is represented in Part 3. After
data reduction, multivariate analysis can be carried out in order to look at differences in the spectral data, here, PCA is shown as an example in
Part 4. This analysis produces a scores plot and a loadings plot which can be used to identify trends across a set of patients (see Multivariate statistical
analysis section, below).
responses since different molecules have different ionization efficiencies, quantification is more difficult, results are less reproducible, there is potential for sample bias and the analysis is destructive to the sample. The advantages of using NMR spectroscopy outweigh the disadvantages, nevertheless these must be acknowledged and in an ideal world NMR data would be complemented by that from parallel mass spectrometric studies (Lindon et al., 2004; Fancy et al., 2006).

**NMR spectroscopy**

NMR spectroscopy exploits a property possessed by some nuclei, called nuclear spin. Hydrogen nuclei (\(^1\)H) are the most commonly investigated nuclei in NMR spectroscopy, however \(^1\)C, \(^15\)N and \(^31\)P are also observable. Such nuclei may be excited by the application of radio waves, the frequency of which not only depends on the type of nucleus but also on its chemical environment; hence molecular compositional information is available. The process of regaining the ground state is called relaxation and this occurs via two main pathways: longitudinal and transverse relaxation, which are characterized by the time constants denoted \(T_1\) and \(T_2\), respectively; differences in these can reveal, for example, mobility differences between molecules/tissues. During relaxation the energy released is detected as a voltage change as a function of time. This signal is converted to a frequency dependent signal following the application of a mathematical protocol (the Fourier transformation). The result is a readable spectrum with intensity on the y-axis and frequency [known as chemical shift, in units of parts per million (ppm)] on the x-axis (Fig. 3). The intensity of the NMR signal is directly related to the concentration of the nucleus giving rise to that signal and this is the key to its utility in the analysis of the complex mixtures characteristic of biological fluids and tissues. Metabolites are small molecules and usually only have a small number of a given nuclei, and these are usually in different chemical environments. Therefore each line (or set of lines) in the spectrum indicates the presence of a particular molecule in the sample.

NMR-based metabonomics studies have been utilized for the analysis of various biofluids such as blood plasma (Nicholson et al., 1995), serum (Soininen et al., 2009), urine (Holmes et al., 2000), bile (Gowda et al., 2009) and more recently, saliva (Bertram et al., 2009). More unusual fluids have also been studied using NMR spectroscopy, such as cerebrospinal fluid (Petroff et al., 1986) as well as reproductive fluids from both the male and the female reproductive tract (as will be reviewed here). The findings of such studies serve as libraries of all the metabolites contained in a given biofluid. Often these biofluids are very similar in composition, for example plasma and FF have almost identical compositions, thus it is possible to analyse the content of the spectrum of one fluid by comparison with that of another. There are a number of metabolite databases that have been developed which are available for reference, such as the Human Metabolome Database set up in 2007 (Wishart et al., 2009).

NMR experiments are available to selectively filter out heavy molecules from the NMR spectra of biofluids, leaving only signals from the LMW molecules of interest (for example, as in the spin echo experiment). In addition, there are techniques and experiments

**Figure 3** 499.97 MHz \(^1\)H-NMR Carr-Purcell-Meiboom-Gill spectrum of human blood plasma. Abbreviations: ala, alanine; glu, glutamate; HDL, high-density lipoprotein; his, histidine; ile, isoleucine; LDL, low-density lipoprotein; met, methionine; phe, phenylalanine; tyr, tyrosine; val, valine; VLDL, very-low-density lipoprotein.
available to help effectively remove the water signal which dominates biofluid NMR spectra (for example pre-saturation: selective irradiation of the water signal). Two-dimensional (2D) NMR spectroscopy can be performed to help identify signals in regions of high overlap. All of these techniques allow different molecules of interest to be analysed selectively, without needing to alter the sample: either by separation techniques or chemical modification processes.

**Multivariate statistical analysis**

NMR spectra of biofluids are extremely complex, and so identifying meaningful trends across a set of spectra by eye is exceptionally difficult and impractical, particularly as metabolite concentrations are expected to change across the set of samples due to natural biological variation as well as in response to a stimulus (Lindon et al., 2004). Therefore it is often necessary to use chemometric pattern recognition (PR) techniques to help interpret the NMR data. A commonly used PR technique is principal components analysis (PCA), which reduces the number of variables in the data into a smaller number of variables called principal components (PCs) which describe the majority of the variation in the spectral data. Each PC is cumulative and orthogonal, with the first PC explaining the greatest source of variation in the data. PCA produces two types of plot which provide information about the variation across a set of samples: a scores plot, which describes how different or similar sample metabolic profiles are, and thus reveals trends and clustering of patients (Fig. 2); and a loadings plot, which indicates the metabolites which are at different or similar concentrations across the samples (in the case of PCA conducted on NMR data, a loadings plot reveals the chemical shifts responsible for the positioning of the samples in the scores plot) (Fig. 2). Prior to multivariate analysis of NMR spectral data, it is necessary to simplify the data into a smaller set of variables and to reduce inter-sample variation. This is achieved via a process known as ‘data reduction’ (Fig. 2).

