Scientific and Clinical Advances Advisory Committee (SCAAC) – Matters arising

Monday 03 June 2024

Date	Action	Responsibility	Due date	Progress to date
06/06/2022	The Executive will amend the treatment add-ons application form and decision tree for considering applications for additional add-ons in line with the updated treatment add-ons rating system. SCAAC can then reconsider the application for Androgen supplementation as a treatment add-on.	Dina Halai, Head of Policy	June 2024	The Executive have amended the treatment add-ons application form and decision tree to reflect recommendations made in the **February 2023** SCAAC meeting. The application for Androgen supplementation as a treatment add-on will be discussed at the June 2024 SCAAC meeting.
03/10/2022	Consider a framework for assessing AI technologies which fall within the regulatory remit of the HFEA. Publish a Clinic Focus article for the sector on developments in the regulation of AI.	Mina Mincheva, Policy Manager	Closed	AI was discussed at the **February 2024** SCAAC meeting. The Executive has initiated a project on AI, robotics and automation in fertility treatment, which includes regulatory mapping. The Executive will communicate with the sector and patients as appropriate.
05/02/2024	Update the wording on the HFEA website to reflect that the review of treatment add-ons ratings will be caried out every five years or when new substantial evidence comes to light.	Molly Davies, Scientific Policy Officer	Closed	The treatment add-ons **webpage** has been updated to reflect the agreed review frequency.

	The standing item ‘Relevant public health developments and research findings’ will be expanded to highlight research developments relevant to add-ons.			
05/02/2024	Update the website information on long-term storage to highlight that there is a lack of evidence regarding the impact of long-term storage on viability of embryos. The wording will be agreed with select members of the Committee.	Molly Davies, Scientific Policy Officer	Closed	Patient **information** relating to freezing of gametes and embryos was updated in May 2024.
05/02/2024	Consider consulting an expert on epigenetics to comment on techniques of modifying the epigenome of the early embryo.	Mina Mincheva, Policy Manager	Closed	In March 2024 an External Adviser with expertise in epigenetics joined the Committee.

Emerging technologies in embryo and gamete testing

Details about this paper

Area(s) of strategy this paper relates to:	Shaping the future and best care
Meeting:	Scientific and Clinical Advances Advisory Committee (SCAAC)
Agenda item:	5
Paper number:	HFEA (03/06/2024) 005
Meeting date:	03 June 2024
Author:	Mina Mincheva, Policy Manager (HFEA)
Annexes	None

Output from this paper

For information or advice?	For advice
Recommendation:	Members are asked to: • Consider the progress of research into embryo and gamete testing • Advise the Executive if they are aware of any other recent developments • Review whether any outputs from the HFEA are required addressing the use of emerging technologies in embryo and gamete testing To note: Given how broad this topic is, the SCAAC can consider splitting the topic of 'Emerging technologies in embryo and gamete testing' into separate priority topics at the February 2025 meeting when the committee will be discussing its 2025/26 workplan.
Resource implications:	N/A
Implementation date:	N/A
Communication(s):	To be determined
Organisational risk:	Low

1. Introduction

1.1. This paper covers new technologies in embryo and gamete testing, including non-invasive methods for PGT-M and PGT-A.

1.2. Preimplantation genetic testing (PGT) allows the testing for hereditary genetic disorders and chromosome abnormalities in embryos before implantation.

1.3. The three main types of PGT currently offered to patients in the UK that are established techniques, and therefore will not be covered in this paper, are:

- PGT for monogenetic disease (PGT-M) tests embryos for the presence of a condition caused by a single gene mutation. As per the Human Fertilisation and Embryology Act (1990) (HFE Act), PGT-M can be offered to prospective parents who know they are carriers of a single gene mutation that could be passed on to their child resulting in them having a serious condition that is untreatable.
- PGT for chromosomal structural rearrangements (PGT-SR) is a treatment that involves looking at the chromosome structure of embryos, and finding where segments may have been deleted, duplicated or inverted. It can be used for people with a known chromosome structural rearrangement, to improve the chance of a healthy pregnancy.
- PGT for aneuploidy (PGT-A) aims to identify embryos carrying an abnormal number of chromosomes, which could lead to miscarriage or IVF failure, and to select euploid (i.e. chromosomally normal) embryos. On the HFEA's treatment add-on list, PGT-A **has been rated 'red'** for increasing the chances of having a baby for most fertility patients. This is because PGT-A is a selection tool that often reduces the number of embryos available for transfer.

1.4. There are a number of different emerging technologies under research aiming to assess embryo ploidy, such as testing cell free DNA in spend culture media or blastocoelic fluid, and time-lapse imaging combined with machine learning algorithms.

1.5. PGT for polygenic disorders (PGT-P), also referred to as polygenic risk score (PRS), has become commercially available over recent years. Polygenic disorders are diseases or characteristics where the phenotype of an individual is influenced by multiple genes, for example cancer, heart disease and diabetes. Embryos are tested and given a 'risk score' of their likelihood of developing a certain disease or characteristic based on their genetic makeup. There are many ethical and practical concerns to consider when determining whether this embryo testing can be introduced in the UK. Currently, PGT-P does not meet the criteria defined in the HFE Act .

1.6. At **the October 2021 SCAAC meeting** the Committee raised particular concerns around the lower treatment efficiency of PGT-P, the aggressive commercialisation of this technology, how it is portrayed to patients, and the lack of adequate counselling with prospective parents to make informed decisions. Further limitations of PGT-P include low accessibility due to the high cost, and that it can result in a low number of embryos available for transfer due to being highly selective.

1.7. At the HFEA's Annual Horizon Scanning Meeting at the European Society for Human Reproduction and Embryology (ESHRE) annual conference in 2021, it was highlighted that the data used for PGT-P is skewed towards those of European descent and there is a potential for both known and unknown pleiotropic effects. That undermines the utility and predictive power of

polygenic risk scores. Further discussion around polygenic risk score at the Horizon Scanning Meeting at ESHRE 2022, highlighted that environmental factors play a greater influence on many diseases than genetic susceptibility. It was also stressed that this technique requires further validation before it can be used in individuals or embryos as a clinical prediction algorithm.

1.8. There has also been an increase in research to develop other non-invasive techniques to assess embryo quality and developmental potential, such as metabolic analysis of spent culture media or using autofluorescence microscopy and combining time-lapse images with machine learning.

1.9. At **the January 2022 SCAAC meeting** the sub-topic of 'gamete testing' was incorporated into the topic of 'Emerging technologies in embryo testing', covering oocyte and sperm testing and selection as well as male infertility.

1.10. 'Metabolomic profiling' was further incorporated into the topic of 'Emerging technologies in embryo and gamete testing' at the **February 2024 SCAAC meeting**. This sub-topic was identified at HFEA's Annual Horizon Scanning Meeting held during the ESHRE conference in 2023. An invited speaker at this meeting described how metabolomic profiling has the scope to become an adjunct tool to embryo assessment for prediction of embryo implantation potential. However, the speaker noted that results from metabolomic profiling have a large dependency on the culture media used. Furthermore, the timeline for commercialisation of metabolomic applications is long, given that the safety of such technology has to be proven before bringing it to market. The speaker highlighted key challenges with metabolomic profiling related to finding technology-driven ways of using these techniques more effectively and improving the technology used. It was further discussed that while these issues are complex, metabolomic profiling does not raise a unique regulatory challenge, especially if it is only used as an assessment or quality control in the laboratory.

1.11. Considering the additions of sub-topics to the topic of 'Emerging technologies in embryo and gamete testing', research highlighted in this paper includes literature covering the following periods: 'Embryo testing' 1st Sept 2021 – 30th April 2024; 'Gamete testing' 1st Jan 2022 – 30th April 2024; 'Metabolomic profiling' 1st January 2023 – 30th April 2024 ; 'Polygenic Risk Score' Jan 2021 – 30th April 2024.

1.12. This paper provides a summary of the results of publications identified in the specified time frame during which the literature search was performed, and it provides a summary of the findings described in published studies and is not an assessment of study validity.

2. Embryo testing

Technologies for non-invasive ploidy testing:

2.1. A meta-analysis by (Huang *et al.*, 2023a) (n=20 studies) assessed the diagnostic value of non-invasive preimplantation genetic testing (niPGT) in patients undergoing IVF. The pooled sensitivity, specificity, and AUC (area under receiver operator characteristic curve [SROC]) for niPGT were 0.84 (95% Confidence Interval [CI] 0.72–0.91), 0.85 (95% CI 0.74–0.92), and 0.91 (95% CI 0.88–0.93), respectively, suggesting its potential as an alternative method for embryo analysis with relatively high detection accuracy. Subgroup analyses indicated that the sensitivity and specificity of niPGT varied based on sample size and the type of genetic material analysed.

In addition, the spent culture media (SCM) subgroup showed higher sensitivity, but lower specificity compared to the subgroup combining SCM with blastocoelic fluid. These findings highlight the need for large-scale studies to determine the detection value of niPGT.

2.2. A number of reviews collectively focus on advancements in PGT methodologies, particularly highlighting the emergence of non-invasive approaches (Rogers *et al.*, 2021; Navarro-Sánchez *et al.*, 2022; Sousa and Monteiro, 2022; Tomic *et al.*, 2022; Cimadomo *et al.*, 2023b; Del Collado *et al.*, 2023; Handayani *et al.*, 2023; Spinelli *et al.*, 2023; Moustakli *et al.*, 2024). They discuss various techniques, such as non-invasive preimplantation genetic testing for both aneuploidy and monogenic conditions (niPGT-A and niPGT-M) using SCM or blastocoel fluid. Additionally, the use of time-lapse imaging in combination with artificial intelligence algorithms to infer the ploidy status of an embryo and thus, to improve embryo selection and IVF outcomes, has also been discussed. While non-invasive methods offer potential benefits, including reduced invasiveness and increased accessibility, challenges such as variability in concordance rates with invasive trophectoderm biopsy, limitations in accuracy, and the need for further validation and standardization remain significant concerns across the studies. Further limitations with niPGT highlighted in these reviews are lower quantity and lesser quality of the cell-free genetic material, and its unknown origin. Investigation of origin of the cell-free media, the percentage of apoptotic events, and the levels of DNA contamination are warranted. Additionally, the emotional and psychological impact on couples and ethical considerations surrounding the use of these advanced techniques, are noted as important areas for future research and consideration in clinical practice.

Time-lapse monitoring (TLM) and Artificial Intelligence (AI) algorithms for inference of ploidy status

2.3. A study by (Zou *et al.*, 2024) explored the morphokinetic characteristics and clinical outcomes of mosaic blastocysts in PGT cycles. The embryos for kinetic analysis were classified as euploid (ie embryos without any chromosomal mosaicism and embryos with less than 20% mosaicism), mosaic (ie embryos containing 20%–80% of aneuploid cells), mosaic with aneuploid (ie aneuploid embryos that implied a meiotic abnormality superimposed with post-zygotic mitotic abnormalities), and uniformly aneuploid embryos. Their study of 923 blastocysts revealed that mosaic embryos reached morula stage more quickly than mosaic with aneuploid, euploid and aneuploid embryos. However, mosaic embryos resulted in lower clinical and live birth rates following transfer compared to euploid embryos. Similarly, mosaic with aneuploid embryos had lower KIDScore (an AI-scoring program from time-lapse incubation system to predict embryo implantation potential) than euploid, mosaic and uniformly aneuploid embryos.

2.4. A study by (Serrano-Novillo *et al.*, 2023) investigated the use of morphokinetic parameters and AI algorithms to predict embryo ploidy status. Analysing 374 blastocysts from PGT cycles, they identified novel morphokinetic parameters like start of t2, "st2" – detected at the beginning of the first cell cleavage – and specific cytoplasmic movement patterns associated with ploidy status. Logistic regression analysis showed a ROC value of 0.69 for ploidy prediction (95% CI, 0.62 to 0.76).

2.5. A retrospective study by (Barnes *et al.*, 2023) developed STORK-A, an automated method for non-invasive embryo evaluation using machine-learning and deep-learning approaches to predict embryo ploidy status. By using a dataset of 10,378 embryos from PGT-A cycles, STORK-A

demonstrated accuracies ranging from 63.4% to 77.6% across different classification tasks, suggesting its potential as a standardized supplement to traditional methods of embryo selection and prioritization for implantation or recommendation for PGT-A.

2.6. A study by (Jiang *et al.*, 2023)investigated the efficacy of combining convolutional neural networks (CNN), support vector machine (SVM), and multi-layer neural networks (NN) with clinical parameters to enhance the accuracy of AI in predicting aneuploidy in blastocysts. Using a cohort of 699 day 5 PGT-A tested blastocysts, they trained a CNN to classify embryos and processed patient characteristics with SVM and NN. The results demonstrated that by combining CNN with patient characteristics, voting ensembles can be created to improve the accuracy of classifying embryos as euploid/aneuploid, suggesting the potential of AI as a non-invasive method for aiding in embryo selection.

2.7. A study by (Cimadomo *et al.*, 2023a) explored the utility of iDAScore v1.0 – a deep-learning model based on a three-dimensional (3D) CNN trained on time-lapse videos from implanted and non-implanted blastocysts – in ranking blastocysts without manual input to aid in embryo selection. In a retrospective, pre-clinical, external validation involving 3604 blastocysts and 808 euploid transfers, iDAScore v1.0 showed significant associations with embryo morphology and competence, although its performance in predicting euploidy and live birth was comparable to embryologists. The study suggests its potential to enhance embryologists' evaluations, although further randomized controlled trials (RCTs) are needed to assess its clinical value.

Spent culture media for ploidy analysis

2.8. A case study by (Kulmann et al., 2021) applied niPGT-A using cell-free embryonic DNA (cfDNA) from SCM (spent culture media). They reported the birth of the first baby in Brazil using niPGT-A, with 5 out of 7 embryos showing concordant diagnoses between niPGT-A and conventional PGT-A, suggesting its potential as a safer method for embryo selection, especially for patients without an indication for conventional PGT-A.

2.9. The feasibility of niPGT-A was assessed by comparing chromosomal analysis of cfDNA in SCM with DNA from whole embryos across different morphological grades (Sonehara *et al.*, 2022). Chromosomal analysis using next-generation sequencing (NGS) was conducted on 46 pairs of embryos and corresponding SCM, revealing concordance rates of 54.5% and 62.5% for niPGT-A in low- and high-grade embryos, respectively, indicating the potential feasibility of niPGT-A for aneuploidy evaluation regardless of embryo morphology.

2.10. A study by (Ou *et al.*, 2022) compared the effectiveness of beta-thalassemia (b-thalassemia) detection using SCM alone versus SCM containing blastocoel fluid in PGT cycles. Data from 10 couples undergoing PGT for b-thalassemia were analysed, with 26 samples collected from SCM with blastocoel fluid (group A) and 33 samples from SCM alone (group B). The study found a higher concordance rate of mutation analysis of beta-globin gene (*HBB*) with biopsy results in group A (73.1%) compared to group B (45.5%), indicating that SCM containing blastocoel fluid improved b-thalassemia detection rates in non-invasive PGT.

2.11. A study by (Shitara Id *et al.*, 2021) assessed the accuracy of niPGT-A compared to traditional PGT-A using trophectoderm (TE) biopsy after extended embryo culture. SCM and TE cells were collected from thawed 20 blastocysts, cultured for up to 10 days post-thaw, and subjected to chromosome analysis using NGS. NiPGT-A demonstrated a higher concordance rate (56.3%)

with outgrowth samples compared to PGT-A (43.8%). Sensitivity, specificity, positive predictive value (PVV) and negative predictive value (NPV) were 100%, 87.5%, 88.9% and 100%, respectively for niPGT-A, while those for PGT-A were 87.5%, 77.8%, 87.5% and 14.3%.

2.12. A study by (Huang *et al.*, 2021) investigated a non-invasive method, NICS (non-invasive implantation capability screening) for preimplantation genetic testing on SCM, outlining the full protocol of NICS, with details on culture medium sampling methods, whole genome amplification (WGA), library preparation, and NGS data analysis.

2.13. A study by (Xu *et al.*, 2023a) assessed whether cfDNA from blastocyst culture media (BCM) could better reflect embryonic chromosome status compared to TE (trophectoderm) biopsy in niPGT-A. They conducted WGA followed by NGS on samples from 35 donated embryos, showing comparable chromosomal ploidy, diagnostic, and sex concordance rates between BCM and TE biopsy.

2.14. A study by (Sialakouma *et al.*, 2021) evaluated the diagnostic performance of niPGT-A by comparing results from TE biopsies and SCM using NGS. The study demonstrated a high overall concordance rate of 81.8% between niPGT-A and conventional PGT-A, with an aneuploidy agreement of 91.66% and euploidy agreement of 76.19%.

2.15. A study by (Olcay et al., 2022) aimed to discriminate aneuploidy in human preimplantation embryos by analysing their amino acid metabolism profile in SCM. Using NGS with trophectoderm biopsy, they evaluated 40 euploid and 71 aneuploid blastocysts from 58 couples, finding significantly higher levels of tyrosine amino acid in the SCM of aneuploid embryos, suggesting altered amino acid turnover as a potential non-invasive method for embryo selection in ART.

2.16. A study by (Yang *et al.*, 2023) investigated the utility of blastocoel fluid and SCM for niPGT compared to control invasive methods, (whole embryo, trophectoderm [TE] and inner cell mass [ICM] biopsy). Using single-cell WGA and NGS, they found higher genomic similarity between SCM and ICM, than that of TE biopsy suggesting SCM as a more effective source of embryonic DNA for niPGT.

2.17. Non-invasive PGT-A (niPGT-A) combined with time-lapse morphokinetics was validated within a routine IVF laboratory workflow, and its concordance with conventional PGT-A was examined (Tsai *et al.*, 2022). Using single-embryo culture time-lapse incubators, 118 SCMs were collected, demonstrating a 67.7% amplification rate for niPGT-A, indicating its potential application in clinical IVF for improved euploidy prediction for transfer.

2.18. A study by (Chow *et al.*, 2024) aimed to optimise niPGT-A efficacy by evaluating two collection timings of SCM (day 5 vs day 6), two embryo rinsing protocols, and conventional insemination versus ICSI. They found that using the sequential rinsing method with SCM collected on day 6 resulted in significantly higher rates of concordance compared to day 5 samples (total concordance: 85.0% vs. 60.0%, $p = 0.0228$), with no significant difference in niPGT-A outcomes between conventional insemination and ICSI, suggesting these optimization steps are crucial prior to future RCTs.

2.19. A study by (Sun *et al.*, 2023a) investigated whether using TE biopsy with NICS (ie analysis of blastocyst culture fluid and blastocyst cavity fluid) can improve clinical outcomes for normal chromosomes and chromosomal rearrangement groups. They found high concordance and

sensitivity rates for NICS in both groups, with higher clinical pregnancy and ongoing pregnancy rates in the euploid TE / euploid NICS group as opposed to the euploid TE / aneuploid NICS group (71.2% and 67.3% versus 54.5% and 51.5%, respectively).

2.20. A study by (Chen *et al.*, 2022) performed chromosome sequencing of 345 paired blastocyst culture medium (BCM) and whole blastocyst samples, and developed a non-invasive embryo grading system based on a rBLASTandom forest machine learning algorithm to predict blastocyst ploidy. Embryos were graded as A, B or C according to their euploidy probability levels predicted by non-invasive chromosomal screening (NICS). Higher live birth rates and lower miscarriage rates were observed in A- and B-grade embryos versus C-grade embryos and higher embryo utilisation rates via the machine learning strategy compared with niPGT-A only.

Other non-invasive methods for embryo testing

2.21. A study by (Tvrdonova *et al.*, 2024) investigated the predictive value of morphokinetic parameters from time-lapse monitoring (TLM) for selecting embryos with high developmental potential in assisted reproduction, analysing 1060 embryos (585 euploid and 475 aneuploid embryos after PGT-A) cultured in a time-lapse incubator. They found that t5 (time of division into 5 cells) and tSB (time of start of blastulation) differed significantly between embryos that resulted in a live birth and those without foetal heartbeat after frozen embryo transfer (FET).

2.22. A study by (Tan *et al.*, 2021) developed a label-free, non-invasive optical imaging by hyperspectral autofluorescence microscopy to discern between euploid and aneuploid cells within inner cell mass (ICM) of mouse preimplantation embryos. They employed primary human fibroblasts with known karyotypes and a mouse model treated with a reversible spindle assembly checkpoint inhibitor during the four- to eight-cell division. Changes in cellular metabolism were determined by quantification of metabolic cofactors (inferred from their autofluorescence signature) such as nicotinamide adenine phosphate dinucleotide dehydrogenase (NAD(P)H) and flavins with the subsequent calculation of the optical redox ratio. Results showed high accuracy in distinguishing between euploid and aneuploid cells, indicating the potential of this method as a new diagnostic tool for embryo analysis.

2.23. A study by (Shah *et al.*, 2022) aimed to detect metabolic differences in euploid versus aneuploid human blastocysts using fluorescence lifetime imaging microscopy (FLIM). Embryo metabolic state was assessed using FLIM to measure the autofluorescence metabolic factors nicotinamide adenine dinucleotide dehydrogenase (NADH) together with NAD(P)H and flavin adenine dinucleotide (FAD). They found significant differences in metabolic parameters between euploid and aneuploid embryos, suggesting that FLIM may be a useful non-invasive clinical tool for identifying the ploidy status of embryos.

2.24. A retrospective study by (Liang *et al.*, 2024) examined the clinical outcomes of patients who underwent PGT and either invasive (n=138 patients) or non-invasive (n=183) prenatal testing after euploid blastocyst transfer. They found no significant differences in clinical outcomes between patients who received invasive versus non-invasive testing, suggesting that non-invasive prenatal testing could be a suitable alternative to detect foetal chromosomal status in PGT cycles.

2.25. A randomized double-blind controlled trial by (Cheng *et al.*, 2023) which is underway aims to compare the efficacy of embryo selection based on morphology alone versus niPGT-A and

morphology in infertile women undergoing IVF (n=500). The primary outcome, live birth per first embryo transfer, will be assessed to determine the effectiveness of niPGT-A in improving IVF success rates.

2.26. A study by (Salih *et al.*, 2023) reviewed 20 articles to compare the performance of AI models, trained on various inputs including images, time-lapse data, and clinical information, with that of standard embryo selection by embryologists in IVF. AI models demonstrated higher accuracy in predicting embryo morphology grade (median accuracy of 75.5% [range 59-94%]) and clinical pregnancy outcomes of (77.8% [range 68-90%]) compared to human embryologists' assessment (65.4% [range 47-75%] for embryo morphology grade, and 64% [range 58-76%] for clinical pregnancy prediction, respectively), indicating its potential to enhance objectivity in embryo selection. Despite the promising results, limitations include the lack of prospective evaluation in a clinical setting, heterogeneity among studies, and the need for external validation of AI models on larger datasets.

PGT for polygenic disease risk (PGT-P)

2.27. The following studies and reviews emphasise the ethical and societal implications of polygenic embryo screening (PES), including concerns about its premature clinical implementation, potential health risks, societal inequalities, and the need for comprehensive guidelines and regulations. Some of them focus on the technical aspects, progress, and potential societal impacts of PES, including discussions on genetic prediction, clinical application, and ethical considerations.

2.28. Ethical concerns have been raised about selecting embryos for non-disease-related socially desirable traits, and potential moral and ethical non-compliance with Islamic law (Chin *et al.*, 2023).

2.29. The potential harms of polygenic embryo screening (PES) are discussed, such as effects of biopsy procedure and possible epigenetic changes on the offspring, on prenatal and postnatal health (Ginod and Dahan, 2023).

2.30. A study by (Siermann *et al.*, 2023) explores healthcare professionals' perspectives on the validity, utility, limitations and potential benefits of PGT-P, highlighting concerns about its prematurity, psychological impact on prospective parents, and ethical considerations related to validity and utility of PGT-P. Results from this study are critically discussed in an opinion piece by (Makrythanasis *et al.*, 2023).

2.31. A study by (Pagnaer *et al.*, 2021) evaluates how information about PGT-P is communicated in press media and explores diverse ethical themes in the public debate. Authors identified five main ethical themes that are also present in academic literature and the broader debate on reproductive technologies: a slippery slope towards designer babies, well-being of the child and parents, impact on society, deliberate choice, and societal readiness.

2.32. Socio-ethical issues of PGT-P from the perspective of healthcare professionals have been discussed, including concerns about selection for medical and non-medical traits, commercialisation, and societal impacts (Siermann *et al.*, 2024b).

2.33. A study by (Barlevy *et al.*, 2024) compared perspectives of reproductive endocrinology specialists and IVF patients on PES. Results highlight a gap between clinician reservations and patient

attitudes. Both groups highlighted potential benefits like embryo selection and preparation for a birth of a predisposed child, but also raised concerns about biases, the probabilistic nature of screening complicating counselling, and the lack of long-term data supporting its clinical use.

2.34. A study (Wu *et al.*, 2023) discuss the technical composition, recent progress, and future prospects of PGT-P, especially how to establish a complete and suitable screening model for the Chinese population.

2.35. A study by (Meyer *et al.*, 2023) analyse public attitudes toward PGT-P and discusses potential societal impacts and inequities. Authors argue that public attitudes towards technology should be considered when informing policymaking, although diverse expert input is crucial for understanding complex issues such as unintended effects, societal impact, equitable access, and regulation, particularly in the context of legal uncertainties surrounding reproductive decisions.

2.36. A review conducted by (Capalbo *et al.*, 2021) explores the potential of preconception genome analysis, particularly in the context of IVF, to detect reproductive and individual health risks, providing couples with increased reproductive autonomy by identifying inheritable genetic conditions and medically actionable secondary findings prior to conception. The study highlights the role of preconception genomic testing in advancing reproductive precision medicine and addressing previously unexplained causes of infertility, paving the way for personalized genomic medicine in reproductive health.

2.37. The utility and ethical considerations of embryo selection based on polygenic risk scores (PRS) have been evaluated by (Polyakov *et al.*, 2022).

2.38. An opinion piece by (Hockings, 2023) advocates for a re-evaluation of the social contract surrounding genomics, emphasising transparency, oversight, and public education about life sciences, particularly in light of increased government involvement and international competition in life science sectors.

2.39. A review by (Siermann *et al.*, 2022b) analysed 38 normative documents by advisory committees at the national, European, and global level to understand what the current consensus and disagreements are on ethical acceptability of PGT-M and how this compares with PGT-P. The study concludes that ethical acceptability for PGT-P is limited, highlighting ethical difficulties regarding severity, risk, autonomy, and informed decision-making.

2.40. A statement by the International Society of Psychiatric Genetics discussed the scientific and ethical challenges of PES (Lenczet al., 2022). It emphasises the need for caution, further research, and oversight, particularly regarding the statistical properties of polygenic risk scores in the context of clinical application.

2.41. A review by (Raben *et al.*, 2022) analysed the present status and future prospects of genomic prediction of complex traits in humans, emphasizing the potential of PRS to predict disease susceptibility and discussing their applications in various contexts, including in IVF for embryo selection and genetic engineering.

2.42. Using a computational approach combining whole-genome sequencing of parental genomes and genotyping of day 3 and day 5 embryos (n=110) a study by (Kumar *et al.*, 2022) achieved accurate prediction of inherited genomes and calculation of PRS across 12 common conditions, with genotype accuracy ranging from 97.2% to 99.4%. Combining rare variants with PRS

revealed substantial differences in predicted risk across sibling embryos, suggesting the potential utility of genome-based PGT in clinical practice for reducing the transmission of common diseases. Results from this study are critically discussed in an opinion piece by (Johnston and Matthews, 2022).

2.43. An opinion piece by (Pereira *et al.*, 2022) explores the results of a roundtable of multidisciplinary expert discussion on PES and identifies associated clinically relevant issues, further emphasising the need for reproductive specialists' perspectives in discussions surrounding PES and its implications.

2.44. A special issue by (Rubio and Simón, 2021) highlights a collection of reviews and original articles on embryo diagnosis and genome editing, focusing on preimplantation genetic testing for chromosomal abnormalities and genetic disorders to improve clinical outcomes in infertility treatment.

2.45. A systematic review by (Siermann *et al.*, 2022a) explored the perspectives of healthcare professionals on the appropriate scope of PGT, highlighting ethical tensions around seriousness, informed decision-making and preventative medicine associated with expanding indications of PGT and the development of PGT-P. They argue that while PGT-P could augment patients' reproductive autonomy, it could also lead to an increased reproductive 'burden' for patients.

2.46. A study by (Lencz *et al.*, 2021) focused on predicting the expected reduction in complex disease risk following PES. They evaluate the impact of various factors on the utility of screening, including selection strategy, disease prevalence, and parental genetic profiles, while discussing potential ethical concerns.

2.47. A study by (Ginod and Dahan, 2024) discuss the commercialization and ethical implications of PES. The study raises concerns about reproductive autonomy, increasing social inequalities, and altering healthcare relationships, emphasizing the need for collaborative approaches between professionals, the public and government to address these issues.

2.48. The European Society of Human Genetics (ESHG) published an opinion piece on using PRSs for embryo selection, highlighting ethical concerns and the need for public debate and further research to ensure proper understanding and oversight (Forzano *et al.*, 2022). It highlights an argument that PRSs only capture a fraction of the total heritable component of a trait and may not be applicable across diverse ethnic groups. Moreover, PRSs are not yet widely used in clinical settings due to their limitations, including the lack of comprehensive assessment of genetic and environmental risks.

2.49. A study by (Siermann *et al.*, 2024a) aimed to assess whether the regulatory approaches for PGT in Europe would accommodate the implementation of PGT-P. Analysing regulatory frameworks in seven European countries, the study identified three models of PGT regulation and found that, regardless of the model, the current legal frameworks and criteria seem to exclude PGT-P, mainly due to healthcare professionals' and scientific organizations' negative stance toward its implementation.

Whole Genome Sequencing (WGS)

2.50. A study by (Li *et al.*, 2023c) developed a pipeline for ultra-low-coverage whole genome sequencing (ulc-WGS) data analysis and benchmarked its effectiveness for genome-wide

association studies (GWAS). Applying this method to samples of 1744 transferred embryos, authors identified 11 genomic risk loci associated with gestational ages and mapped 166 genes to these loci, providing insights into the genetic variations of foetal embryos from the Chinese population and their relationship with gestational age-related outcomes.

2.51. Data from ~10,000 PGT-A biopsies analysed by ulc-WGS was used to impute genotype likelihoods of genetic variants in embryo genomes, enabling a GWAS of aneuploidy incidence (Sun *et al.*, 2023b). One locus on chromosome 3 significantly associated with meiotic aneuploidy risk was identified, with CCDC66 identified as a candidate gene involved in chromosome segregation during meiosis, confirmed through functional evaluation in a mouse oocyte system. The results provide insights into the research utility of PGT-A ulc-WGS data of genetic contribution to maternal meiotic aneuploidy risk and introducing a generalisable method for similar association studies leveraging ulc-WGS data.

2.52. A study by (Chien *et al.*, 2024) used digitally estimated mitochondrial copy number (mtCN) and telomere length (TL) to identify associations with the implantation potential of blastocysts (n=965) in PGT-A FET cycles (n=232). Using low-pass WGS data from PGT-A and machine learning models, authors found a significant positive association between TL and pregnancy outcomes, with decision tree and random forest models achieving classification accuracies of 0.82 and 0.91, respectively. The results suggest the importance of TL in predicting embryo survival post-transfer and suggesting potential improvements in clinical infertility treatment strategies.

2.53. A study by (Chavli *et al.*, 2024) used single-cell whole-genome sequencing (scKaryo-Seq) to detect mosaicism involving numerical and structural chromosome abnormalities in 82% of 55 good-quality surplus blastocysts, with most abnormalities affecting less than 20% of the cells. Structural abnormalities, potentially arising from replication stress and DNA damage, were observed in 69% of the embryos, suggesting that mosaicism is prevalent in good-quality blastocysts and may not be adequately identified by current bulk DNA-Seq techniques used for PGT-A.

2.54. The method of ucl-WGS was applied on embryo biopsies to develop an approach to infer sex-specific recombination landscapes (Ariad *et al.*, 2024). Results demonstrated high accuracy even at coverages as low as 0.02×. Applying this method to PGT-A data from 18,967 embryos, authors mapped 70,660 recombination events with ~150 kbp resolution, revealing chromosome-specific alterations in crossover distributions and providing insights into the role of aberrant meiotic recombination in the origin of aneuploidies.

2.55. A case study by (Neumann *et al.*, 2021) reports on the use of a low cost PGT-M approach with WGA templates and NGS for detecting propionic acidemia (PA) in embryos obtained from a confirmed heterozygous propionyl-CoA carboxylase alpha subunit (PCCA) couple. This approach identified a wild-type PCCA male embryo and a heterozygous PCCA variant female embryo, leading to a clinical pregnancy and delivery of a healthy male newborn without PA.

Novel methods for embryo testing

2.56. A study by (Pan *et al.*, 2021) investigated cell-free DNA integrity (cfDI) in follicular fluid (FF) and spend culture media (SCM) as a potential biomarker for high-grade embryo selection in IVF/ICSI cycles. Results showed significantly lower cfDI in FF corresponding to subsequent high-grade embryos, while cfDI in SCM was positively correlated with high-grade embryos. ROC curves of

the analysis of cfDI in FF showed great potential in predicting subsequent embryogenesis and embryo grade (AUC > 0.927).

2.57. A single-centre study by (Zhong *et al.*, 2023) investigated whether the trophectoderm (TE) of human reconstituted embryos after spindle transfer (ST) accurately represents the ICM in terms of chromosome copy number variations (CNVs) in each cell (22 embryos from ICSI and 23 from ST), using single-cell multi-omics sequencing of blastocysts from both conventional ICSI and ST. Results showed good chromosomal concordance between TE and ICM in the ICSI blastocysts (kappa = 0.659, P < 0.05), but not in ST blastocysts (P = 1.000), suggesting that the TE in reconstituted embryos is not representative of ICM.

2.58. A new system, Morphological Analysis and Genetic Integrality Criterion (MAGIC), was developed to correlate the morphology of biopsied TE cells to their quality and subsequent genetic testing outcomes in PGT (Kuo *et al.*, 2023). Biopsied TE cells were first evaluated according to the MAGIC procedure, followed by WGA and library construction, and then sequenced using the Illumina X Ten Platform. Results demonstrated that selecting high- or good-quality TE cells for genetic testing significantly reduces allele drop-out rates and false-positive mosaicism, thereby enhancing the accuracy and reliability of PGT results.

2.59. A retrospective multicentre study by (Pons *et al.*, 2023) developed a novel blastocyst scoring system, using 1044 Day 5 blastocysts. They ranked the blastocysts for their likelihood of live birth using a standardised grading system. Through morphological and morphometric assessments of blastocyst expansion degree, TE and ICM quality, cut-off points were established for blastocyst expansion degree and TE grade, with logistic regression showing TE grade as the sole predictor of live birth likelihood, further confirmed by a decision tree analysis.

2.60. A nested case-control study by (Wang *et al.*, 2023a) investigated whether the glycan profile in blastocyst culture media (BCM) could predict implantation outcomes in IVF/ICSI cycles (n=78). Glycosylation patterns revealed differences between successful and failed implantations, with increased glycans binding to certain lectins in successful cases, suggesting the potential of glycan profiling as a non-invasive biomarker for embryo viability assessment.

2.61. A review by (Sciorio *et al.*, 2023) explores the benefits of time-lapse technology for enhanced insight in dynamic events of embryo development, such as blastocyst collapse events and morphometric blastocyst assessment and their association with embryo viability and implantation potential.