**History of NMR spectroscopy and female fertility**

In 1956, Scandinavian gynaecologist Odeblad was the first physician to utilize NMR spectroscopy for the analysis of a reproductive fluid. He retrieved cervical mucus from apparently healthy women and using 1H-NMR spectroscopy demonstrated changes in an unknown signal at different stages of the menstrual cycle (Odeblad and Bryhn, 1957). The authors postulated that this change was in response to estrogenic changes (Odeblad and Bryhn, 1957). Subsequent to this work, Odeblad has studied other reproductive tracts using NMR spectroscopy, including the development of a micro-NMR technique ideal for the analysis of small amounts of cervical secretions (Odeblad, 1966, 1968a, b) and a comparison of NMR spectra in women taking oral contraceptives to ovulatory women (Odeblad, 1968a, b). As well as contributions to the field of cervical mucus, Odeblad has studied other reproductive tracts using NMR spectroscopy, for example vaginal contents and epithelial cells (Odeblad, 1959). In 1973, Odeblad and Rudolfsson suggested the existence of seven distinct types of human cervical mucus and postulated a micelle structure of the mucus based on NMR-findings (Odeblad and Rudolfsson, 1973). Later, Morrill carried out various NMR spectroscopic studies of amphibian oocytes (Morrill et al., 1983, 1984; Gupta et al., 1985a, b; Morrill et al., 1985a, b). These were mainly studies of insulin action on oocytes, studies of intracellular sodium and magnesium ions in oocytes and the role of calcium in oocyte pH regulation. Since these works, many other NMR spectroscopic studies have been conducted on amphibian oocytes (Sakai et al., 2006; Bodart et al., 2008). NMR studies of male fertility are widely available, as reviewed by Deepinder et al. (2007), however fewer female studies exist.

Odeblad demonstrated that NMR spectroscopy can be successfully implemented for analysis of cell material and hypothesized its application in clinical practice. Fifty years later, his propositions are becoming a reality. The purpose of this review is to examine the more recent literature regarding the use of NMR spectroscopy for the identification and quantification of metabolites, specifically in the female reproductive tract.

**Methods**

Literature searches for publications written in the English language were performed in PubMed, MEDLINE®. SciFinder® Scholar 2007 and ISI Web of Knowledge™. Key words used for the searches were: ‘NMR’, ‘NMR spectroscopy’, ‘metabonomics’ and ‘metabolomics’. Each of these keywords was paired with several reproduction-related terms, as summarized in Table 1. Papers were only considered for this review if they involved the use of NMR spectroscopy for the analysis of biofluid composition or specific metabolite identification within female reproductive tracts/biofluids. Studies involving the use of NMR spectroscopy for structural determination of molecular components from the female reproductive tract were excluded, as were magnetic resonance imaging and NMR micro-imaging studies. Animal studies were included as well as human studies, however papers focused on male subfertility were excluded, as were

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Search terms performed for the compilation of literature for this review.</th>
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</thead>
<tbody>
<tr>
<td><strong>Keywords</strong></td>
<td><strong>Paired terms</strong></td>
</tr>
<tr>
<td>‘NMR’</td>
<td>Subfertility/infertility</td>
</tr>
<tr>
<td>‘Nuclear magnetic resonance spectroscopy’</td>
<td>‘Fertility’</td>
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<tr>
<td>‘Metabonomics’</td>
<td>‘Reproduction’</td>
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<tr>
<td>‘Metabolomics’</td>
<td>‘IVF’</td>
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<tr>
<td>‘Embryo culture/embryo culture medium’</td>
<td>‘Follicular fluid’</td>
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<tr>
<td>‘Preservation of ovarian tissue/ovarian tissue’</td>
<td>‘Ovaries’</td>
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<tr>
<td>‘Cervical mucus’</td>
<td>‘Oocytes’</td>
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<tr>
<td>‘Peritoneal fluid’</td>
<td>‘Embryo culture’</td>
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<tr>
<td>‘Pelvic inflammatory disease/PID/Chlamydia’</td>
<td>‘Embryo culture medium’</td>
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<tr>
<td>‘Polycystic ovary syndrome/PCOS’</td>
<td>‘Preservation of ovarian tissue’</td>
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<tr>
<td>‘Reduced ovarian reserve/premature ovarian failure’</td>
<td>‘Reproduction’</td>
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<tr>
<td>‘Endometriosis’</td>
<td>‘Fertility’</td>
</tr>
<tr>
<td>‘Uterine disorders/adenomiosis’</td>
<td>‘IVF’</td>
</tr>
</tbody>
</table>

Each of the four keywords was paired with each of the terms in the right-hand column in the search engines used, for example ‘NMR AND subfertility’.
Results and discussion

NMR studies of cervical mucus

Cervical mucus acts as a barrier to infections (Brunelli et al., 2007) and also as a transport medium for spermatozoa (Sahrbacher et al., 2002). The mucus is made up of an aqueous layer and a viscus layer containing mucin glycoproteins (Sahrbacher et al., 2002; Brunelli et al., 2007).

Since the pioneering work of Odeblad into analysis of cervical mucus, there have been further developments in this field. In 1978, the movement of water molecules within cervical mucus was investigated by Katz and Singer using pulsed-gradient NMR spectroscopy (Katz and Singer, 1978). A pulsed gradient spin echo NMR procedure was employed in order to measure the self-diffusion coefficients of the $^1$H nuclei in the water of bovine cervical mucus (Katz and Singer, 1978). A simple mathematical model was used to assess the extent to which the self-diffusion of water molecules was inhibited by the mucous microstructure (Katz and Singer, 1978). The authors did not observe a significant reduction in the sperm-free bovine mucus water diffusiveness at any phase of the oestrous cycle, suggesting that cervical mucus contains little solid surface area to attach water molecules via hydration bonds and generally hinder water mobility (Katz and Singer, 1978). It was calculated that that <5% of the water in the mucus existed in hydration bonds (Katz and Singer, 1978), which agreed with the findings of Odeblad and Rudolfsson (Odeblad and Rudolfsson, 1973). Using further mathematical computations Katz and Singer went on to investigate the effect of a motile spermatozoon, known to produce fluid convective forces that contribute to the movement of water molecules in the cervical mucus (Katz and Singer, 1978). They observed significant hydrodynamic interaction between a single spermatozoon and the surrounding microfilaments of the cervical mucus, explaining why spermatozoa do not undergo an irreversible loss of motility and metabolic activity when diluted in cervical mucus, unlike in other simple media (Katz and Singer, 1978).

By 1983 the literature regarding the physiochemical state of cervical mucus was contradictory and the facts were unclear. The work of Odeblad and Rudolfsson and Katz and Singer had contributed to the information available, but did not always agree with findings from non-NMR studies. At this time Charles Merilan used an alternative approach to the problem of determining the infrastructure of bovine cervical mucus in relation to the oestrus cycle (Merilan, 1983a, b). This study focused on measuring the width of the water peak in the mucous NMR spectrum at half the height of the peak ($W_{50}$) and at 20% of the peak height ($W_{20}$) (Merilan, 1983a, b). Ideally, NMR signals should be a symmetrical Lorentzian curve, therefore changes in the line width indicate chemical change in the system (Merilan, 1983a, b). From these spectral measurements, it was possible to compute asymmetry indices to identify changes in chemical characteristics of the mucus (Merilan, 1983a, b). Merilan observed that the cervical mucus followed a cyclic change of NMR properties in relation to the oestrous cycle: with a distinct change in lineshape between the early to mid-oestrus stages, reflecting increased hydration of the mucus; and a second change at 6–15 days post-oestrus, reflecting a decrease in water content of the mucus and conformational changes of the mucus polymers, or the presence of a new luteal phase-specific chemical species (Merilan, 1983a, b). In a separate study, but using this same approach, Merilan also investigated the relationship between change in asymmetry indices (obtained from $W_{50}$ and $W_{20}$ measurements of bovine cervical mucus) and reproductive status, and developed a potential NMR-based method for detecting pregnancy in bovines (Merilan, 1983a, b).

Following on from some of the first cervical mucus compositional analysis conducted by Odeblad using micro-NMR, Rohr et al. (1992) used the then more up to date high-resolution NMR methodologies to demonstrate the potential of NMR spectroscopy for the qualitative and quantitative examination of the properties of low- and medium-molecular weight cervical mucus components. Using selective saturation to suppress the large water signal, and replacing the water solvent with a deuterium oxide ($D_2O$) solvent, Rohr et al. obtained one-dimensional (1D) and 2D $^1$H-NMR spectra of cervical mucus from women presenting with infertility (Rohr et al., 1992). With the water peak suppressed, many other $^1$H-NMR signals corresponding to low- and medium-molecular weight mucus constituents could be observed (Rohr et al., 1992). These signals were assigned, based on their chemical shifts and splitting patterns and supported by the 2D spectra, to reveal the presence of metabolites such as amino acids, glucose, lactate, creatinine, acetate and even ethanol (this was unexpectedly observed in high quality spectra of untreated cervical mucus, i.e. not dissolved in $D_2O$ in the cervical mucus (Rohr et al., 1992)). This work was presented only as a short communication to demonstrate the potential of the then more sophisticated, and ever improving technique of NMR spectroscopy, and so no further investigations into cervical factors influencing infertility, or the mechanisms involved in the sperm-mucus interaction were included (Rohr et al., 1992). However, the authors discussed potential applications of high-resolution NMR spectroscopy including the above, and described the suitability of NMR spectroscopy for the analysis of cervical mucus in terms of its ability to examine samples in their native state, provide a more differentiated analysis, identify one type of nucleus at a time and provide structural information about the mucus constituents (Rohr et al., 1992). This preliminary work was followed by the same group with a more detailed analysis of the molecular composition of the aqueous phase of human cervical mucus, and the development of a method for determining the absolute concentrations of the metabolites (Sahrbacher et al., 2002). In this most recent NMR study of cervical mucus, it was found that concentrations of the mucus components varied greatly across individuals despite being pre-treated for 7 days with ethinylestradiol in an attempt to standardize the mucus properties (Sahrbacher et al., 2002). Furthermore, these concentrations were not related to values described for blood plasma, suggesting that cervical mucus is not just a transudate (Sahrbacher et al., 2002). A strong correlation of all mucus components to the water content for each individual patient over time provided evidence that constituents are regulated in a constant manner, however the opposite trend was seen when different patients were compared (Sahrbacher et al., 2002).