2.62. A study by (Zeng *et al.*, 2023) investigated the association between follicular FF perfluoroalkyl acid (PFAA) concentrations and embryo quality in 729 women undergoing IVF treatment, utilizing ultra-performance liquid chromatography coupled to tandem mass spectrometry to measure 32 PFAA. The high-quality embryo rates at the 50th percentile of linear perfluoro-1-octanesulfonate acid (n-PFOS), all branched PFOS isomers (Br-PFOS) and linear perfluoro-n-octanoic acid (n-PFOA) were −6:34% [95% confidence interval (CI): −9:45, −3:32%], −16:78% (95% CI: −21:98, −11:58%) and −8:66% (95% CI: −11:88, −5:43%) lower, respectively, than the high quality embryo rates at the reference 10th percentile of PFAA. Branched PFOS isomers showed a stronger negative effect than linear PFOS isomers, suggesting a potential adverse impact of PFAA exposure on IVF outcomes.

2.63. A stud by (Farias *et al.*, 2023) present the development of an automatic method for segmenting morphological structures of blastocysts, such as the zona pellucida (ZP), TE, blastocoel (BC), and ICM, during various developmental stages. They used 2132 raw images for training and 55 for validation. After validation against a public repository of 249 images, the method resulted in accuracies of 0.96 and 0.93 and Dice similarity coefficient (DSC) of 0.67 and 0.67 for ICM and TE, respectively.

2.64. (Goswami *et al.*, 2024) introduced EVATOM, a machine-learning assisted embryo health assessment tool utilizing artificial confocal microscopy (ACM) for label-free nucleus detection and novel quantitative embryo health biomarkers. EVATOM achieves high accuracy in grading embryos into healthy/intermediate (H/I) or sick (S) classes, with weighted F1 scores of 1.0 and 0.99 on in-distribution test sets of 72 fixed embryos and 0.9 and 0.95 on out-of-distribution test datasets 19 time-instances from 8 live embryos, respectively.

2.65. (Yang *et al.*, 2022) introduced a novel procedure, TAGs-seq PGT-A/M, for simultaneous detection of monogenic diseases and genomic imbalances in embryos during IVF treatment. The library preparation method involved integrating multiplex polymerase chain reaction (PCR) into the WGA process, enabling subsequent one-step low-pass whole genome sequencing (WGS) and high-depth target enrichment sequencing (TES) using the resulting library. The method, validated both with genomic DNA and clinical samples, achieved over 90% coverage of the whole-genome region and identified embryos with genomic imbalances and β-thalassemia with high consistency to conventional PGT-M methods, demonstrating its potential as a universal approach for embryo screening.

2.66. (Xie *et al.*, 2022) used 188 embryo samples to develop a comprehensive PGT method, HaploPGT, capable of detecting various genetic disorders simultaneously. By combining reduced representation genome sequencing, read-count analysis, B allele frequency and haplotyping analysis, HaploPGT demonstrated accurate identification of genetic abnormalities in embryos, achieving 100% concordance with reference methods for PGT-A, PGT-M, PGT-SR (PGT for structural rearrangements), and PGT-HLA. Despite its benefits, HaploPGT requires additional family members for haplotype deduction, and limitations exist in distinguishing between haploid and diploid embryos and detecting partial genetic disorders.

2.67. (van Dijk *et al.*, 2022) developed an embryo tracking system (ETS) to track embryos right after WGA to full genome haplotype profiles to increase scalability and efficacy of PGT. Authors used 322 whole-genome amplified (WGAed) DNA samples derived from IVF embryos as well as 563 bulk DNA isolated from peripheral blood of prospective parents. To determine possible interference of the ETS in the NGS-based PGT workflow, barcoded DNA fragments were added to DNA samples prior to library. The new ETS-PGT approach had one step only in the entire PGT procedure that needed the four-eyes principal as compared to six manual control steps in PGT. Furthermore, ETS-PGT had no effect on the genomic landscape of preimplantation embryo. Results suggest that ETS-PGT could easily be adapted to any sequencing-based diagnostic method, including PGT-SR and aneuploidies by low-pass sequencing as well as non-invasive prenatal testing.

2.68. (De Witte *et al.*, 2022) developed a pipeline for comprehensive PGT for blastocysts that is suitable for parents-only haplotyping and third party reproduction. Samples (n=104 blastocysts) were WGAed and processed by GENType. Quality metrics, genome-wide haplotypes, b-Allele

frequencies (BAFs) and copy number profiles were generated by Hopla. PGT-M results were deduced from relative haplotypes, while PGT-SR/PGT-A results were inferred from read-count analysis and BAF profiles. For both PGT-M and PGT-SR/PGT-A the technology demonstrated 100% concordance with reference PGT methods for diverse WGA methods. Equally, for parents-only haplotyping and single-parent haplotyping (of autosomal dominant disorders and X-linked disorders), PGT-M results were fully concordant.

3. Metabolomic profiling

3.1. A study by (Zhao *et al.*, 2023a) argued that dynamic nutrient requirements during pre-implantation embryo development are essential to support the energetic and biosynthetic needs of early embryos. The authors proposed the concept of a new class of 'developmental metabolites' and hypothesised that they may play an important role not only in metabolism but also in regulating development. The criteria to define such metabolites are:

- metabolites should be specifically present at a certain stage or in a specific type of cell during early embryo development, while rarely present during other physiological contexts; or metabolites may be more broadly present but should exhibit a specific function related to development at a specific time.
- metabolites should be involved in regulating development through a clearly defined mechanism or process.

3.2. A study by (Xu *et al.*, 2023b) used metabolome and transcriptome analysis to evaluate the global metabolomic profiles of follicular fluid (FF) from women with polycystic ovarian syndrome (PCOS). The authors demonstrated that PCOS women exhibited distinct metabolic features in follicles, such as the increase in fatty acid utilization and the downregulation in amino acid metabolism. A review by (Minasi *et al.*, 2023) provides an overview of different approached to evaluate oocyte quality and competence including metabolomic analysis of spent culture medium.

3.3. A review by (Dai *et al.*, 2024) summarises the evidence on alterations in FF composition in PCOS. Evidence demonstrates a pronounced proinflammatory milieu characterized by increased inflammatory cytokine levels and dysregulation in immune cells. Additionally, PCOS FF presents with dysregulation in reactive oxygen species production, antioxidant defences, microRNAs, proteomic pathways to immune responses, and metabolites, suggesting potential adverse effects on oocyte quality and fertility outcomes.

3.4. A study by (Martínez-Moro *et al.*, 2023) performed metabolomics analysis on cumulus cells (CC) from cumulus–oocyte complexes (COCs) of IVF/ICSI cycles with known reproductive outcome. The abundance of malonate, 5-oxyproline, and erythronate in CC was significantly higher in COCs that ultimately established a pregnancy, providing clues on the pathways required for oocyte competence.

3.5. A study by (Liang et al., 2023) combined metabolomic profiling of spent embryo culture medium and clinical variables to create an implantation prediction model as an adjunct to morphological screening of day 3 embryos (42 embryos from 34 IVF patients) with an accuracy of 0.88. Similarly, (Cheredath *et al.*, 2023) used metabolomic data from spend culture medium and embryological data of day 5 blastocysts (from 56 infertile couple undergoing ICSI) to develop custom artificial neural network model for prediction of embryo implantation potential.

3.6. A study by (Liu *et al.*, 2023b) performed a targeted metabolomics study in plasma from early embryonic development arrest (EEDA) patients (n = 27) and normal pregnant women (NPW, n = 27) to identify potential diagnostic marker metabolites. The authors suggest that S-methyl-5'-thioadenosine, kynurenine, leucine, and malate could be used as a panel of metabolites for EEDA diagnosis, with area under the curve (AUC) of 0.941.

3.7. A study by (Molina *et al.*, 2023) analysed receptive-phase endometrial metabolome profiles among women from couples with infertility of different aetiology and the associations of these profiles with Mediterranean diet (MD). The authors found lower levels of polyunsaturated fatty acids in women with endometriosis and recurrent implantation failure compared to those with no clear endometrial alterations. Moreover, MD adherence seemed to be associated with the endometrial metabolomic profile in a manner dependent on the health status of the uterus.

3.8. A study by (Venturas *et al.*, 2022) performed metabolic imaging via fluorescence lifetime imaging microscopy (FLIM) on 215 discarded embryos measure the autofluorescence of two central coenzymes, NAD(P)H and FAD. The results demonstrated significant metabolic variations between discarded human blastocysts, influenced by developmental stage, time since fertilization, and individual differences. Metabolic heterogeneity was observed within individual blastocysts, including between cell types and regions, highlighting FLIM's potential for non-invasive assessment of embryo viability and quality.

3.9. A single-centre retrospective cohort study was conducted to investigate the impact of post-warming culture time on blastocyst metabolism and pregnancy outcome in FET cycles (Ardestani *et al.*, 2024). They analysed outcomes from 11,520 FET cycles and performed non-invasive metabolic imaging of discarded human blastocysts using FLIM. Results showed that differences in post-warming culture time did not significantly affect live birth or miscarriage rates, suggesting minimal impact on pregnancy outcomes.

4. Oocyte selection and testing

4.1. Two novel methods for oocyte testing have been developed. (Liu *et al.*, 2021a) assessed the ability to use RNA sequencing of granulosa cells as a method to assess oocyte quality. Authors highlighted that further studies are needed to assess whether the genes that were found to associate with developmental outcomes could be used to predict oocyte quality and embryo development. Similarly, (Daei-Farshbaf *et al.*, 2021) calcineurin levels to predict oocyte fertilisation potential, but further study is required before it is considered as an oocyte selection method.

4.2. A study by (Liu *et al.*, 2023a) investigated the role of ATP-dependent Lon peptidase 1 (LONP1) in oocyte meiotic defects associated with advanced maternal age. The findings reveal that decreased expression of LONP1 disrupts meiotic progression, mitochondrial function, and increases DNA damage in oocytes, suggesting LONP1 as a potential therapeutic target to improve aged oocyte quality.

4.3. A retrospective by (Li *et al.*, 2023a) analysed 10,878 IVF PGT-A cycles to identify if Anti-Müllerian hormone (AMH) could predict markers of oocyte quality. AMH levels independently predicted the likelihood of obtaining ≥ 1 euploid embryo for transfer, adjusting for age and number of embryos biopsied. However, neither age nor AMH were predictive of live birth once a euploid embryo was

identified by PGT-A, suggesting AMH's role in predicting aneuploidy risk, but not live birth per transfer following PGT-A.

4.4. A deep learning image analysis was performed using a model that was developed and validated to predict blastocyst development from static images of 37,133 denuded mature oocytes (Fjeldstad *et al.*, 2024). The model achieved favourable performance metrics (AUC of 0.64, balanced accuracy of 0.60, specificity of 0.55, and sensitivity of 0.65) on the test dataset (n = 7807). Subgroup analyses revealed highest performance for the age group 38-39 years (AUC 0.68), negligible impact of male factor, and good generalisability across geographical locations, with model predictions correlating with blastocyst quality on a converted scoring scale.

4.5. A study by (Wang *et al.*, 2022) compared expression patterns in follicular fluid (FF) and cumulus cells of diminished ovarian response (DOR) patients and control subjects undergoing ICSI, identifying 31 differentially expressed proteins between mature and immature oocytes. Gene ontology (GO) enrichment analysis revealed enrichment trends in 'cell population proliferation', while KEGG (Kyoto Encyclopaedia of Genes and Genomes) enrichment classification revealed significant pathways such as phagosome process and the PI3K-Akt signalling pathway. Additionally, proteins such as prostatic acid phosphatase (ACPP) and CD5 antigen-like (CD5L) were validated as indicators of oocyte quality, with ACPP showing lower levels in DOR patients and CD5L upregulation in their FF, providing valuable insights into assessing oocyte maturation and quality.

4.6. A prospective study by (Skowrońska *et al.*, 2022) investigated the relationship between intrafollicular vitamin D and AMH concentrations in 208 FF samples from 33 IVF patients. The findings suggest that vitamin D concentration in FF correlates with oocyte developmental stage and embryo development status on day 3, while such correlation was not observed for AMH concentration in FF.

4.7. A review by (Anagnostopoulou *et al.*, 2022) discusses the importance of gamete and embryo assessment in assisted reproduction techniques, emphasizing the impact of oocyte maturity on embryo quality and subsequent fertilization rates. Various methods for assessing embryo quality, including morphology assessment, preimplantation genetic testing, morphokinetics, proteomics, and metabolomics, are compared to identify the most effective approaches for selecting embryos with the highest implantation potential, aiming to optimize the success of pregnancy outcomes in ART treatments.

5. Sperm selection and testing

Reviews covering various selection and testing approaches

5.1. An editorial by (Li *et al.*, 2023b) presents 13 articles, including original research and reviews that cover diverse topics such as identifying gene variants associated with abnormal sperm parameters, discovering protein biomarkers of sperm quality, evaluating different sperm preparation techniques for ICSI, and discussing emerging trends in sperm selection methods and sperm function evaluation.

5.2. The potential of precision medicine approaches was emphasised, including WGS and proteomic analyses, to optimise outcomes for male infertility management (Nixon *et al.*, 2023). Advanced

imaging technologies coupled with machine learning hold promise in identifying infertility biomarkers and providing new avenues for diagnostics and treatment of male infertility.

5.3. Four reviews by (Itoi *et al.*, 2022), (Baldini *et al.*, 2021), (Gallagher *et al.*, 2023) and (Pareek *et al.*, 2023) collectively highlight various novel techniques and advancements in sperm selection for ART and discuss potential pitfalls and advantages of each technique. These reviews focus particularly on artificial intelligence; microfluidics; automated computer-based methods to assess sperm head size, shape and acrosome status; micro swim-up directly on the ICSI dish and laser-assisted selection of immotile sperm (LAISS).

Selection with zona pellucida binding

5.4. A study by (Ganeva *et al.*, 2024) compared outcomes between ICSI using sperm selected based on their ability to adhere to solubilised and immobilised ZP (zona pellucida) and conventionally selected sperm. Implantation rate, clinical pregnancy rate and live birth rate were significantly higher, with lower incidents of miscarriage in the group with sperm selected by zona adhesion.

5.5. A study by (Izadi *et al.*, 2024) investigated embryo morphokinetics following ICSI with ZP-bound sperm selection versus conventional methods. There was a significant difference between control in ZP-bound groups for t8 (time of cleavage to eight cells) and cc3 (duration of third cell cycle) The ZP-bound group also exhibited higher rates of Grade A embryos, chemical pregnancy, clinical pregnancy, and implantation.

5.6. Additionally, (Leung *et al.*, 2023) explored the role of spermatozoa-ZP interaction in selecting fertilization-competent spermatozoa. The results showed that ZP-bound spermatozoa had significantly higher expression levels of heat shock 70 kDa protein 2 (HSPA2) and sperm acrosome associated 3 (SPACA3) - protein markers associated with the sperm ZP-binding ability - along with higher levels of normal morphology, DNA integrity, chromatin integrity, protamination, and global methylation compared to unbound spermatozoa.

5.7. A study by (Handzhiyska *et al.*, 2024)compared the efficacy of conventional swim-up and cumulus matrix (CM) sperm selection methods on sperm motility, morphology, and DNA fragmentation (n= 60 normozoospermic men). CM sperm selection significantly increased sperm motility and reduced morphologically abnormal spermatozoa and DNA fragmentation rates in comparison to the conventional swim-up preparation.

Microfluidics technology

5.8. Three reviews summarise how microfluidic technology can complement and optimise current sperm sorting and IVF protocols. (Ma *et al.*, 2024) highlight the potential for improving sperm selection and evaluation for IVF and ICSI, discussing the structural design of microfluidic platforms, their screening results, and their integration into various steps of the IVF process. (Bouloorchi Tabalvandani *et al.*, 2024) underscore the utility of microfluidics in infertility diagnosis and treatment, focusing on sperm separation and analysis. The review also discusses recent advancements in on-chip fertilisation. (Olatunji and More, 2022) outline the use of microfluidics technology in selecting and assessing sperm parameters and how it affects male infertility. Additionally, (Jahangiri *et al.*, 2023) reviewed microfluidic sperm sorting (MSS) techniques for treating infertility. Authors highlight the predominance of motility-based properties in current sperm selection methods and identified low throughput as a major limitation. They highlight the

need for further research to optimise these platforms, particularly in utilising properties like chemotaxis and rheotaxis.

5.9. A meta-analysis by (Aderaldo *et al.*, 2023) found only marginal positive outcomes when using MSS compared to standard sperm selection techniques for couples undergoing ICSI, with no statistical significance in the results. The study underscores the need for further validation through large-scale clinical trials to determine the true efficacy of MSS in enhancing ART outcomes.

5.10. 3D device to be inserted above semen samples in a test tube that densely packs thousands of channels to optimise the isolation of sperm was developed (Simchi *et al.*, 2021). Results found that the technique outperformed current clinical methods by improving DNA integrity of the selected sperm subpopulation up to 95%, whilst reducing the sperm preparation time 3-fold.

5.11. MSS and density gradient centrifugation (DGC) techniques for sperm sorting were compared in couples undergoing ICSI in oocyte donation cycles (Srinivas *et al.*, 2022). While pregnancy rates were similar between groups, the DG group had higher clinical pregnancy rates and lower miscarriage and biochemical pregnancy rates, indicating no additional benefit of MSS on clinical pregnancy rates.

5.12. A randomized controlled trial conducted by (Quinn *et al.*, 2022)compared microfluidic sperm preparation to standard DGC for IVF with ICSI (n=386 patients). The authors found no significant differences in embryo quality or clinical and ongoing pregnancy rates between the two groups, suggesting similar performance in unselected populations.