Thus far, the majority of the NMR-based investigations of cervical mucus have been concerned with observing its composition and changes within it during the oestrous cycle, or reproductive state.
There are no cervical mucus NMR-based studies to our knowledge concerned with looking for biomarkers of reproductive diseases, evidence of oxidative stress or specific correlations of physiological state to fertility. Furthermore, no metabonomics studies of cervical mucus (involving NMR spectroscopy or otherwise) were discovered during the literature search. Sahrbacher et al. (2002) mentioned that their observation of a strict regulation of cervical mucus constituents may be relevant for the control of its function in fertility, helping to understand the mechanisms of sperm–mucus interaction, but did not discuss or research this further (Sahrbacher et al., 2002). As mentioned previously, cervical mucus acts as a barrier and a transport medium to spermatozoa and therefore understanding the mechanisms underlying these actions is of upmost importance in understanding fertility. NMR spectroscopy offers an excellent alternative method to conventional methods for analysis of highly viscous cervical mucus, without the need for sample pretreatment (Sahrbacher et al., 2002). Metabonomics allows the comparison of biological samples taken from individuals at different times and at different physiological states and therefore could be a potentially powerful technique in understanding reasons for subfertility within cervical mucus.

NMR studies of uterine matter

One of the problems encountered by traditional techniques attempting to assess metabolites in the uterus is that of partial degradation of the analyte, thus variability in reported results was common (Oliver and Kellie, 1970). The development of NMR spectroscopy has opened up the opportunity for non-invasive studies of function and metabolism in cell suspensions and isolated perfused tissues. Localized spectroscopic studies using 31P, 13C and 1H were introduced as a technique to observe regional differences in chemical composition and variations in tissue metabolism. In the late 1980s, these techniques were beginning to become more frequently used and Dawson et al. (1988) reviewed the literature at that stage, in particular concentrating upon the use of NMR spectroscopy in assessing the differences in myometrial metabolism of pregnant and non-pregnant patients. Degani et al. (1984) studied the effects of estrogen on the high-energy phosphates of the immature rat uterus using 31P-NMR spectroscopy. Using the same technique, Shivaji et al. (1995) demonstrated changes in the concentrations of phosphorous containing metabolites in the uterus of hamsters during the oestrous cycle. Degani’s team later utilized 13C-NMR spectroscopy to assess in vitro estrogen induction of glucose metabolism in the immature rat uterus. They were able to demonstrate, using this non-invasive technique, a specific and rapid estrogen stimulation of glycolysis (Shinkarenko et al., 1994). Developing our understanding of metabolic pathways taken by the uterine tissue when stimulated with estrogen provides further information with respect to subfertility diagnosis and therapeutics.

Adenomyosis is a benign pathological disorder whereby endometrial glands and stroma invade the myometrium. It is thought to play a role in certain cases of subfertility, as well as causing menorrhagia and dysmenorrhoea. Izuka et al. (1996) have tried to assess whether there are metabolic differences in uterine fluid in the presence of adenomyosis. The purpose of this study was to identify a marker for adenomyosis within uterine fluid (Izuka et al., 1996). Utilizing 1H-NMR spectroscopy in mice with experimentally induced adenomyosis, Izuka et al. identified citrate as being markedly different when compared to controls, with high levels in the uterine tissue and low levels in the uterine fluid (Izuka et al., 1996). Since glycolysis is a major metabolic process in the mouse uterus and citrate is a significant factor involved in the regulation of uterine glycolysis, they postulated that abnormal citrate levels in mice with adenomyosis could reflect a depression of citrate acid consumption by way of the citric acid cycle (Izuka et al., 1996). This in turn may be associated with subfertility in females with adenomyosis (Izuka et al., 1996).

Griffin et al. (2003) describe the technique of high-resolution magic angle spinning (HRMAS) 1H-NMR spectroscopy as a method to overcome many of the problems associated with observation of metabolites within intact tissue using straightforward 1H-NMR spectroscopy, such as dipolar couplings, chemical shift anisotropy and magnetic susceptibility changes. This technique, which utilizes rapid spinning of samples at the ‘magic angle’ (54.7°) with respect to the direction of the external magnetic field, minimizes these problems and greatly improves the resolution of broad signals in solids. However, resonances from one metabolite may be co-resonant with those from another, which is a particular problem with lipid resonances which are broad, and spectral editing may be required (Griffin et al., 2003). In this metabonomics study, the metabolic profile of the human Ishikawa cell line, derived from endometrial adenocarcinoma, was determined and the metabolic action of a selective estrogen receptor modulator used in the treatment of breast cancer, tamoxifen, was examined (Griffin et al., 2003).