5.13. The microfluidic-based device ZyMot®ICSI was assessed by (Pujol *et al.*, 2022) to determine whether it can reduce the proportion of sperm with double-stranded sperm DNA fragmentation (dsSDF) in ICSI couples with male partner having ≥60% dsSDF (n=163), assessed via neutral Comet assay. They demonstrated a significant reduction in dsSDF with ZyMot®ICSI compared to fresh ejaculate or swim-up methods, leading to improved laboratory and clinical outcomes in ICSI cycles of patients with high dsSDF.

5.14. A study by (Mirsanei *et al.*, 2022) evaluated sperm parameters, DNA fragmentation, and gene expression of phospholipase C zeta (PLC-ζ) and transition nuclear proteins 1 (TNP1) in sperm selected by MSS versus conventional DGC in ICSI patients with a history of fertilization failure. The use of MSS resulted in higher sperm quality, increased gene expression of PLC-ζ and TNP1, and higher fertilisation rates and embryo quality compared to DGC.

5.15. A study by (Lara-Cerrillo *et al.*, 2023) evaluated the clinical utility of ZyMot®ICSI microfluidic device in ICSI treatments for couples with male partners exhibiting high values of double-strand breaks (DSB) in sperm DNA (n=28) assessed through neutral Comet assay. MSS improved reproductive outcomes compared to conventional methods, with higher biochemical, clinical pregnancy rates, and live births. Similarly, (Zaha *et al.*, 2023) compared MSS with ZyMot®ICSI with classic DGC for sperm preparation in IVF. Although no significant difference was found in fertilisation rates, ZyMot®ICSI showed higher blastocyst rates and clinical pregnancy rates.

5.16. Microfluidic sperm sorting (MSS) was compared to DGC in couples with repeated implantation failure (RIF) in PGT-A cycles and high sperm DNA fragmentation (SDF) (Keskin *et al.*, 2022). MSS yielded a higher number of top-quality blastocysts compared to DGC, though no improvement in euploidy or live birth rates was observed, suggesting the need for further research on factors affecting embryonic genomic status in the presence of high SDF.

5.17. A stdy by (Aydın *et al.*, 2022) compared traditional swim-up procedures to the Fertile Chip for sperm selection in ICSI cycles (n=128), finding no difference in fertilization rates or embryo quality but significantly higher implantation, pregnancy, and live birth rates with the Fertile Chip. Similarly, (Feyzioglu and Avul, 2023) found no difference in clinical pregnancy and live birth rates between control (swim-up) and treatment (MSS) group in couples with unexplained infertility undergoing IUI (n=326).

5.18. A retrospective cohort study by (Banti *et al.*, 2024) showed significantly higher blastocyst, utilization, and euploidy rates in ICSI couples (n=53) when FERTILE PLUS™ sperm sorting chip was used compared to DGC.

5.19. A study by (Kocur *et al.*, 2023a) identified couples with a history of high embryo aneuploidy undergoing ICSI / PGT-A cycle, initially using DGC for sperm selection. Subsequent ICSI / PGT-A cycles (n=71) with MSS in these couples showed significant reductions in sperm chromatin fragmentation and dsSDF fragmentation, resulting in improved embryo euploidy rates, implantation rates and clinical pregnancy rates compared to DGC previous cycles.

5.20. A study by (Huang *et al.*, 2023b) devised a Progressive Sperm Sorting Chip based on rheotaxis behaviour of sperm for high-quality sperm isolation (n=10), achieving >90% of isolated sperm exhibiting high motility (> 25 µm/s) and low DNA fragmentation rates (<5%). Similarly, (Huang *et al.*, 2024)and (Zeaei *et al.*, 2023) designed MSS devices based on thermotaxis and rheotaxis or rheotaxis and boundary-following behaviour, demonstrating increased improvement in sperm motility and DNA integrity.

5.21. As an alternative method to hyaluronic acid (HA) for physiological selection of spermatozoa in PICSI, the microfluidic sperm sorting (MSS) technique was tested by (Anbari *et al.*, 2021). Authors found MSS to successfully utilise channels that mimic the female reproductive environment, such that higher quality spermatozoa were selected. An increasing in quality embryo formation, implantation and clinical pregnancy (n=95) were reported.

5.22. (Kocur *et al.*, 2023b) evaluated the role of sperm chromatin fragmentation (SCF) in guiding treatment for couples with unexpectedly initial poor outcomes after ICSI (n=76 couples with 168 cycles). Couples with prior history of poor clinical outcomes following ICSI, underwent a subsequent ICSI cycle using either ejaculates processed by MSS (microfluid sperm sorting) or spermatozoa retrieved from the testis, and clinical outcomes were compared between history and treatment cycles. In couples using autologous oocytes, there was an increase in implantation and pregnancy rates, while miscarriage rate decreased after using MSS or testicular sperm extraction.

Selection with Hyaluronic Acid (HA)

5.23. The Hyaluronic Acid Binding sperm Selection (HABSelect) clinical trial by (West *et al.*, 2022) investigated the efficacy of pre-ICSI treatment using HA-binding/selection. Findings indicate that older women randomised to the experimental arm (selection of sperm bound to immobilized, solid-state HA) exhibited comparable live birth rates to younger counterparts, potentially attributed to enhanced avoidance of sperm with compromised DNA integrity. The authors provide evidence that lower HA-binding score and DNA quality are associated with poorer sperm quality that compromised treatment outcomes throughout the gestational timeline.

5.24. A study by (Scaruffi *et al.*, 2022) investigated retrospectively whether HA sperm selection improved ICSI outcome of couples with previous ICSI cycle failure. HA sperm selection prior to second ICSI significantly improved pregnancy and implantation rates compared to standard ICSI in couples with previous cycle failure.

5.25. A study by (Abd Elraouf *et al.*, 2023) evaluated the effect of SpermSlow™-ICSI as an alternative product for slowing sperm motility that contains HA on clinical outcomes of ICSI cycles as compared to standard procedure (polyvinylpyrrolidone, PVP). They found positive drift in embryo quality and pregnancy rates in the SpermSlow-ICSI group compared with PVP-ICSI.

5.26. A study by (Emirdar *et al.*, 2023) compared the outcomes of morphologically selected sperm for conventional ICSI cycles (n=2415) with PICSI cycles (n=400). There was no significant difference in fertilization rate, embryo quality, clinical pregnancy rate, biochemical pregnancy rate, or miscarriage rate between the two methods, suggests that the PICSI does not offer superior outcomes in compared to conventional ICSI.

Magnetic-activated cell sorting (MACS)

5.27. There are conflicting results on the benefits of using MACS over DGC for sperm selection to improve semen parameters and reproductive outcomes in MAR (medically assisted reproduction) (donor IUI, ICSI autologous or donor oocytes) such as quality of embryos, embryonic development, miscarriage rates, live birth rates and cumulative live birth rates per embryo transferred (Gil Juliá *et al.*, 2022; González-Ravina *et al.*, 2022; Salehi Novin *et al.*, 2023).

5.28. A narrative review by (Garrido and Gil Juliá, 2024) critically evaluated the evidence regarding the potential benefits of using MACS for sperm selection, highlighting controversial results in in vitro fertilization outcomes and emphasizing the need for methodologically sound research on specific populations before clinical implementation. Despite promising theoretical advantages, the unclear benefits of adding MACS underscore the necessity for further investigation and refinement of this technique.

Artificial Intelligence (AI)

5.29. Using AI, (Ito *et al.*, 2021) developed the first tool to use automated machine learning to determine spermatogenesis activity of testis samples based on Johnsen scores. The two datasets, with positive predictive values of 82.6% and 99.5% (n=275), were deemed to be helpful to support pathologists' evaluations. Similarly, (Li *et al.*, 2021) developed an intelligent nomogram to predict the success of different methods of fertilisation of men with borderline semen. The model was deemed to be clinically useful to select optimal fertilisation methods.

5.30. Reviews by (Cherouveim *et al.*, 2023; Lustgarten Guahmich *et al.*, 2023; Si *et al.*, 2023) overview different applications of AI – particularly and video and image processing – for gamete and embryo assessment and selection, discussing current challenges and development directions. They all highlight the potential of AI algorithms as assisting tools to improve the selection processes through fast and accurate analysis of large datasets, yet emphasising the need to validate the clinical benefit these algorithms might have in improving reproductive outcomes.

5.31. A study by (Montjean *et al.*, 2024) developed a computer-assisted software SiD (sperm ID) program for real-time sperm assessment and selection based on motility characteristics. Their study compared ICSI outcomes between embryos fertilized with embryologist-selected sperm

(ICSI group, n=320) and those selected using the SiD software (ICSI-SiD group, n=326), revealing comparable fertilization rates, embryo development, and pregnancy outcomes, suggesting the efficacy of automated sperm selection.

Other methods for sperm selection

5.32. A variety of techniques have been developed to optimise sperm selection or predict the success of ART cycles. Researched biomarkers that lacked sufficient evidence to support their predictive ability were: sperm DNA fragmentation testing, testis-specific actin capping proteins, and sperm morphology in inseminated sample (Stanhiser *et al.*, 2020; Fuentes Ávila *et al.*, 2021; Inagaki *et al.*, 2021; Le *et al.*, 2021).

5.33. Conversely, evidence has been found to support the use of sperm motility before preparation as a predictor for ART success (Jeong *et al.*, 2021). (Dearing *et al.*, 2021) found the computer-assisted sperm analyser (CASA- Mot) system reduced, but did not eliminate, sperm motility measurement uncertainty compared to the WHO manual method. The researchers reasoned that this technique could optimise IVF fertilization success predictions.

5.34. A study by (Nassir *et al.*, 2022) developed a multidisciplinary approach to predict the 3D swimming behaviour of sperm cells by integrating 3D refractive-index profiles acquired with high-resolution optical diffraction tomography with numerical mechanical modelling. They demonstrated that abnormal sperm cells can exhibit faster swimming speeds than normal ones, challenging the traditional assumption of selecting sperm based solely on progressive motion for fertility treatments.

5.35. A case report by (Rossi *et al.*, 2023) investigated the efficacy of sperm selection using the hypo-osmotic swelling test (HOST) to increase the proportion of chromosomally balanced spermatozoa in male carriers of complex chromosomal rearrangements (CCRs). They reported a significant reduction in the proportion of unbalanced spermatozoa from 88% to 15% in a 36-year-old male t(4;7;14)(q12;p21;q11.2) carrier undergoing infertility treatment.

5.36. The expression and localisation of PLC-ζ (phospholipase C zeta) was investigated in individual spermatozoa across seven different hypo-osmotic swelling test (HOST) tail patterns (‘a’-‘g’ according to WHO 2010 criteria), with the aim to enhance routine sperm selection for ICSI (Allahveisi and Yousefian, 2023). Authors found that sperm from HOST grade 'd' exhibited significantly higher PLC-ζ staining patterns compared to other grades, suggesting that HOST could serve as a useful diagnostic tool for selecting sperm with elevated PLC-ζ expression.

5.37. A case-control study by (Ribeiro *et al.*, 2023) investigated the impact of sperm selection based on head birefringence on intracytoplasmic sperm injection (ICSI) outcomes in couples with various infertility factors as a cost-effective and easily implementable technique to enhance reproductive success. They demonstrated significantly higher cleavage and clinical pregnancy rates in the birefringence-ICSI (n=74 cycles) group compared to conventional ICSI (n=107 cycles).

5.38. A pilot study by (Cabello *et al.*, 2023) investigated the impact of novel non-centrifugation method (Io-Lix) for sperm selection on outcomes in autologous and donor oocyte ICSI cycles. Io-Lix protocol resulted in an increase in pregnancy rates and a reduction in miscarriages in the autologous ICSI programme when compared to swim-up sperm selection, an effect which was not observed in the donor oocyte ICSI programmes.

5.39. A study by (Conflitti *et al.*, 2023) investigated SDF (sperm DNA fragmentation) and sperm-borne miRNAs, miR-34c-5p and miR-449b-5p, as biomarkers for semen quality and ART outcomes. They found that higher SDF levels post-sperm selection correlated positively with the percentage of low-quality embryos and negatively with viable embryos. Additionally, there were associations between miRNA levels and sperm concentration, with miR-449b-5p positively associated with SDF.

5.40. A study by (Luongo *et al.*, 2023) investigated the effectiveness of a modified swim-up procedure for sperm selection (ie with exposure to cumulus cell secretome, SUC) compared to conventional swim-up (SU). They found that SUC resulted in the recovery of high-quality spermatozoa with enhanced mitochondrial functionality and motility and significantly reduced sperm DNA damage compared to SU-selected sperm.

5.41. A multi-centre study by (Michailov *et al.*, 2023) was conducted to evaluate a novel non-invasive microscope system utilising quantitative phase microscopy (QPM) for label-free sperm-cell selection for use in ICSI cycles. They found that QPM allowed for precise measurement of sperm morphology, with significantly higher repeatability compared to conventional sperm selection by embryologists using a manipulator and high power bright field microscopy (BFM). Additionally, QPM was in good agreement with the measurements performed on the reference method of stained cells (according to World Health Organization (WHO) 2021 criteria) imaged through BFM.

5.42. A study by (Gómez-Torres *et al.*, 2023a)investigated the distribution of izumo sperm-egg fusion protein 1 (IZUMO1) – an acrosome transmembrane protein implicated in the adhesion and fusion of gametes – in human sperm under various physiological conditions including capacitation, induced acrosome reaction (AR) and HA selection tests. Dotted fluorescence in the acrosomal region was the major staining pattern (~70%) in non-capacitated, one-hour capacitated, and mature sperm, while a new distribution pattern was found in HA-bound spermatozoa. The results show diffusion of the IZUMO1 protein during different physiological conditions that could contribute to the improvement in sperm selection techniques.

5.43. A multi-centre study by (Shapouri *et al.*, 2023) compared a novel electrophoretic system (Felix™) for sperm isolation with conventional DGC (density gradient centrifugation). Results showed that Felix™ led to isolation of highly motile spermatozoa with significant improvement in DNA integrity relative to DGC, despite yielding significantly lower sperm recovery.

5.44. A study by (Bibi *et al.*, 2023) evaluated four sperm selection techniques (DGC, swim-up, DGC-swim-up, and DGC-MACS) for ICSI couples (n=385) with male partners having teratozoospermia. Analyses showed superiority of DGC-MACS yielding better results for sperm with normal morphology, DNA integrity and chromatin maturity. Embryo cleavage, clinical pregnancy, and implantation rates were also improved compared to other techniques.

5.45. A study by (Jamil *et al.*, 2023) compared density gradient centrifugation (DGC) with extended horizontal swim-up for sperm isolation and a combination of both methods in IVF-ICSI couples (n=97). The combination of both methods yielded the lowest rates of sperm DNA fragmentation and chromatin de-condensation, however with no difference in fertilisation rates or day 3 embryo development between groups.

5.46. The effect of sperm isolation techniques (swim-up, DGC and electrophoretic separation using Felix™ device) on sperm vitality and quality post-thaw was investigated (Hungerford *et al.*, 2023).

Use of Felix™ resulted in improved sperm vitality, significantly less DNA damage and lower levels of lipid aldehyde formation compared to direct swim-up and DGC. However, there was no difference between techniques with respect to sperm morphology and mitochondrial reactive oxygen species generation.

6. Male infertility

Reviews on male infertility investigation and management

6.1. A number of reviews cover different aspects of male infertility investigation and management such as genetic and epigenetic analysis, the role of sperm DNA integrity and developments in the gold standard methods for semen analysis:

6.2. (Goss *et al.*, 2023) focus on the role of seminal plasma small extracellular vesicles (S-EVs) in sperm physiology suggesting therapeutic and diagnostic potential. However, challenges in isolating pure populations of S-EVs from bodily fluids hinder their clinical application, with microfluidic technology emerging as a promising approach to overcome these obstacles.

6.3. (Caroppo and Skinner, 2024) provide a comprehensive review on the significance of sperm epigenome abnormalities in male infertility, discussing epigenetic mechanisms in gene expression regulation, the impact of environmental factors on the human germline, and the diagnostic and prognostic efficacy of evaluating sperm epigenome, particularly in cases of severe spermatogenic dysfunction.

6.4. (Barratt *et al.*, 2022) discuss the evolution of semen evaluation methods over the past 40 years, focusing on the changes introduced in the sixth edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen. They explore emerging technologies and diagnostic approaches that may enhance the precision and interpretation of semen analysis, providing insights into potential advancements. (Schardein *et al.*, 2023) further discuss the sixth edition of the WHO manual in the context of emerging technologies like point-of-care (POC) testing and microfluidics for sperm processing, possibly coupled with machine learning, which have the potential to enhance fertility care.

6.5. (Farkouh *et al.*, 2022) review the pathophysiological aspects of SDF, different assessment tools for SDF and potential therapeutic options to manage infertile men with high SDF.

6.6. (Sengupta *et al.*, 2022) reviews the role of oxidative stress (OS) as a common mechanism inducing idiopathic male infertility (IMI) and the use of antioxidants to treat OS. The study also explores the potential of omics technologies, such as next-generation sequencing (NGS), to address IMI, including the incorporation of oxidation-reduction potential (ORP) as a clinical biomarker.

Genetic studies

6.7. (Kyrgiafini *et al.*, 2023) investigated male infertility, focusing on teratozoospermia, using whole-genome sequencing and RNA expression analysis. They identified 1166 unique mutations in teratozoospermic men within differentially expressed long-noncoding RNAs (lncRNAs), distinguishing them from normozoospermic men. Functional analysis revealed potential regulatory roles of these variants, shedding light on novel genetic factors associated with teratozoospermia.

6.8. Three studies investigated genetic factors underlying multiple morphological abnormalities of sperm flagella (MMAF). (Tang *et al.*, 2023) identified a novel CFAP69 frameshift variant in MMAF, achieving successful fertility outcomes through ICSI. (Long *et al.*, 2023) found 18 different DNAH1 variants in MMAF in 11 unrelated families, observing successful fertility outcomes post-ICSI. (Ma *et al.*, 2023) discovered seven cilia- and flagella-associated protein 43 (CFAP43) mutations in MMAF, achieving pregnancy through ART, expanding the mutant spectrum of CFAP43 for genetic diagnosis and counselling. Similarly, (Jin *et al.*, 2023) identified CFAP52 mutations implicated in asthenoteratozoospermia, revealing sperm structural abnormalities in head-tail connection and flagella development, consistent with findings in mice. This study underscores CFAP52's role in sperm development, suggesting its potential as a diagnostic target for infertility.

6.9. Two studies explored total fertilization failure (TFF) in infertile men, identifying homozygous or compound-heterozygous variants of paternal-effect genes ACTL7A and PLCZ1 associated with TFF (Wang *et al.*, 2021; Zhao *et al.*, 2023b). They showcased ultrastructural defects in spermatozoa and proposed interventions like ICSI with assisted oocyte activation (ICSI-AOA) for TFF, underscoring the significance of genetic screening. Similarly, (Wang *et al.*, 2023b) explored the genetic landscape of MMAF Han Chinese men, revealing novel DNAH1 gene variations associated with MMAF and successful pregnancies post-ICSI, suggesting a broader scope for genetic diagnosis and ICSI in addressing male infertility.

6.10. (Mottola *et al.*, 2023) explored the association between polymorphic rearrangements of chromosome 9 and male infertility in an Italian cohort, observing such rearrangements in infertile patients with abnormalities in sperm quality. They found that chromosome 9 rearrangements might be linked to incorrect spermatogenesis regulation, leading to abnormal sperm quality.

6.11. (Dai *et al.*, 2023)examined deaminase domain-containing, including ADAD1 and ADAD2, variants in male infertility (n=337 infertile men), identifying novel variants in these genes associated with human male infertility. They demonstrated disrupted expression of ADAD1 and ADAD2 in spermatozoa of affected individuals, suggesting a potential role of ADAD variants in human male infertility.

6.12. (Gao *et al.*, 2024) identified androglobin (ADGB) gene variants in infertile men of Han Chinese families (n=105) with severe asthenoteratozoospermia, highlighting their role in male infertility. They demonstrated structural defects in spermatozoa of affected individuals and successful fertilisation through ICSI, providing insights for genetic counselling and clinical treatment.

Other studies

6.13. Multiple methods have been evaluated for assisting in the diagnosis of male infertility. A meta-analysis by (Liu *et al.*, 2021b) found the use of multiple miRNAs and seminar plasma-derived

miRNAs gave high sensitivity for male infertility diagnosis. Similarly, (Dutta *et al.*, 2021) compared the accuracy of assays for diagnosing infertility using sperm chromatin integrity and (Da Costa *et al.*, 2021) saw promise with simultaneous detection of sperm membrane along with DNA fragmentation with flow cytometry.

6.14. Another technique that has shown early promise is the MiOXSYS system for measuring the overall oxidation-reduction potential (ORP) in semen samples (Karabulut *et al.*, 2021). ORP was found to reliably distinguish between normal and impaired semen parameters. However, further study is needed before this is used to predict ART success or diagnose infertility clinically (Panner Selvam *et al.*, 2021).

6.15. (Peng *et al.*, 2023) investigated the combined effect of sperm DNA fragmentation index (DFI) and routine semen parameters on IVF outcomes (n=1258 couples). In multivariable adjustment, increased sperm DFI and decreased sperm motility and semen concentration levels were associated with reduced odds of favourable IVF outcomes. There was no significant difference in IVF outcomes between the group with low sperm DFI levels and high sperm motility and semen concentration levels and that with low DFI levels and moderate sperm motility and semen concentration levels. These two groups were associated with more favourable IVF outcomes (live birth rate) when compared to low sperm parameter levels, even when DFI values remain low.

6.16. (Gómez-Torres et al., 2023) analysed the expression and spatial location of the heat shock protein A2 (HSPA2) on human spermatozoa based on its HA (hyaluronic acid) binding capacity. The results suggested that the interaction with HA may induce the unmasking of HSPA2 epitopes. Authors suggest that HA-selection and HSPA2 biomarker could be considered candidates to complement the classic seminal analysis when considering appropriate ART.

6.17. (de Lima Rosa *et al.*, 2023) developed cytometric assays for assessing sperm integrity, acrosome status, mitochondrial potential, and superoxide anion production using flow cytometry, cytometric assays using cytometers equipped with 2 and 3 lasers.

6.18. (Castleton *et al.*, 2023) investigated the natural fluctuations in semen redox indicators (MiOXSYS® and OxiSperm® II) their association with markers of sperm oxidative stress. Results showed that high redox-potential levels were associated with lower sperm motility and morphology, and higher DNA fragmentation, but neither MiOXSYS® nor OxiSperm® II assays were predictive of sperm function or oxidative stress, suggesting limited diagnostic potential for these systems.

6.19. A meta-analysis by (Yuan *et al.*, 2022) assessed the accuracy and clinical value of sperm telomere length (STL) as a diagnostic marker for male infertility and predicting embryonic development (n=12 studies, involving 1700 patients). There was a positive linear correlation between STL and semen parameters with an optimal cut-off value of 1.0, sensitivity and specificity of 80%. Longer STL was associated with higher clinical pregnancy rates, suggesting STL as a potential biomarker for male infertility diagnosis and embryonic development prediction.

6.20. (Cheung *et al.*, 2023) categorised couples with unexplained infertility based on success of ICSI outcomes, with ancillary sperm function tests and sperm DNA whole exome sequencing conducted. Lower sperm aneuploidy was observed in fertile couples, and mutations associated with sperm-egg fusion (ADAM3A) and acrosomal development (SPACA1) were found

irrespective of reproductive outcome. Subsequent categorization of the infertile cohort based on reasons for reproductive failure revealed specific sperm genetic profiles.

6.21. (Juchnewitsch *et al.*, 2024) investigated the prevalence of congenital defects in Ras/mitogen-activated protein kinase (MAPK) pathway genes, known as RASopathies, in men seeking infertility management. They conducted exome sequencing on 521 idiopathic spermatogenic failure (SPGF) patients, including 155 with cryptorchidism (CR), and 323 normozoospermic controls. The study identified likely pathogenic or pathogenic variants in RASopathy-linked genes, particularly enriched among patients with a history of CR, highlighting the association between undiagnosed RASopathies and male infertility, with implications for further evaluation of associated health concerns, including rare malignancies.

6.22. (Garcia-Segura *et al.*, 2022) characterised the seminal microbiota in a western Mediterranean population of sperm donors (n=14) and men with idiopathic infertility (n=42) using full-length 16S rRNA gene sequencing. They also evaluated microbiota association with sperm DNA integrity and oxidative stress. Authors observed microbial diversity in samples from sperm donors and infertile idiopathic patients, with specific genera found to correlate with seminal quality defects. Particularly Moraxella, Brevundimonas, and Flavobacterium were negatively associated with sperm global DNA fragmentation.

6.23. (Janiszewska *et al.*, 2022) examined the association between the sialylation degree of glycoprotein clusterin (CLU) and markers of oxidative-antioxidant balance in infertile men. They found that decreased expression of sialic acid (SA) in seminal plasma was associated with multiple sperm disorders, and reduced concentrations of Sirtuin-3 (SIRT3) were linked to teratozoospermia and oligoasthenoteratozoospermia. Additionally, they identified potential markers, such as the relative reactivity of CLU glycans with specific lectins and levels of SIRT3 and SIRT5, which could help differentiate between infertile patients with different sperm deficits.

6.24. A single centre study by (Gianzo *et al.*, 2022) investigated the association between human sperm aminopeptidase N (APN) levels and embryo development in 81 couples undergoing oocyte-donation cycles. The cumulative probability of having well-developed blastocysts increased by 1.38-fold at Day 5 and 1.90-fold at Day 6 of embryo development, and the likelihood of having usable embryos increased by 1.48-fold, when semen samples with low APN levels were used during the ICSI technique.

6.25. A cross-sectional pilot study by (Omes *et al.*, 2022) investigated calprotectin concentration in seminal fluid of 45 men undergoing semen quality evaluation. Results indicated that higher calprotectin levels were associated with better seminal fluid quality (ie normozoospermic vs samples with at least one abnormal semen parameter). Further, calprotectin concentration was significantly different between seminal fluids with normal sperm morphology and those of teratozoospermic samples. ROC curves showed diagnostic accuracy around 67% using calprotectin concentration (threshold value: 0.121 µg/ml) as a preliminary test to discriminate subjects with and without abnormal semen parameters, especially morphology.

6.26. (Laganà *et al.*, 2022) aimed to evaluate structural, functional, and molecular sperm biomarkers in total globozoospermia with successful embryo development after ICSI. Using field-emission scanning electron microscopy (FE-SEM), transmission electron microscopy (TEM), and fluorescent microscopy, authors identified eight morphological patterns and observed high percentages of coiled forms with cytoplasmic retentions around the head and midpiece. Less

than 1% of sperm cells displayed tyrosine phosphorylation in the flagellum and 85 % lacked Heat shock-related 70 kDa protein 2 (HSPA2). These findings offer valuable insights into globozoospermia and its implications for refining sperm selection methods in ART.

6.27. (Vickram *et al.*, 2022) aimed to identify a novel protein biomarker for the diagnosis and prognosis of male infertility by isolating prostasomes from ejaculates and characterising them using biochemical, molecular, and in silico methods. In MALDI (matrix-assisted laser desorption ionization) results, the maximum hit was obtained against Clusterin in prostasomes, potentially contributing to sperm capacitation and motility, thus suggesting its utility as a diagnostic biomarker for male infertility.

7. Conclusions

7.1. There is an increase in research to develop non-invasive methods for embryo testing to determine embryo quality, implantation potential and embryo ploidy status, all aiming to maximise the chances of a healthy live birth. These include analysis of cfDNA in spent culture media and blastocoelic fluid; machine learning (ML) algorithms combining time-lapse imaging and clinical data; metabolomics profiling of spent culture media.

7.2. Collectively, studies show the potential benefits of non-invasive methods, including reduced invasiveness and increased accessibility, but challenges linked to variability in concordance rates, limitations in accuracy, and the need for further validation and standardisation remain significant concerns across the studies. Further large scale studies to confirm the origin of the cell-free genetic material and levels of DNA contamination are warranted.

7.3. Additionally, there is a need for further research around the accuracy of ML-algorithms to make predictions, as well as the wider ethical and practical considerations needed for the implementation of AI in the sector. Specifically, it is essential that ML-based algorithms are externally validated on large datasets and diverse target patient populations.

7.4. PGT-P has become commercially available outside the UK, however it remains controversial with concerns around ethical and societal implications, its premature clinical implementation, potential health risks and the need for development of comprehensive guidelines and regulations. Further research is needed on statistical properties of polygenic risk scores in the context of clinical application. Due consideration around counselling and the impact of its use, especially with non-disease traits such as intelligence, is also crucial.

7.5. Regarding gamete testing, there is an increased interest in using metabolomics profiling of spent culture media and follicular fluid to evaluate oocyte quality and competency, and to identify distinct metabolomic features associated with PCOS. Emerging trends in sperm testing and selection include testing for new gene variants associated with abnormal sperm parameters and protein biomarkers of sperm quality, HA-binding and microfluidic technology to optimise selection of sperm of high quality, DNA integrity and fertilising potential. There has also been a substantial increase in effort to identify genetic cause of male infertility to ultimately improve infertility management and provide effective treatment.

8. Recommendations

8.1. Members are asked to:

- Consider the progress of research into embryo and gamete testing
- Advise the Executive if they are aware of any other recent developments.
- Review whether any outputs from the HFEA are required addressing the use of emerging technologies in embryo and gamete testing.

8.2. To note: Given how broad this topic is, the SCAAC can consider splitting the topic of 'Emerging technologies in embryo and gamete testing' into separate priority topics at the February 2025 meeting when the committee will be discussing its 2025/26 workplan.

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Artificial wombs for early or whole gestation (ectogenesis)

Details about this paper

Area(s) of strategy this paper relates to:	Shaping the future
Meeting:	Scientific and Clinical Advances Advisory Committee (SCAAC)
Agenda item:	6
Paper number:	HFEA (03/06/2024) 006
Meeting date:	03 June 2024
Author:	Molly Davies, Scientific Policy Officer (HFEA)
Annexes	N/A

Output from this paper

For information or advice?	For advice
Recommendation:	Members are asked to: • consider the progress of research into ectogenesis; • advise the Executive if they are aware of any other recent developments; and • consider whether the research to date on the topic of ectogenesis shows advances relevant to the remit of the HFEA. and to note that: • the topic of artificial wombs for early or whole gestation (ectogenesis) will remain on the SCAAC prioritisation list until February 2025 for the next prioritisation discussion so that it can be considered alongside the committee's consideration of its workplan for 2025/2026.
Resource implications:	N/A
Implementation date:	N/A

Communication(s):	N/A
Organisational risk:	Low

1. Introduction

1.1. Artificial wombs offer the possibility of creating an environment where a fetus can be sustained and developed to full gestation outside of a 'natural' womb. Partial or complete gestation of an embryo or fetus in an artificial environment is also known as ectogenesis.

1.2. Artificial womb technologies can be broadly categorised into those designed for whole gestation and those designed for partial gestation. Whole gestation artificial womb technology aims to support the development of an embryo from implantation through to full-term gestation. This involves creating a completely synthetic environment that replicates the functions of a natural womb throughout the entire pregnancy. Partial gestation artificial womb technology is designed to support the development of fetuses that are born prematurely. This technology attempts to mimic a natural pregnancy at a point where the fetus is viable outside the mother's womb (~22 weeks gestation) but still requires significant support to develop fully. Artificial womb technologies for partial gestation are often conflated with artificial placenta technologies, sharing a common goal of improving outcomes for extremely premature infants initially gestated in utero.

1.3. Both artificial womb and artificial placenta technologies are currently experimental and are not yet available clinically. However, one of the most promising applications of artificial womb technologies is to support the development of preterm infants in place of an incubator. By mirroring the conditions of the natural womb more closely, artificial womb and placenta technologies have shown the potential to improve developmental outcomes and reduce complications associated with extreme prematurity in animal models.

1.4. The topic of artificial wombs was first added to the SCAAC's horizon scanning prioritisation list as a medium priority topic in January 2022. It has since been established that the use of artificial wombs would fall within the HFEA remit where the technology was applied for complete gestation of human embryos created in vitro. At this time, this is both technically infeasible and limited by legal restrictions such as the 14-day rule.

1.5. The topic was deprioritised to low priority in February 2023, being considered out of remit and unlikely to impact on research or treatment in the near future, and has remained low priority since.

1.6. This paper provides an overview of technical developments in artificial womb technology since 1st January 2022, including ethical-legal concerns relevant to the potential use of these technologies. The Executive notes that the current paper provides a summary of the findings described in published studies and not an assessment of study validity.

2. Technological developments

2.1. The review by (Khulbe *et al.*, 2023) highlights the current working models of artificial womb technology and the mechanisms underlying them. These include the Ex-Vivo uterine Environment (EVE) by Tohoku University and the University of Western Australia, and the EXTra-uterine Environment for Neonatal Development (EXTEND) developed by the Children's Hospital of Philadelphia. Development and potential clinical translation of the EXTEND system is further discussed in the reviews by (Larson *et al.*, 2022) and (Flake, 2022) where its earlier success in supporting fetal growth in premature lamb models is reviewed. Advances in artificial placenta

technology and its potential use in extremely premature infants are summarised in the reviews by (Spencer and Mychaliska, 2022a; Usuda *et al.*, 2023b).

2.2. An experimental study by (Sanchez-Martinez *et al.*, 2023) followed 12 fetal sheep that were transferred to an artificial placenta system consisting of a pumpless circuit with umbilical cord connection at 109-117 days gestation. To understand the cardiovascular adaption of fetuses following connection to the artificial placenta, ectocardiographic assessments and measurements of blood flow were recorded. Connection to the artificial placenta system was found to result in a transient fetal hemodynamic response that normalised over hours. Cardiac structure and function were preserved but a non-physiologically elevated venous pressure and pulsatile flow was observed.

2.3. A study by (Usuda *et al.*, 2023a) reported on the successful maintenance of six extreme preterm sheep fetuses using artificial placenta therapy for up to two weeks ex-utero. At delivery, no differences in birth weight, brain weight or femur length were reported between experimental and control groups, however organ weight and humerus lengths were significantly reduced in the therapy group. This indicates a need to focus on refining the artificial placenta system for optimisation of fetal growth and organ development, alongside cardiovascular stability.

2.4. In their experimental study (Trad *et al.*, 2023) report their experience using an ex-utero support system for fetal pigs of a gestational age equivalent to 32-week in humans. Of the two animal models used, one fetus was successfully cannulated and survived for seven hours. Failure at cannulation of fetus two indicates the need for further studies to refine technique before artificial placenta technology can be effectively translated to the clinic.

2.5. The qualitative study by (Verrips *et al.*, 2023) explored clinical considerations for umbilical cord cannulation in extremely premature infants transitioning from the uterus to a liquid-based perinatal life support system. Analysis of interviews and focus groups with twelve medical specialists revealed that a side-entry cannulation technique, automatic microsurgery instrument sutures for fixation, and localized medication for vasospasm and anticoagulation are preferred to minimize vessel damage, enhance stability, and maintain blood flow.

2.6. The Preemie-Ox, developed by (Omecinski *et al.*, 2023), introduces a novel approach to artificial placenta support for periviable infants. This device utilises a hollow fiber membrane bundle to provide pumpless respiratory support via umbilical cord cannulation. In vitro evaluations of the prototype demonstrated gas exchange performance exceeded predicted results, indicating its potential for safe and efficient respiratory support in artificial uterine environments.