NMR studies of the pelvis

The literature searches performed failed to identify any studies investigating endometriosis or endometriotic tissue using NMR spectroscopy, nor were there any studies found pertaining to peritoneal fluid or hydrosalpinx fluid. There has, however, been research performed using NMR spectroscopy to understand the pathogenesis of C. trachomatis, an obligate intracellular bacterial parasite of eukaryotic cells that infects the mucosal columnar epithelium, and is a major cause of PID. Peeling et al. (1989) studied the intracellular energy requirements of this organism using high-resolution 31P-NMR spectroscopy. Their findings suggest that the major outer membrane protein may be the major route through which ATP accesses ATPase (Peeling et al., 1989). Later studies incorporated 31P- and 13C-NMR spectroscopy to determine ATP levels and glycolysis rates throughout the course of infectivity. This research concluded that chlamydiae are able to stimulate glucose transport in the host cell sufficiently to compensate for the extra energy requirements as a result of the infection (Ojcius et al., 1998). From these studies, a deeper understanding of the infectivity processes can be gleaned and, utilizing this information, it may be possible to develop a strategy for disease intervention.

NMR studies of ovarian tissue

The use of NMR studies on ovarian tissue is sparse, and mainly concentrated on research into ovarian cancer. There have, however, been a few small studies investigating metabolism within the ovary. Early studies utilized 31P-NMR spectroscopy to characterize non-invasive changes in the levels of phosphates and in their interconversion rates in perfused rat ovaries. In particular, Haseltine et al. (1986) studied the effect of added adenosine on the concentration of ATP...
in rat luteal cells and demonstrated a loss of ATP followed by acidification. The authors propose that adenosine may regulate ATP levels in the ovaries during periods of hypoxic challenge (Haseltine et al., 1986). More than a decade later Tanaka et al. (1999) related these findings to the ovulatory process and in their study, also utilizing 31P-NMR spectroscopy in the perfused rabbit ovary, demonstrated decreased ATP prior to ovulation. They suggest that protein phosphorylation through the activation of the cyclic adenosine monophosphate-dependent protein kinases prior to induction of various chemical reactions is correlated to the instigation of ovulation (Tanaka et al., 1999). Bentley et al. (1990) employed the technique of NMR spectroscopy on isolated granulosa cells incubated with or without follicle-stimulating hormone (FSH). This study demonstrated lower relative intensities of fructose 6-phosphate to ribose 5-phosphate after exposure to FSH, implying that FSH increases the activity of the pentose pathway and ultimately can activate metabolic pathways, which provide the substrates for steroid synthesis and cholesterol mobilization. This has crucial implications in the process of ovarian stimulation regimens in the treatment of subfertility.

With respect to utilizing NMR spectroscopy as a means of identifying epithelial ovarian cancer (EOC) there has been more extensive research, as the drive to develop a reliable biomarker of the disease, which frequently presents at late stages, increases. Ferretti et al. (2002) suggested the use of NMR spectroscopy to understand biological functions in varying ovarian cancer cell lines, and in their study identified and quantified previously unidentified carcinoma cell lines. 1H-NMR analysis in conjunction with metabolomics of serum has been found to reliably distinguish between EOC and healthy controls and has been proposed as a potential novel strategy for the early detection of EOC (Odunsi et al., 2005). More recently, following NMR studies in malignant tumours elsewhere in the body, Ricci et al. (2008) studied intracellular levels of the metabolites of phosphatidylcholine, the major phospholipid of eukaryotic cell membranes in the progression from healthy ovarian surface epithelial cells to ovarian cancer cell lines. They demonstrated differing metabolite levels in cancer cells (Ricci et al., 2008).

As NMR spectroscopy has been successfully implemented in the identification of metabolite levels characteristic of cancer, we postulate that the same technique could be employed in distinguishing between disease states within the ovary resulting in subfertility and healthy individuals. The literature search however failed to retrieve any research performed investigating reduced ovarian reserve or premature ovarian failure by utilizing the technique of NMR spectroscopy and metabolomics. A single study was identified which had used NMR analysis to investigate the effects of moderate-intensity exercise without weight loss on lipoprotein profiles taken from plasma in women with PCOS (Brown et al., 2009).

**NMR studies of FF**

FF fills the antral cavity surrounding the oocyte in follicles in the ovaries, and provides the in vivo microenvironment for a developing oocyte, containing all of the substances essential to follicle growth, oocyte maturation, ovulation and protection of the oocyte during transit into the oviduct (Edwards, 1974). The composition of FF has been extensively studied, mostly by non-NMR methods, as reviewed by Edwards (Edwards, 1974), and more recently by Revelli et al. (2009) who discussed the components of FF in terms of predictors of oocyte quality. Like most biofluids, FF is known to contain a high concentration of water as well as lipids, carbohydrates, proteins (and enzymes), inorganic salts, mucopolysaccharides, peptide hormones, anticoagulants and unusually high concentrations of steroids (Edwards, 1974).

Gosden et al. (1990) were the first group to employ NMR spectroscopy for the analysis of ovarian FF. Prior to this study, little work had been carried out with the aim of examining the non-steroidal, small organic molecular composition of FF (Gosden et al., 1990). This study utilized the spin-echo pulse sequence designed to selectively identify signals from non-protein bound, LMW metabolites only, filtering out any broad signals from larger molecules such as proteins (Gosden et al., 1990). This pulse sequence, and modifications of it, are now common place in biofluid NMR analysis; the first example was by Brown et al. for the analysis of human erythrocyte metabolism (Brown et al., 1977). 1H-NMR spectra were obtained for Graafian FF samples aspirated from sheep, pigs and cows and quantitative estimates of the molar concentrations of several metabolites with well-resolved signals were deduced by reference to the concentration of lactate (measured using a lactate dehydrogenase assay) (Gosden et al., 1990) (Table II). The concentration of glucose in the fluids was too difficult to quantify due to the large overlap of signals in the region where the glucose signals reside (Gosden et al., 1990).