2.7. The study by (Inatomi *et al.*, 2023) also presents a novel artificial placenta system. Using a mechanical mock circulation system and a fetal animal experiment, researchers tested a loop circuit configuration for extracorporeal membrane oxygenation (ECMO) designed to be applied to the fetus in the form of an umbilical arterial-venous connection. The system was found to be feasible in both in vitro and in vivo conditions, however simulation revealed challenges in maintaining fetal hemodynamic with high ECMO flow. In vivo experiments demonstrated feasibility, with one fetal goat successfully maintained on the artificial placenta system for 12 days and allowed to grow to term.

2.8. Support of 13 preterm fetal pigs using a centrifugal pump system was reported by (Charest-Pekeski *et al.*, 2022). Compared to a pumpless artificial placenta circuit, the pumped system was

found to extended survival time yet also induced supraphysiologic circuit flows, tachycardia, and hypertension, with a progressive decline in umbilical vein flow and oxygen delivery. This study indicates that ensuring cardiovascular stability remains an important challenge for the translation of artificial placenta technology to clinic.

2.9. To understand the impact of venovenous artificial placental support, (Li *et al.*, 2023) measured the myocardial performance index, cardiac output and blood biochemical parameters in five fetal goats supported on the stem for nine hours. Results showed an initial increase in right ventricular cardiac output followed by a significant decrease, along with an increasing right ventricular Tei index and elevated plasma cTnI and arterial lactic acid levels. Suggesting that special attention should be paid to right ventricular dysfunction during artificial placenta support.

2.10. The study by (Spencer *et al.*, 2024) aimed to evaluate the effects of changing fetal haemoglobin percent on physiology and circuit function during artificial placentation support in a sheep model. As more adult blood transfusions were given to maintain haemoglobin, the percentage of fetal haemoglobin declined, reaching 40% by post operative day seven. This decrease affected flow rates, requiring higher flows to maintain oxygen levels and blood circulation. Methods to minimise priming volume or the establishment of fetal blood banks to provide blood with higher fetal haemoglobin concentrations are required prior to clinical translation.

2.11. A study by (Usuda *et al.*, 2022) explored whether the use of synthetic red cells as the basis of a priming solution for artificial placenta therapy could address challenges in using maternal blood. In doing so two sheep fetuses were successfully maintained on an artificial placenta for 72 hours with controllable anaemia and methemoglobinemia indicating a method to refine artificial placenta technology for clinical translation.

2.12. Perfluorocarbons have also been proposed as red blood cell substitutes for artificial placenta procedures by (Nocentini *et al.*, 2023). In their review article they discuss the application of these organic liquids as a means to improve donor organ perfusion during the ex vivo assessment.

2.13. Hepatic outcomes in a lamb model of extreme prematurity supported for seven days by an artificial placenta system were investigated by (Harvey *et al.*, 2022). Lambs received total parenteral nutrition with either SMOFlipid or intralipid lipid emulsions. Results indicate that lambs maintained normal hepatic function with minimal injury during artificial placenta support with total parenteral nutrition, with the use of SMOFlipid emulsions associated with decreased cholestasis and hepatic injury when compared to Intralipid.

2.14. The impact of artificial placenta therapy on nuclear receptor expression was investigated by (Ikeda *et al.*, 2024). Six ovine fetuses at 95 days gestation were maintained on an artificial placenta platform for 120 hours. Liver tissue and blood samples were then compared to age-matched in utero control fetuses. After loss of placental-maternal support a number of nuclear receptors (HNF4α, LRH1, LXR, ESR1, PXR, CAR, and PPARα/γ) in the fetal liver were significantly elevated. Expression of target transporter genes appeared to be insufficient to compensate role of the placenta and maternal liver and avoid fetal liver damage.

2.15. The study by (Fallon *et al.*, 2023) tested a novel non-thrombogenic extracorporeal circuit, known as the Nitric Oxide Surface Anticoagulation (NOSA) system, using premature lambs connected to an artificial placenta. The intention of the study was to reduce the risk of intercranial haemorrhage associated with artificial placenta technology. The system was found to support the

lambs for seven days without the need for systemic anticoagulation, thus addressing some limitations in the translation of this technology to clinic.

2.16. The study by (Eixarch *et al.*, 2023) describes the gradual development of an artificial placenta system in sheep, aiming to achieve survival up to one week while addressing learning curve challenges and main bottlenecks. A total of 28 fetal sheep were transferred to an artificial placenta system at 110-115 days gestation. Results indicate a progressive reduction in cannulation complications, improvement in initial pH, and an increased rate of experiments reaching the survival goal, highlighting the complex and time-consuming nature of achieving reproducible transition and extended survival in artificial placenta systems.

2.17. The use of artificial womb technologies has also demonstrated wider application in research whereby it has been used to investigate complex gene expression in animal models of chronic hypoxemia (Moon *et al.*, 2022; Omann *et al.*, 2022) and proposed as a useful environment to establish beneficial microbial communities (Nami *et al.*, 2023).

2.18. Work to explore the clinical application of artificial womb technology for the support of extremely premature infants (<28 weeks gestation) in human patients has also begun with the application of high-fidelity medical stimulation protocols. In 2023, (van Haren *et al.*, 2023) developed a stimulation protocol to conceptualise a step-by-step plan for transference of a human fetus between 24-28 weeks gestation from the maternal uterus to an artificial placenta and womb system. These findings offer a starting point for further development of the transfer procedure, paving the way for the translation of artificial womb technology to clinical trials. A follow up review by the same group, has since identified further considerations for the obstetric procedure (van Haren *et al.*, 2024).

2.19. The report by (Hunter, 2024) summarises the advancements seen in artificial womb technology and considerations relevant to its therapeutic applications in humans, including for the potential treatment of congenital defects that may only be treated effectively through early pregnancy intervention. As described in this paper, developments in artificial womb technology led the US Food and Drug Administration (FDA) to hold a seminar in 2023 to discuss the safety and effectiveness of artificial womb technology, “including regulatory and ethical considerations for first-in-human studies”. (Shah, 2023) discusses the potential approval of human trials in the US.

2.20. In their review article, (Shah and Mychaliska, 2023) discuss the clinical indications, current approaches in development, ongoing challenges, remaining milestones and ethical considerations prior to clinical translation of artificial womb or placenta technologies. Though inherently different approaches with their own ethical challenges (Kukora *et al.*, 2023a), authors conclude these systems will likely prove to be complementary therapies for the treatment of extreme prematurity. To move towards clinical application, key milestones including miniaturization, anticoagulation strategies, risk stratification, critical care protocols, regulatory pathways, and technology translation strategies are being addressed by various research groups (Spencer and Mychaliska, 2022b).

2.21. The paper by (van Haren *et al.*, 2024) provides a consensus framework for the research and development of liquid-based perinatal life support technology. It highlights the importance of addressing technical, socio-ethical, and legal considerations, incorporating input from healthcare professionals, designers, ethicists, researchers, and patient representatives to co-create a system that promotes physiological growth and development in extremely preterm infants.

2.22. The impact of introducing artificial placenta technology to clinics is discussed by (Romanis and Adkins, 2024), who make recommendations for care pathways surrounding the artificial placenta. These include the need for counselling and careful consideration of the language used when these technologies are introduced. A roadmap for the ethical development and implementation of artificial amniotic sac and placenta technology in clinical practice is suggested by (Verweij *et al.*, 2021).

2.23. To investigate how participants may be protected in the first human trials of artificial womb technologies (Cavolo and Pizzolato, 2024) compared randomized controlled trials and single arm trials to understand which trial design best balances the interests of science and participant. Researchers concluded that a single arm trial could prevent some of the methodological and ethical challenges of randomised trails. Ethical challenges unique to designing the first in-human trials of the artificial placenta and artificial womb have also been examined by (Kukora *et al.*, 2023b), who make recommendations to guide ethical study design for initial human translation of these technologies.

Ex-utero culture

2.24. Developing in vitro platforms that accurately replicate the physiological and pathological conditions of complex organisms is crucial for both applied and translational research.

2.25. The review by (Francés-Herrero *et al.*, 2022) summarises the evolving landscape of bioengineering strategies, platforms, and therapies in female reproductive medicine, aiming to enhance understanding of reproductive biology and offer new avenues for fertility restoration. These include scaffold free approaches, hydrogels, decellularised extracellular matrix and polymer scaffolds, bioprinting, organoid, and microfluidic strategies.

2.26. The review by (Yao *et al.*, 2023) highlights the importance of understanding cellular and molecular processes during human early post-implantation development. Using non-human primates as a species for investigating mechanisms underlying human embryonic development, recent advances in vitro culture systems for the growth of non-human primate embryos are discussed in this review, along with potential optimization strategies and applications.

2.27. The review by (Oldak *et al.*, 2022) discusses the latest ex-utero embryo-culture systems for rodents, nonhuman primates, and humans, emphasizing their technical aspects, developmental timeframes, and potential contributions to understanding natural and synthetic mammalian embryogenesis, as well as the stem-cell field.

2.28. The study by (Xu *et al.*, 2023) describes a three-dimensional "sandwich" vascular niche culture system utilizing human placenta perivascular stem cells and human umbilical vein endothelial cells to support mouse embryo development from embryonic day 3.5 to 7.5 in vitro. The constructed vascularization microenvironment was found to significantly enhancing embryo development, providing a valuable platform for investigating the physical and molecular mechanisms of embryo implantation in vitro.

2.29. The study by (Ichikawa *et al.*, 2022) introduces an ex vivo Matrigel-collagen-based culture system capable of recapitulating mouse development from embryonic day 4.5 to 6.0. The system demonstrates successful replication of embryonic growth, axis initiation, and overall three-dimensional architecture in approximately 49% of cases, facilitating detailed cellular dynamics

observation through automatic cell segmentation via light-sheet microscopy. This study establishes a cell culture system that can be utilized to investigate extraembryonic mesoderm during human peri-gastrulation development, both in monolayer cultures and more complex models.

2.30. In the study by (Govindasamy *et al.*, 2021), a three-dimensional biomimetic culture environment mimicking the murine implantation niche was established, allowing direct analysis of trophoblast invasion and early interactions with the maternal vasculature. This biomimetic platform provides insights into the hidden dynamics of early interactions at the implantation site, shedding light on mechanisms mediating embryo-maternal interconnection.

2.31. A protocol for establishing a three-dimensional biomimetic environment based on synthetic hydrogels which harbor key biomechanical properties of the uterine stroma is presented in the paper by (Govindasamy *et al.*, 2023). From describing the steps for isolating and culturing embryos in PEG/DexMA hydrogel, authors detail the co-culture of embryos and endothelial cells in a microfluidic device.

2.32. The protocol set out by (Sun *et al.*, 2024) outlines a method for culturing cynomolgus monkey embryos in vitro for up to 25 days post-fertilization, allowing for the study of gastrulation and early organogenesis events. It includes procedures for culturing using a three-dimensional system, immunofluorescence analysis, single-cell RNA sequencing, and subsequent bioinformatic analysis.

2.33. The study by (Bondarenko *et al.*, 2023) engineered a uterus-like microenvironment to mimic peri-implantation development of whole mouse embryos ex vivo, shedding light on the essential roles of physical embryo-uterine interactions during implantation.

2.34. The study by (Gong *et al.*, 2023) describes the development of an embedded three-dimensional culture system that allows for the extended ex utero culture of cynomolgus monkey embryos for up to 25 days post-fertilisation. Morphological, histological, and single-cell RNA-sequencing analyses demonstrate that ex utero cultured embryos largely recapitulated key events of in vivo development.

2.35. Recent advances and possible future directions in the development of in vitro models is discussed in the review by (Zhai *et al.*, 2022). At present, culturing non-human primate embryos beyond the gastrulation stage is one of the best strategies to understand human gastrulation. However, reconstructions of stem cell–based embryo models and advancements in single-cell multimeric studies and imaging provide insight into the mechanisms underlying human embryogenesis.

3. Ethical and legal considerations

3.1. The developments in artificial womb technology for the support of premature babies have raised a variety of ethical and legal questions concerning the potential future application of these technologies for complete ectogenesis.

3.2. In their scoping review, (De Bie *et al.*, 2023) summarise the existing and emerging ethical considerations in relation to artificial womb technology. By breaking these down into a framework, the authors consider the ethical concerns regarding the application of artificial womb technologies at different stages of gestation in relation to the: (1) potential benefits and harms for each

stakeholder group, (2) decision making authority of parents, (3) legal statuses and protections, and (4) fairness of access. Although application of the technology prior to 22 weeks (the current limit of viability) will require overcoming significant developmental and technical challenges with the technology, the authors highlight the most discussed ethical questions in relation to complete ectogenesis. These include the moral status of embryos and fetuses, the impact on parental roles, child welfare, societal implications, regulatory frameworks, legal status, and intellectual property concerns.

3.3. In response to this article numerous commentaries have been published (Brown and Watson, 2023; Browne *et al.*, 2023; Cordeiro, 2023; De Proost and Zuijdwegt, 2023; Esquerda *et al.*, 2023a, 2023b; Gavina *et al.*, 2023; Gulino *et al.*, 2023; Holmes and Hosford, 2023; Kennedy and Nelson, 2023; Kimberly and Quinn, 2023; Lomotey-Nakon and Lanphier, 2023; Mercurio and Werner, 2023; Roesner, 2023a, 2023b; Takashima *et al.*, 2023; Verweij and Kingma, 2023). Amongst these the legal and ethical arguments in relation to the application of artificial wombs have been discussed, including implications for abortion rights (Anderson, 2023; Räsänen, 2023; Simkulet, 2023; Stratman, 2023), the future role of surrogacy (Romanis, 2022), the moral status and appropriate terminology for offspring (Segers and Romanis, 2022; Werner and Mercurio, 2022), wider reproductive rights (Hooton and Romanis, 2022; Kendal, 2022; Bidoli, 2024), the impact on familial structure and the value of gestational ties (Accoe and Pennings, 2024; Ferreira, 2022; Kendal, 2023; Kennedy, 2024), and religious considerations (Muhsin *et al.*, 2023).

3.4. As stated by (Medori *et al.*, 2023) extensive further research is required for the practical implementation of artificial placenta and artificial womb technologies as healthcare procedures for humans, particularly in relation to complete ectogenesis. It is therefore important to differentiate between technologies which are feasible and those which remain highly theoretical. Ethical concerns pertaining to the application of these technologies will need to be addressed and resolved prior to their introduction to the public sphere.

4. Conclusions

4.1. While the use of artificial womb technology for whole gestation remains theoretical, significant strides have been made towards developing this experimental technology for late-gestation support. Current research is predominantly focused on refining the technology and ensuring its safety and efficacy ahead of clinical application. As research progresses, clinical trials for its application as a clinical tool to support premature human babies will be necessary to determine how this technology can improve outcomes and to address any ethical, medical, and logistical challenges.

4.2. As above, the use of artificial wombs would fall within the HFEA remit where the technology was applied for early to complete gestation of human embryos created in vitro. At this time research is limited to the development of ex utero culture systems, predominantly focused on understanding implantation and early embryogenesis in human or stem cell-based embryo models.

4.3. Significant advancements in research and a radical extension to the 14-day rule would be required before research on and clinical applications of artificial wombs for complete gestation can be considered.

5. Recommendations

5.1. Members are asked to:

- consider the progress of research into ectogenesis;
- advise the Executive if they are aware of any other recent developments; and
- consider whether the research to date on the topic of ectogenesis shows advances relevant to the remit of the HFEA.

5.2. and to note that:

- the topic of artificial wombs for early or whole gestation (ectogenesis) will remain on the SCAAC prioritisation list until February 2025 for the next prioritisation discussion so that it can be considered alongside the committee's consideration of its workplan for 2025/2026.

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Application to include androgen supplementation as a new add-on in the HFEA's rated list

Details about this paper

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Author:	Mina Mincheva, Policy Manager (HFEA)
Annexes	Annex A: Add-ons application form on androgen supplementation Annex B: SCAAC treatment add-on application decision tree

Output from this paper

For information or advice?	For advice
Recommendation:	Members are asked to: • advise whether androgen supplementation meets the criteria set out by the treatment add-ons decision tree to be eligible for a HFEA rating Note: we are not asking the committee to make a recommendation at this meeting on the rating itself.
Resource implications:	Medium
Implementation date:	To be determined
Communication(s):	To be determined
Organisational risk:	Low

1. Introduction

1.1. Treatment add-ons are often non-essential treatments that may be offered in fertility clinics in addition to routine treatment with the claim that they can improve treatment outcomes. As with all new treatments or technologies being introduced into reproductive medicine, we expect the introduction of treatment add-ons into clinics to be preceded by good quality scientific research into the effectiveness and safety of these interventions. However, some treatment add-ons are being offered to patients without this evidence base for effectiveness at increasing live birth rate, safety, or other treatment outcomes. They are frequently offered outside of a research setting and are charged for at an additional cost.

1.2. Medical professionals, academics or patient organisations can propose that we review the evidence base for a treatment add-on if they are concerned that it is being offered to patients in a UK licensed clinic:

- with the claim that it will increase the live birth rate or improve other treatment outcomes;
- without conclusive evidence of its effectiveness at improving the live birth rate or other treatment outcomes;
- it is not already listed in our the HFEA's rated list of add-ons
- there is evidence that an add-on treatment may reduce treatment effectiveness or there are potential safety concerns.

1.3. In **October 2021** the SCAAC recommended that androgen supplementation should be considered a separate treatment add-on from immunological tests and treatments.

1.4. An application was taken to the **June 2022** meeting, where it was agreed that androgen supplementation did not meet the criteria set out by the treatment add-ons decision tree in place at the time.

1.5. Following revisions to the treatment add-ons application form and decision tree, recommended by the committee in **February 2023**, the committee agreed at the **July 2023 meeting** that androgen supplementation should be reconsidered as a treatment add on.

1.6. The executive have updated the add-ons application form for androgen supplementation that was previously considered by the committee (Annex A) for reconsideration.

2. Recommendation

2.1. Members are asked to:

- Advise whether androgen supplementation meets the criteria set out by the treatment add-ons decision tree (Annex B) to be eligible for a HFEA rating.

2.2. Note: we are not asking the committee to make a recommendation at this meeting on the rating itself. Should members consider androgen supplementation to meet the criteria, an expert literature review will be commissioned by the Executive and be bought for discussion to a future SCAAC meeting.

Annex A - Add-ons application form on androgen supplementation

Proposed treatment add-on

What is the name of the treatment?

Androgen supplementation

Most commonly either dehydroepiandrosterone (DHEA) or testosterone.

Please provide some background about the treatment and include how the treatment is used and how it claims to improve live birth rate or other treatment outcome(s). (max. 600 words)

DHEA and testosterone are steroid hormones used in assisted reproduction (they are used within standard treatment for those with poor ovarian response (POR) or diminished ovarian reserve (DOR)), due to their suggested role in improving pregnancy rates and increasing live birth rates.

Testosterone is administered transdermally, through either patches or gel, or orally. DHEA is ingested as a 75mg pill once per day, up to 12 weeks prior to ovarian stimulation and during ovarian stimulation. DHEA is an androgen pre-hormone and a precursor to multiple hormones (including testosterone), which decreases as a women's age increases (Nagels et al., 2015).

Animal studies and human studies have demonstrated that androgens have an essential role in the regulation of ovarian function (Walters & Handelsman 2018) in particular in pre-antral and small antral follicular health (Sunkara et al., 2012). Other suggestions for the role of androgens includes their acting as ligands for androgen receptors, also promoting follicular growth (Fouany & Sharah 2013, Nagels et al., 2015). High levels of androgens are observed in the development of polycystic ovarian syndrome (PCOS) (Walters, 2015).

Studies have investigated the role of androgens in increasing the effect of FSH on follicular growth (Vendola et al., 1999, Weil 1998), and animal models have been used to investigate the method by which androgens regulate 'follicle health, development and ovulation' (Anderson et al., 2012 and Walters, 2015). However, much of the scientific mechanism of androgens continues to be 'elusive' (Nagels et al., 2015), and the mechanism of androgens for increasing the follicular pool in patients with POR is still being investigated (Montoya-Botero, 2019, Founay & Sharara 2013).

Research into androgens has investigated their role in increasing the expression of androgen receptors (Hu et al., 2017), their positive effect in ovarian follicular development, recruitment, and growth (Polyzos et al., 2018, Vendola et al., 1998 & 1999, Weil 1998), and their role in increasing the number of primary and pre-antral follicles (Triantafyllidou, 2017). Animal and human research has shown that the use of androgen supplementation can increase the number of oocytes produced, which in turn could assist in increasing pregnancy rates (Barad 2007, Gleicher 2011, Nagels et al., 2015)

Androgen supplementation has been suggested as beneficial for women with DOR or POR (that is, not for all patient groups) as a means to improve ovarian function via increasing ovarian response to stimulation, ovarian reserve, and follicular development and recruitment (Kim C H 2013, Fanchin et al., 2011, Lossl et al., 2020, Noventa et al., 2019, Nagels et al., 2015). Androgen supplementation has also been suggested to reduce aneuploidy rates, thereby decreasing miscarriages in older women resulting in higher live birth rates (Gleicher et al., 2009, 2010, 2015, Fouany & Sharara 2013).

A large number of RCTs and meta-analyses call for further RCTs and studies to continue to further establish the role of androgen supplementation in IVF.

Please demonstrate that this treatment is being offered to or requested by patients in a UK fertility clinic with the claim that this treatment increases live birth rate or improves other treatment outcome. This could

be contained in patient information leaflets, website content or anonymised conversations between patients and fertility clinic staff. (max. 300 words)

DHEA and Testosterone are available in the UK with a prescription. In the UK DHEA is also readily available as a tablet supplement through health supplement websites (eg **Biovea**, **iHerb**). However, these supplements are marketed for other health benefits, but not marketed for fertility treatment. In the USA they are available as an over-the-counter supplement.

Academic papers have claimed that approximately a quarter of fertility clinics worldwide use DHEA for poor responder patients to improve pregnancy chances (Fouany & Sharara 2013).

To the best of the Executive's knowledge, androgen supplementation is not discussed on many HFEA licensed fertility clinic websites, nor is there extensive discussion of the topic on UK fertility forums.

The HFEA has not received any recent patient enquiries regarding the use of androgen supplementation.

DHEA and testosterone are listed in the price lists of several UK fertility clinics. However, DHEA and testosterone are offered within standard treatment plans for some subsets of patients, including patients with DOR or predicted POR.

To the Executive's best knowledge, there is no indication that, in the UK, androgen supplementation is being offered outside of these patient groups to claim to increase clinical pregnancy or live birth rates.

Please provide any recommendations made by professional bodies, eg NICE, ESHRE, RCOG, BFS or ASRM, for or against the use of this treatment in fertility patients. (max. 500 words)

ESHRE published recommendations in 2019 in which the use of either DHEA or testosterone in IVF was not recommended for use in poor responders.

As regards to DHEA, in this report they stated that the evidence for its use is 'inconsistent' for improving live birth rate and ongoing pregnancy rate. The studies they considered used varying durations of DHEA treatment, which may have contributed to the inconsistencies in the results.

Similarly, ESHRE stated that the evidence for testosterone pre-treatment is currently 'inconsistent' in terms of improving number of oocytes retrieved and clinical outcomes of live birth rates in poor responders undergoing IVF treatment.

Due to this, ESHRE recommended that large RCTs should take place for DHEA supplementation and testosterone supplementation in order to establish further information on dosage, administration duration, and safety. Furthermore, ESHRE's recent good practice recommendations on add-ons in reproductive medicine do not support the use of adjuncts during ovarian stimulation: "Adjuncts (metformin, growth hormone, testosterone, DHEA, aspirin, indomethacin, and sildenafil) before or during ovarian stimulation are not recommended."

NICE added recommendations about DHEA to their 'Fertility problems: assessment and treatment' clinical guidelines in 2013. In this they stated that DHEA should not be used as an adjuvant treatment for controlled ovarian stimulation in IVF. There are no recommendations on the use of testosterone.

Current evidence base

Effectiveness

To be included in the HFEA add-on review list, a treatment needs to lack published evidence about its effectiveness. Please provide peer-reviewed published evidence that this treatment add-on **is or is not effective at increasing live birth rate or improving other treatment outcomes**, i.e. the extent to which this treatment is or is not able to deliver the promised benefits. Please include references to any relevant published data as appendices to this form. For example, you may wish to include references to data from animal studies, large data studies, research on human embryos, or clinical trial data. Study outcomes should include live birth rate as a primary or secondary outcome. (max. 500 words)

There is limited research in androgen supplementation for women who are not poor responders or who do not have diminished ovarian reserve.

Live birth rates

A 2015 **Cochrane review** analysed 17 RCTs reporting on DHEA or testosterone supplementation in IVF (12 DHEA, 5 testosterone) (Nagels et al., 2015). It included 1496 participants with most trials focussing on '"poor responders"' to standard IVF protocols' (Nagels et al., 2015). Pre-treatment with DHEA was associated with higher live birth rates. However, when trials with a risk of performance bias were removed, the results no longer reached significance. Similarly, pre-treatment with testosterone was only associated with higher live birth rates when studies at a high risk of performance bias were included in the analysis. Reasons for defining the evidence from these RCTs as 'moderate' included low sample sizes, lack of blinding, and the imprecise/poor reporting of study methods (Nagels et al., 2015).

Two further RCT meta-analyses found that women with POR or DOR receiving testosterone showed higher live birth rates (Noventa et al., 2019, Bosdou et al., 2012, Neves et al., 2022). Similarly, patients with POR and DOR receiving DHEA (Xu et al, 2019) had improved live birth rates. However, Sunkara et al., 2011, found that androgen supplementation did not show significant differences in live-birth rates. A number of other meta-analyses showed no improvement in live birth rates following DHEA pre-treatment supplementation in women with POR or DOR undergoing IVF or ICSI (Zhang et al., 2023; Neves et al., 2022; Wang et al., 2023).

Zhang et al., 2021, investigated DHEA use in women with endometriosis and found that live birth rate was higher in the DHEA supplementation group. However, there were only 44 study participants. Another RCT investigating DHEA supplementation in women (52 participants) with POR undergoing IVF found that pre-treatment DHEA supplementation did not improve live birth rates (Narkwichean et al., 2017). Similarly, an RCT by Wang et al., 2022 including randomising 410 women to DHEA pre-treatment before IVF did not find an increase in cumulative live birth rates.

Other outcomes

One RCT found that DHEA slightly increased the number of oocytes retrieved and increased the fertilization rate in women with DOR (Kara et al., 2018). An increase in number of oocytes retrieved was reported in a network meta-analysis by Zhu et al., 2023. An RCT by Wang et al. 2022 found that DHEA did not increase the number of retrieved oocytes in women with POR undergoing IVF. Similarly, two RCTs investigating testosterone supplementation in women with POR found that testosterone did not increase the number of mature oocytes retrieved (Subirá et al., 2021, Hoang et al., 2021). Hoang et al., 2021, reported pre-treatment of testosterone for 4 or 6 weeks increased clinical pregnancy rates.

Further trials have also noted increased clinical pregnancy rates and functional ovarian reserves through the use of DHEA (Singh et al., 2013, Barad et al., 2014, Li et al., 2015). Clinical trials have reported the effectiveness of DHEA for women with DOR or POR varies by age and FMR1 genotypes (Gleicher, 2013 and Weghofer et al., 2012).

One meta-analysis found that DHEA improves pregnancy rates in young women with DOR, and reduces miscarriage rates in older women with DOR as it decreases age-related aneuploidy (Fouany & Sharara 2013). Two other meta-analyses showed improved clinical or ongoing pregnancy rates in women with POR or DOR (Zhu et al., 2023; Wang et al., 2023). A meta-analysis by Neves et al., 2022 reported an increase in clinical pregnancy rate after testosterone supplementation in DOR or POR.

In their 2021 meta-analysis Richard & Jayaprakasan found that DHEA conferred no benefit to women with DOR on IVF outcome. They also reported testosterone use in women with DOR or POR improved IVF outcomes, but only when including low quality studies with a high risk of bias.

Other meta-analyses found that DHEA treatment did not result in a significant difference in clinical pregnancy rates in women with DOR or POR (Narkwichean et al., 2013; Neves et al., 2022). Qin et al., 2017 noted that DHEA only increased clinical pregnancy rates for women with DOR when non-RCT studies were included within their meta-analysis.

Safety

If there is evidence that this treatment is **not** safe or there is risk of harm, for either the patients or the children born after the use of this treatment or may reduce treatment effectiveness, please outline it here. Please include references to any relevant published data as appendices to this form. For example, you may wish to include references to data from animal studies, large data studies, research on human embryos, or clinical trials data. (max. 500 words)

Studies have shown little discussion and research into the safety of DHEA and testosterone.

As noted in Polyzos et al., 2018, an excess of testosterone is likely to 'induce adverse events' and could be either 'ineffective' or 'detrimental'. Additionally, as excess androgens show a key role in the polycystic ovary syndrome, further research is required (Walters et al., 2019).

A meta-analysis by Xu et al., 2019, found that DHEA supplementation did not cause any adverse effects and that miscarriage rates did not differ between control and DHEA groups. Similar findings were reported in an RCT by Wang et al., 2022.

Side effects in women undergoing DHEA treatment have included acne, hair loss, excess hair growth, dizziness and voice deepening (Tartagni et al., 2015, Wiser et al., 2010, Zhang et al., 2014). Limiting treatment at 75mg/day of DHEA reduces these effects (Kroboth et al., 1999, Artini et al., 2012). The Cochrane review has noted that patient's medical history, administration methods, and dosage of both DHEA and testosterone require further research and studies (Nagels et al., 2015). Additionally, further research is needed into the effect of androgen supplementation on the embryo (Sir-Petermann et al., 2002, Nagels et al., 2015) to establish whether any risk of harm exists for children born after use of this treatment.

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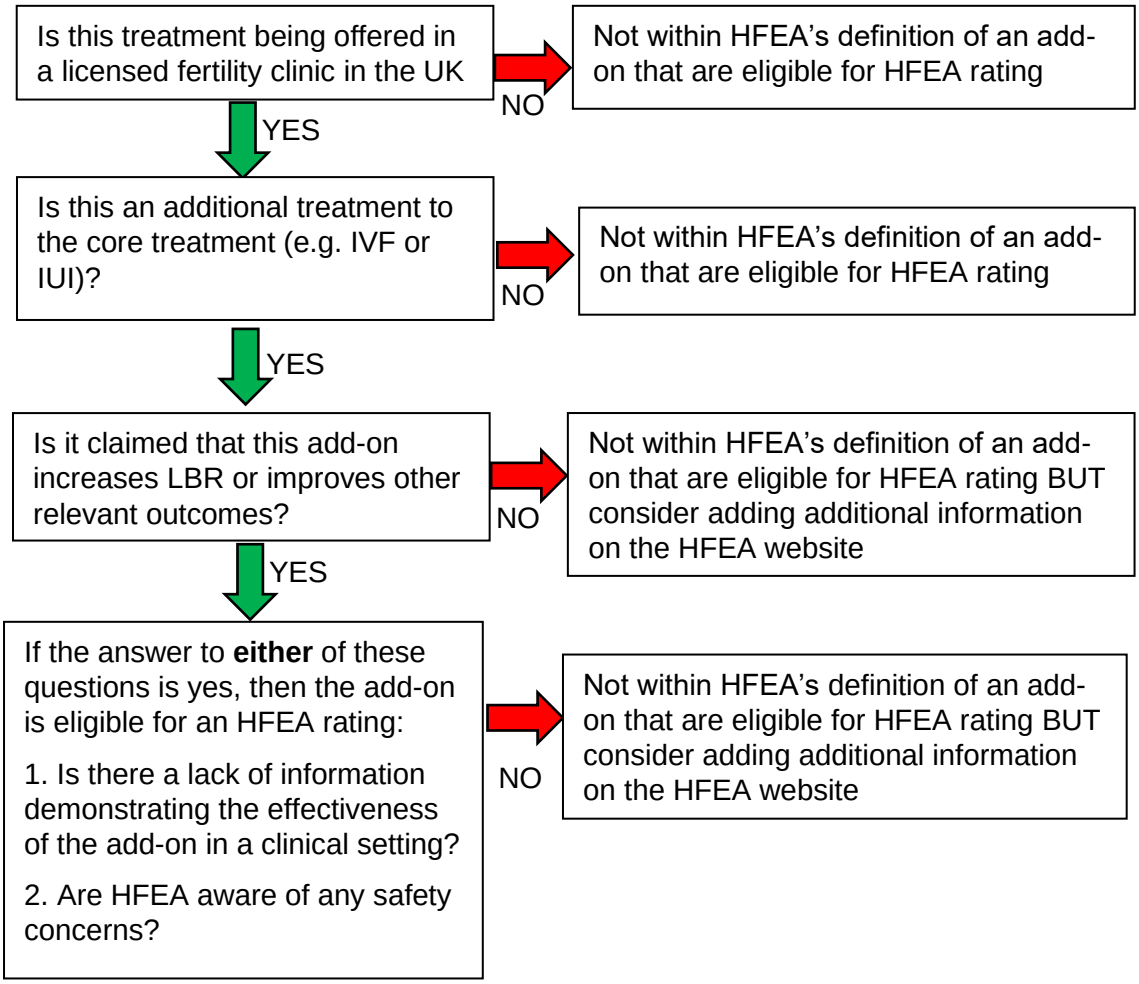
Annex B - SCAAC treatment add-on application decision tree



Alternative methods to derive embryonic and embryonic-like stem cells

Details about this paper

Area(s) of strategy this paper relates to:	Shaping the future
Meeting:	Scientific and Clinical Advances Advisory Committee (SCAAC)
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Annexes	N/A

Output from this paper

For information or advice?	For advice
Recommendation:	Members are asked to: • consider the progress of research into alternative methods to derive embryonic or embryonic-like stem cells; • advise the Executive if they are aware of any other recent developments; and • review whether any outputs from the HFEA are required.
Resource implications:	N/A
Implementation date:	N/A
Communication(s):	N/A
Organisational risk:	Low

1. Introduction

1.1. Human embryonic stem cells (hESCs) are a type of pluripotent stem cell derived from the inner cell mass of a blastocyst. These cells are capable of differentiating into any cell type in the body, which makes them a valuable resource for disease modelling, drug testing and development, and basic research. They additionally have potential application in therapies for patients.

1.2. The use of hESCs is subject to ethical debate as their derivation can involve the destruction of a viable human embryo which has been donated to research. To address this, researchers are investigating alternative methods of deriving hESC, or hESC-like cells, that do not involve the use of viable embryos, which for some, may raise fewer ethical concerns. These methods aim to either obtain pluripotent stem cells without destroying embryos or to create conditions where hESC-like cells can be derived in a way that would raise fewer ethical concerns.

1.3. In the UK, research involving the derivation of hESC from embryos or hESC-like cells from human gametes is licenced under a HFEA research licence. Section 3A(1)(c) of Schedule 2 of the Human Fertilisation and Embryology Act 1990 (as amended) requires embryo research to be "necessary or desirable" for defined purposes. If in future alternative methods of deriving hESC or hES-like cells become fully developed, a question may be raised whether it may become less 'necessary' for licensed research groups to use viable embryos in all of the research purposes for which they are currently used. Therefore, it is important for the Authority to keep up to date with developments regarding these alternative methods so that the HFEA Licence Committee can bear them in mind when considering research licence applications in line with the Act.

1.4. Alternative methods to derive hES-like cells has been brought to SCAAC as a standing high priority item for several years. The last update was discussed at the January 2022 meeting where the committee considered recent developments in methods to derive primed and naïve pluripotent stem cell lines alongside the 2021 updates to Guidelines for the Field of Stem Cell Research and Regenerative Medicine developed by the International Society for Stem Cell Research (ICSSR).