Gosden et al. confirmed that the concentrations of metabolites in ovine and porcine ovarian FF were similar to those in ovarian venous plasma samples, which were also obtained and analysed by 1H-NMR spectroscopy (though these data were not shown and the authors stated that there were few blood samples available for study) (Gosden et al., 1990). By further comparison of the above concentrations with literature on plasma/serum, the authors were able to conclude that concentration gradients of LMW molecules between FF and blood/lymph are extremely small, and that the concentrations of sugars, amino acids and fatty acids in blood perfusing the ovary are indicative of those in FF (Gosden et al., 1990). An exception to this rule was lactate, which appeared at much higher concentrations in FF than recorded in blood (Gosden et al., 1990). It was suggested that the variable concentrations of lactate and ketone bodies could be due to differences in anaesthetic or diet (Gosden et al., 1990). Hypoxanthine was present at much lower concentrations (in fact, below the detection limit) than had been previously reported in porcine FF (Gosden et al., 1990). Although the authors of this study had determined that the composition of FF, in terms of LMW compounds, was very similar to that of plasma, there was no attempt to describe the significance of these metabolites and their concentrations in oocyte development and quality (apart from the provision of energy for the oocyte by lactate (Gosden et al., 1990)). However this study demonstrated the power of NMR spectroscopy in the analysis of the chemical composition of ovarian FF, without the need for sample pre-separation or destruction.

In a later study, Gerard et al. (2002) used 1H-NMR spectroscopy to study changes in the composition of equine FF during follicular growth and maturation. This was the first study to report the composition of FF in relation to the oestrous cycle of any species (Gerard et al., 2002). A small volume of FF was aspirated from dominant follicles at an early and a late dominant stage and at the pre-ovulatory stage (34 h after
induced ovulation) (Gerard et al., 2002). Two unknown peaks (at 3.10 ppm (singlet) and at 3.25 ppm (doublet)) were observed in the $^1$H-NMR spectra, which had not been observed in human plasma $^1$H-NMR spectra or FF $^1$H-NMR spectra from sheep, pigs or cows (as reported by Gosden et al. (1990)). However, similar observations had been made in stallion seminal plasma studies, which allowed the authors to conclude that these peaks corresponded to compound(s) specific to horses (Gerard et al., 2002). The concentration of sugars decreased in FF between the early and late dominant stages, and then greatly decreased following induced ovulation, which the authors suggested may result from either a lower follicular production or from a local increase in carbohydrate catabolism (Gerard et al., 2002). Similarly, trimethylamine and acetate concentrations significantly decreased from the late dominant stage to the pre-ovulatory stage (Gerard et al., 2002). The authors linked the change in trimethylamine to polyamine metabolism (no change in FF polyamine content was observed) since both are degradation products of cellular metabolism (Gerard et al., 2002). FF concentrations of alanine and lipoproteins decreased significantly between the early and late dominant stages, and the latter then increased between the late dominant and pre-ovulatory stages (Gerard et al., 2002). The authors linked the change in alanine to steroidogenic activity of the follicular cells, since progesterone followed the same profile as the lipoproteins (Gerard et al., 2002). This was the first time that lipoprotein concentration variation during follicular development had been documented (Gerard et al., 2002). This study took NMR analysis of FF above and beyond composition determination by looking at the dynamics of FF content. The authors were able to characterize follicle concentrations to the outcome of the insemination of the mares. All mares involved in the study were inseminated the day following induced ovulation and 3 out of 10 tested positive for pregnancy. Therefore, there was a good opportunity to correlate the FF content findings to the outcome of the insemination.

In 2006, Sarty et al. (2006) carried out a similar FF dynamics study in which $^1$H-NMR was employed to study the physical properties of bovine FF aspirated from the dominant and subordinate follicles at four different stages of the oestrous cycle [Day 3 and 6 of the first wave of follicular growth (D3W1) and (D6W1), and Day 1 of the second wave (D1W2)], and on a day in the pre-ovulatory period of at least 17 days following ovulation ($D \geq 17$) (Sarty et al., 2006). Inversion recovery experiments were carried out in order to determine the longitudinal spin relaxation time constants, $T_{1}$, for the two lactate signals to give a measure of viscosity (Sarty et al., 2006). Significant differences in the $T_{1}$ values were observed between the dominant and subordinate follicles at D1W2, which the authors suggested may be reflecting the known difference in viscosity of FF for follicles that are likely to ovulate from those that are not, and led them to conclude that the intensity of the lactate NMR signal reflects to some degree the physiological status of the follicle (Sarty et al., 2006). The subordinate follicles were the most different at D6W1 where $T_{1}$ was at its highest, and the dominant follicles showed the biggest change at D1W2 where $T_{1}$ was at its lowest (Sarty et al., 2006). PCA of the ratios of the spectral peak heights followed by two-way multivariate analysis of variance of some of the resulting components (those with eigenvalues greater than 1), revealed a significant difference between dominant and subordinate follicles (Sarty et al., 2006). In most cases, this difference was at a maximum along PC1 at $D \geq 17$, supporting the expectation that the dominant and subordinate follicles would be physiologically different at $D \geq 17$ since the former will ovulate while the subordinate follicles will undergo atresia (Sarty et al., 2006). This is the only study so far to utilize NMR spectroscopy and advanced multivariate analytical techniques to try to understand more about the physiology of follicles with respect to time and status (dominant or subordinate). The main

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$^1$H shift/ppm</th>
<th>Multiplicity</th>
<th>Assignment</th>
<th>Concentration range/mmol l$^{-1}$</th>
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<tr>
<td></td>
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<td></td>
<td>Pig</td>
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<tr>
<td>Acetate</td>
<td>1.94</td>
<td>s</td>
<td>CH$_3$</td>
<td>0.34–1.50</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.48</td>
<td>d</td>
<td>CH$_3$</td>
<td>0.24–0.88</td>
</tr>
<tr>
<td>Creatinine</td>
<td>3.06</td>
<td>s</td>
<td>CH$_3$</td>
<td>0.71–1.50</td>
</tr>
<tr>
<td>Dihydroxyacetone</td>
<td>4.43</td>
<td>s</td>
<td>CH$_2$</td>
<td>–</td>
</tr>
<tr>
<td>α-glucose</td>
<td>5.26</td>
<td>d</td>
<td>H$_1$</td>
<td>–</td>
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<td></td>
<td>3.20–3.90</td>
<td>m × n</td>
<td>H$_2$–H$_6$</td>
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<tr>
<td>β-glucose</td>
<td>4.65</td>
<td>d</td>
<td>H$_1$</td>
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<td></td>
<td>3.20–3.90</td>
<td>m × n</td>
<td>H$_2$–H$_6$</td>
<td>–</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.58</td>
<td>s</td>
<td>CH$_2$</td>
<td>0.26–1.40</td>
</tr>
<tr>
<td>3-hydroxybutyrate</td>
<td>1.24</td>
<td>d</td>
<td>CH$_3$</td>
<td>0.00–4.20</td>
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<tr>
<td>Lactate</td>
<td>1.34</td>
<td>d</td>
<td>CH$_3$</td>
<td>2.30–15.5</td>
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<td></td>
<td>4.13</td>
<td>q</td>
<td>CH</td>
<td>–</td>
</tr>
<tr>
<td>Valine</td>
<td>1.02</td>
<td>d</td>
<td>CH$_3$</td>
<td>1.10–2.70</td>
</tr>
<tr>
<td></td>
<td>1.04</td>
<td>d</td>
<td>CH$_3$</td>
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* s, singlet; d, doublet; m × n, several multiple peaks; q, quartet.
focus of this paper was to examine whether there was an identifiable change in the FF physiology due to follicle status and cycle time point and not so much about how the FF changed, or why any changes would be biologically relevant. Other than lactate, little information was provided on the change in the concentrations of the FF constituents with cycle time and follicle status. The authors do however report a significant difference in the concentrations of alanine and acetate between dominant and subordinate follicles at D ≥ 17, but were unable to provide any indication of how these metabolites changed between other time points, or in what directions any change would occur.

Most recently, Piñero-Sagredo et al. (2010) have used a range of 1D and 2D NMR techniques to exhaustively profile human FF from oocyte donors. Using specialized NMR pulse sequences, the authors demonstrated that light and heavy components of FF can be detected separately, without requiring any chemical change to the fluid, and that 2D NMR analysis assists in the identification of signals in regions of high overlap in 1D NMR spectra (Piñero-Sagredo et al., 2010). The concentrations of glucose and lactate were estimated using a linear combination of reference spectra for both metabolites, resulting in a 2:1 ratio, supporting the presence of anaerobic metabolism in hyperstimulated follicles (Piñero-Sagredo et al., 2010). Furthermore, high correlations were observed between glucose, β-hydroxybutyrate, lactate, pyruvate, acetocacete and acetate when the data were grouped according to age, normalized fertilization rate and IVF outcome (Piñero-Sagredo et al., 2010). These findings indicate that FF may play a role in providing lactate and pyruvate as a source of energy to the follicle, and hence oocyte maturation (Piñero-Sagredo et al., 2010). Interestingly, a relationship was observed between the glycolytic pathway and fatty acid metabolism, which was particularly elevated in young donors and high fertilization rate groups (Piñero-Sagredo et al., 2010). The authors conclude by discussing the potential of metabolic profiling of FF in the search for biomarkers to predict oocyte quality, and suggest that such findings could also influence culture conditions of oocytes and embryos in infertility treatments (Piñero-Sagredo et al., 2010). This is the first publication to profile human FF using NMR spectroscopy, and serves as a library for human FF constituents, and the chemical shifts and multiplicities of their signals in various types of NMR spectra.

**Concluding remarks**

NMR has a history of revealing interesting features; when used in the global ‘hypothesis generating’ area of metabonomics the potential is even greater. Outside the field of female subfertility, metabonomics has been recognized as an independent and frequently utilized technique (Goldsmith et al., 2010). The more recent literature detailed in this review has begun to incorporate metabonomics methods, but as yet its practice is not being widely used in the context of female subfertility and many of the studies discussed in this review utilized the approach of targeted metabolite analysis. In particular, NMR-based metabonomics studies in this field are rare. Thus far metabonomics has been employed in gynaecological cancer research (Oduusi et al., 2005; Denkert et al., 2006; Guan et al., 2009). NMR spectroscopy itself (without the incorporation of metabonomics techniques) has also played an important role in understanding gynaecological cancer (Klimek, 1990; Celik et al., 2005). A further application where metabonomics has been used extensively is in the analysis of embryo culture medium with a view to identification of biomarkers to predict oocyte and embryo quality as reviewed by Bromer et al. (2008). NMR spectroscopy has been used in such studies, (Seli et al., 2008), however the most commonly used analytical techniques have been Raman spectroscopy (Seli et al., 2007; Scott et al., 2008) and near infra-red spectroscopy (Seli et al., 2007; Vergouw et al., 2008; Seli et al., 2010). The findings of these studies, along with others, are discussed in the review by Botros et al. (2008).

Fewer metabonomics studies have been carried out on FF or granulosa cells which surround an oocyte during its development in the ovaries and these may contain vital clues about the quality of the corresponding oocyte. Recently, Revelli et al. (2009) reviewed the use of metabonomics in the analysis of FF and concluded that most of the studies aiming to find biomarkers of oocyte quality have been on a small-scale, mostly utilizing animal models and have predominantly involved univariate analysis, identifying only certain classes of metabolite, rather than multivariate analysis. As a result, there are currently no reliable biomarkers for oocyte quality or likelihood of fertilization (Revelli et al., 2009). One of the main problems in studying FF using metabonomics techniques is the complexity of the fluid, meaning that often only one metabolite class can be studied at a time. However, NMR spectroscopy is capable of analysing all the metabolites in the intact, untreated fluid at once, and so is the ideal solution to this problem. Metabonomics or targeted metabolite analysis in conjunction with NMR spectroscopy could be applied to such samples to reveal metabolites that are indicative of good or bad oocyte quality, or to monitor changes during oocyte development. Although metabolic changes during different stages of the oestrus cycle has been previously examined in animal FF (Gerard et al., 2002; Sarty et al., 2006), human cervical mucus (Merilan, 1983a, b) and the human endometrium (Sarac et al., 2004) using NMR spectroscopy, application of the sophisticated modern metabonomics techniques to the problem may provide a more comprehensive and detailed description of changes within these fluids/tissue than has been previously possible.

It has been postulated that oxidative stress may play a role in female subfertility (Agarwal et al., 2005), yet few metabonomics studies searching for markers of oxidative stress within the female reproductive tract have been conducted. Oocyte quality will most likely be affected by oxidative stress in the surrounding FF. Evidence of oxidative stress can be identified using NMR spectroscopy since many observable metabolites are involved with oxidative stress processes and increased or decreased concentrations of these molecules can suggest the occurrence of such stress. Despite this, there has not been any NMR research investigating oxidative stress in FF, although studies have utilized non-NMR methods (Jozwik et al., 1999; Attaran et al., 2000; Oyawoye et al., 2003; Pasqualotto et al., 2004, 2009; Das et al., 2006; Basini et al., 2008). In many of these studies the use of FF oxidative stress markers in predicting oocyte quality has been investigated, but the results are conflicting and it is not certain whether oxidative stress is solely detrimental to an oocyte or whether there is a threshold level of oxidative stress that is essential for oocyte viability, but above which there are deleterious effects.

This review has shown that few metabonomics studies exist which examine the effects of various reproductive diseases such as PCOS, endometriosis, PID and C. trachomatis. Metabonomics is an ideal technique for examining disease states since healthy and diseased
individuals can be compared. Furthermore peritoneal fluid has not yet been examined and potentially may contain a vast amount of metabolic information about diseased states.

Since headway is being made into the use of NMR spectroscopy and metabolomics in the field of subfertility and assisted conception the question remains, how practical would it be to introduce NMR into a typical non-hospital associated IVF programme? This is an area for debate in all ‘health-related’ applications of metabolomics. A model is currently being developed in the USA [e.g. MatrixBio (http://www.matrix-bio.com/)] in which dedicated laboratories are being established housing NMR and MS instrumentation and expertise to provide a high-throughput service in the analysis of biological samples. A similar model may be appropriate in the UK, perhaps as an add-on to existing NHS and private clinical pathology laboratories. Cost-effectiveness of rapidly transported samples, timeliness of reporting and its impact on success rates in prospective randomized controlled trials are important areas for further investigation.

Subfertility is a significant problem with multiple aetiologies. Developing techniques to assist in a comprehensive understanding of the reproductive tract environment and nutritional status of the developing oocyte and embryo has implications for diagnostics as well as therapeutics. NMR spectroscopy may be employed within the research of subfertility to gain a better understanding of pathological conditions. It may also be used to develop subfertility therapeutic techniques for optimization of folliculogenesis and oocyte maturation during ovulation induction and supra-physiological ovarian stimulation as for IVF. Furthermore, NMR spectroscopy has the potential to be a useful tool in assisting in the selection of the embryo for transfer in IVF.

References