1.5. The current paper provides a summary of the available research on alternative methods to derive embryonic and embryonic-like stem cells published between 1st January 2022 and 31st April 2024. The Executive notes that this paper is not an assessment of study validity.

2. Methods to create and maintain human pluripotent stem cells

2.1. One way to derive hESC-like cells is by reprogramming human somatic cell lines into induced pluripotent stem cells (iPSC). Conventional iPSC resemble cells of the post-implantation epiblast, existing in a 'primed' pluripotent state for germ layer specification. 'Naïve' pluripotency occurs in cells derived from the pre-implantation stage when there is more differential capacity. It is now possible to induce a naïve state in human pluripotent stem cells in primed cells, enhancing the utility of pluripotent cells for research and clinical applications.

2.2. Producing iPSCs at a scale suitable for clinical applications remains a challenge to the translation of human iPSC to clinical practice. To address this, (Tian *et al.*, 2022) describe a protocol for the creation of clinical-grade iPSC from small populations of banked umbilical cord blood, which can

be maintained in a feeder-free, xeno-free environment using commercially available reagents. Resultant iPSC lines were found to display pluripotency, genomic integrity and stability vital for translation to their use in clinical trials.

2.3. A step-by-step protocol for the generation of human iPSC using progenitor cells derived from umbilical cord blood and adult peripheral blood is also described in the study by (Gao *et al.*, 2022). Here, reprogramming endothelial progenitor cells into iPSCs was achieved through ectopic expression of four transcription factors, OCT4, KLF4, SOX2, and c-MYC, using self-replicative RNA technology.

2.4. The use of peripheral blood mononuclear cells to generate iPSC lines has also been described by (Singh *et al.*, 2022). Using an optimized protocol, researchers were able to derive clinical-grade iPSC lines from erythroid progenitor cells expanded from peripheral blood in a feeder-free and xenofree conditions. Peripheral blood therefore represents an easily accessible and minimally invasive somatic cell source for the derivation of large numbers of iPSC lines, which may be obtained from individual healthy donors, diseased patients, or donors with homozygous human leukocyte antigen (HLA) for haplobanking.

2.5. The study by (Sokka *et al.*, 2022) describes the high-efficiency reprogramming of human somatic cells into iPSC through targeted clustered regularly interspaced short palindromic repeats (CRISPR) activation of endogenous pluripotency factors. CRISPR activation was found to reprogramme cells into a pluripotent state with higher fidelity than conventional methods utilising the ectopic expression of transcription factors, supporting the use of CRISPR activation to improve accuracy when generating human iPSC.

2.6. The study by (Cuesta-Gomez *et al.*, 2023) presents a 3D-suspension cell-expansion protocol for improved expansion capacity, genetic integrity, pluripotency phenotype, and in vitro and in vivo pluripotency potential of iPSCs. The study demonstrated a nearly 100-fold iPSC expansion over 5-days when using the 3D protocol in comparison to 2D models, with 3D expanded cells showing enhanced pluripotency phenotypes. Demonstrating an efficient scale-up strategy for movement towards clinical implementation.

2.7. Using small molecules for chemical stimulation (Guan *et al.*, 2022) demonstrate an alternative method to derive human iPSC from somatic cells. In doing so the group define an intermediate plastic state during which chemical-induced dedifferentiation occurred and identify the c-Jun N-terminal kinase (JNK) pathway as a major barrier to chemical reprogramming.

2.8. Understanding the initial populations of human iPSC has been argued to instruct proficient lineage commitment. To address this (Pei *et al.*, 2023) generated human iPSC from somatic cells by transduction using Sendai viral vectors. Genome-wide DNA methylation analysis and transcriptional analysis were performed to evaluate the pluripotent capacity and somatic memory state of hiPSCs, revealing that human umbilical arterial endothelial cell-derived iPSCs exhibit pluripotency comparable to human embryonic stem cells and hiPSCs derived from other tissues (such as umbilical vein endothelial cells, cord blood, foreskin fibroblasts, and fetal skin fibroblasts). Despite this, human umbilical arterial endothelial cell-derived iPSCs retain a transcriptional memory typical of their parental cells and share a DNA methylation signature similar to umbilical cord blood-derived iPSCs, suggesting that somatic cell data may predispose iPSC to differentiate more amenable into specific cell fates.

2.9. The study by (Ren *et al.*, 2023) reports on the feasibility of using ethanol as a fixative for preparing feeder cells for human pluripotent stem cell culture, addressing concerns regarding the toxicity associated with methanol previously used for this purpose. Feeder cells treated with ethanol sustain the self-renewal and undifferentiated potential of various types of human pluripotent stem cells over an extended duration. This observation holds true for primed, naïve, and extended human embryonic stem cells, as well as induced pluripotent stem cells.

2.10. A review by (Sharp *et al.*, 2022) discusses the present status of RNA viral vectors technology for reprogramming of somatic cells, highlighting Paramyxoviruses, Sendai and measles virus as emerging vectors for non-integrated delivery of reprogramming factors. Application of a Sendai vector delivery system to generate transgene-free naïve human iPSC by (Kunitomi *et al.*, 2022) is described below.

2.11. A study by (Nimonkar *et al.*, 2023) reports on the generation and characterisation of human iPSC line, NIMHi007-A, derived from peripheral blood mononuclear cells. Cells were reprogrammed using the non-integrating Sendai virus consisting of Yamanaka reprogramming factors- SOX2, cMYC, KLF4, and OCT4. iPSCs displayed a normal karyotype, express pluripotency markers, and could generate into three germ layers, endoderm, mesoderm, and ectoderm, in-vitro.

2.12. A study by (Lyra-Leite *et al.*, 2023) develop a simplified basal medium and memory B cell (BMEM) supplement to enhance the growth rate of human iPSC. By refining the media, authors highlight the essential and optimal concentrations for growth of human iPSC which can be derived into multiple lineages.

2.13. Numerous basic science studies have also been conducted to improve understanding of molecular and genetic factors vital to reprogramming. In categorising unestablished molecular mechanisms, researchers are able to better refine existing methods to generate iPSC.

2.14. The challenge of heterogeneity among primed and naïve pluripotent stem cell lines is addressed in the study by (Kunitomi *et al.*, 2024). By introducing a fusion protein of the maternal-specific linker histone H1FOO with a destabilizing domain (H1FOO-DD), alongside OCT4, SOX2, KLF4, and LMYC, into human somatic cells, authors enhance reprogramming to both primed and naive pluripotency. Notably, H1FOO-DD generates naive induced pluripotent stem cells with lower variation in transcriptome and methylome among clones and a more uniform and superior differentiation potency.

2.15. A study by (Wille and Sridharan, 2022) describe how inhibition of telomeric silencing 1-like (DOTL1), the histone 3 lysine 79 (H3K79) methyltransferase, increased efficiency when reprogramming somatic cells into iPSC. Promoting iPSC generation beyond the mesenchymal to epithelial transition, DOTL1 inhibition was found to be effective in iPSC generation from established epithelial cell types. As regulation of single genes was unable to substitute DOTL1 inhibition, authors demonstrated that H3K79 methylation has pleiotropic effects in maintaining cell identity and the complex role of DOTL1 in cell reprogramming.

2.16. The expression of lineage markers, isolated neural lineages and the related microRNAs involved in iPSC reprogramming was identified in the study by (Sun *et al.*, 2022). Using genome-wide microRNA analysis and molecular network analysis, researchers identified 45 differentially regulated microRNAs with 6,047 validated mRNA targets, mostly related to genes of neurogenesis. Key proteins involved in neural lineage reprogramming included Sall1, Foxa2, Nf2,

Ctnnb1, Shh, and Bmpr1a, suggesting that transcription factors can drive the reprogramming of somatic cells towards pluripotency via the neuroectoderm.

2.17. The study by (Sevinç *et al.*, 2022) shows that inhibition of the bromodomain-containing protein 9 (BRD9), an essential component of the ncBAF (non-canonical barrier to autointegration factor) SWI/SNF (SWItch/sucrose non fermentable) chromatin remodelling complex, increases the efficiency of human somatic cell reprogramming and can replace transcription factors KLF4 (Krüppel-like factor) and c-MYC (cellular myelocytomatosis). Mechanically, BRD9 inhibition downregulates fibroblast-related genes and decreases chromatin accessibility at somatic enhancers. Consequently, its inhibition may be utilised to enhance various reprogramming methods.

2.18. Through genome-wide DNA methylation profiling (Buckberry *et al.*, 2023) characterised the persistence and emergence of epigenetic difference between human iPSC throughout primed and naïve reprogramming of somatic cells. They found that reprogramming-induced epigenetic aberrations emerge midway through primed reprogramming, whereas DNA demethylation begins early in naive reprogramming. Using this knowledge, they developed a transient-naive-treatment reprogramming strategy that emulates the embryonic epigenetic reset without disrupting genomic imprinting. In correcting epigenetic memory and aberrations, transient-naive-treatment reprogramming was found to enhance differentiation of human iPSC derived from multiple cell types.

2.19. Further research performed by (De Los Angeles *et al.*, 2024) challenges this strategy, highlighting that Buckberry et al. (2023) characterized bulk human iPSCs instead of isolating clonal lines and overlooked the persistent expression of Sendai virus carrying exogenous Yamanaka factors. In the follow up analysis, authors state that Sendai virus genes were expressed in most control pluripotent stem cell samples, including human embryonic stem cells (hESCs), and were particularly high in samples treated with naive media. These findings challenge conclusions about transient-naive-treatment reprogramming efficacy in the context of delivery methods.

2.20. A study by (Kristiansen *et al.*, 2022) compared mitochondrial function and mtDNA replication in human embryonic stem cells and iPSCs at three different stages - pluripotent, neural progenitor and astrocyte. Authors found that, while embryonic stem cells and iPSCs have a similar mitochondrial signature, at the neural stem stage, iPSC derived cells displayed decreased adenosine triphosphate production and a reduction in mitochondrial respiratory chain (MRC) complex IV compared with those derived from embryonic stem cells. In addition, iPSC derived astrocytes showed increased mitochondrial activity including elevated ATP production, MRC complex IV expression, mtDNA copy number and mitochondrial biogenesis relative to those derived from embryonic stem cells. Mitochondrial remodelling during neural differentiation from human iPSC has further been investigated by (Chen *et al.*, 2022).

2.21. Advances in understanding the molecular mechanisms underlying pluripotency are driving the development of innovative techniques for inducing and maintaining naive pluripotent stem cells. As naïve pluripotent stem cells can give rise to any cell type in the body, they offer a more versatile model for research and clinical applications.

2.22. The study by (Zorzan *et al.*, 2022) presents protocols for high-efficiency generation of either conventional or naïve iPSCs by delivery of messenger RNAs (mRNAs) using a microfluidic

system, describing both the production of the microfluidic system itself and the application of such devices to reprogram human somatic cells into naïve and primed states.

2.23. The study by (Onfray *et al.*, 2022) describe a protocol to reprogram human fibroblasts into naive pluripotent stem cells by overexpressing the transcription factors OCT4, SOX2, KLF4, and c-MYC using Sendai viruses. Resulting cells represent an earlier stage of development that corresponds to pre-implantation epiblast.

2.24. The study by (Haraguchi and Nakamura, 2022) showed that preferentially expressed antigen of melanoma family member 12 (Pramef12), which is highly expressed in oocytes, enhances the generation of iPSC from mouse fibroblasts. In addition, overexpression of Pramef12 during early phase reprogramming was found to enhance expression of naïve pluripotency-associated genes and promote mesenchymal-to-epithelial transition during iPSC generation.

2.25. The study by (Iwatsuki *et al.*, 2023) presents optimal culture conditions to derive and propagate post-implantation epiblast-derived PSCs in rat models, showing how epiblast-derived iPSC can be reset towards a naïve pluripotent state with exogenous Klf4, but not Nanog, Klf2, Esrrb, Tfcp2l1 and Tbx3. These cells retain competency to produce authentic primordial germ cell-like cells that undergo functional gametogenesis and can lead to the birth of viable offspring.

2.26. Enhance naïve PSC generation through the development of a chimeric super-SOX factor, Sox2-17, is described by (MacCarthy *et al.*, 2024). A swap of alanine to valine at the interface between Sox2 and Oct4 delivered a gain of function by stabilizing Sox2/Oct4 dimerization and enabling generation of high-quality iPSCs. Transient expression of Sox and Klf4 restored dimerization and boosted the developmental potential of pluripotent stem cells across species, providing a universal method for naive reset in mammals.

2.27. An alternative feeder-free culture system for the maintenance of naïve human PSC is presented by (Isono *et al.*, 2023). Using two inhibitors to establish the culture system, cells undertook stable cell proliferation and were positive for naïve cell markers. In addition, they were found to differentiate into the three germ layers.

2.28. In their review (Diamante and Martello, 2022) summarise our current understanding of the metabolic requirements of PSC, describing the instructive role of different metabolites on proliferation, differentiation, and epigenetic profile of PSCs. Fatty acid oxidation, for example, is strictly required for energy production in naive PSCs, but becomes dispensable in more advanced, or primed, PSCs. Pluripotency regulators and nutrient availability in PSCs actively shape the metabolic profile of PSCs. In response to alterations, developmental progression of pluripotent cells can be paused both in vitro and in vivo.

2.29. The study by (Kunitomi *et al.*, 2022) reports on the modification of the Sendai viral system to generate transgene-free naïve human iPSC from a range of somatic cell types in a feeder free culture. The naive iPSCs generated by this method show better differentiation to trilineage and extra-embryonic trophectoderm than those derived by conventional methods.

2.30. The study by (Romayor *et al.*, 2022) used both primed human iPSC and human embryonic stem cells to evaluate the successful establishment and maintenance of a naïve cell stage using three different naïve-conversion medias. In general, naive culture medium, NHSM, (in both feeder and feeder-free systems) was found to confer greater viability and the highest expression of naive

pluripotency markers. NHSM medium also allowed for better cell differentiation cells toward endoderm and mesoderm.

2.31. The study by (Fischer *et al.*, 2022) detail a protocol for inducing naïve pluripotency in primed human embryonic stem cells and iPSC, applying five kinase inhibitors and two growth factors (5i/L/A). Authors additionally outline the use of two fluorescent reporter systems to track acquisition of naïve identity in live cells: (a) a green florescent protein (GFP) reporter linked to an endogenous OCT4 allele in which the primed-specific proximal enhancer has been deleted (OCT4-ΔPE-GFP); and (b) a dual-colour reporter system targeted to both alleles of an X-linked gene that reports on the status of the X chromosome in female cells (MECP2-GFP/tdTomato).

2.32. By establishing that naive, but not primed, hiPSC colonies are characterized by a self-organized extracellular matrix-rich microenvironment, (Cesare *et al.*, 2022) developed a three-dimensional (3D) culture system that supports robust long-term, feeder-free, self-renewal of naive human iPSCs. This provides new opportunities for using naive human iPSCs to explore critical stages of human development in a 3D context.

2.33. The paper by (Chowdhury *et al.*, 2024) revealed that a medium stiffness polyacrylamide hydrogel ~100 kPa) significantly improved human cell reprogramming, resulting in up to a 10-fold increase in the number of reprogrammed cells, accelerated reprogramming kinetics, and enhanced quality of iPSCs with more naïve characteristics and lower remnant transgene expression compared to traditional tissue culture polystyrene methods. This study introduces a novel culture protocol and substrate for hydrogel-based cell reprogramming, offering insights into the mechanisms underlying substrate stiffness reprogramming modulation and iPSC quality outcomes.

2.34. The study by (Ji *et al.*, 2024) demonstrates that inhibition of protein kinase C increased PR domain-containing 14 (Prdm14) levels to promote self-renewal of mouse embryonic stem cells through reducing Suv39h-induced H3K9 methylation. Results provide novel insights into the pivotal association between PKC inhibition-mediated self-renewal and epigenetic changes, which improve understand the regulatory network of stem cell pluripotency.

2.35. The study by (Okubo *et al.*, 2024) reports genetic and non-genetic approaches to generate authentic hypoblast cells, termed naive human PSC-derived hypoblast-like cells (nHyCs). nHyCs spontaneously assemble with naive human PSCs to form a three-dimensional bilaminar structure (bilaminoids) with a pro-amniotic-like cavity. In the presence of additional naive human PSC-derived analogues of trophectoderm, the efficiency of bilaminoid formation was found to increase from 20% to 40%, with epiblast development continuing in response to trophectoderm-secreted IL-6. This research demonstrates how extraembryonic tissue guides stage-specific growth of the epiblast.

Expanded and extended potential stem cells

2.36. More recently new populations of pluripotent stem cells have been developed. These include expanded and extended potential stem cells (EPSCs) which have the unique capacity to differentiate into both embryonic and extraembryonic lineages (Yang *et al.*, 2017a, 2017b; Gao *et al.*, 2019). However, the notion that EPSCs have increased totipotent potential relative to conventional embryonic stem cells remains controversial (Posfai *et al.*, 2021).

2.37. The study by (Chen *et al.*, 2023a) describes direct derivation of human expanded potential stem cells from the human pre-implantation embryo. Embryo-derived human expanded potential stem

cells were found to show a unique open chromatin conformation, possessing a higher proportion of H3K4me3 bound broad domain with enriched Hippo signalling than human expanded potential stem cells derived from naive and primed embryonic stem cells. This is associated with an enhanced trophoblast differentiation potency.

2.38. The study by (Hao *et al.*, 2023), establishes a novel a robust EPSC culture medium, OCM175. Using the medium authors successfully converted integration-free iPSCs from human Urine-Derived Cells iPSCs into EPSC, demonstrating that ESPCs had the ability to form both intra- and extra- embryonic chimerism, and could contribute to the trophoblast ectoderm lineage and three germ layer cell lineages.

2.39. Developmental potency and metabolic traits of extended pluripotency were found to be transferred to somatic cells via cell fusion induced reprogramming from EPSCs. In the study by (Song *et al.*, 2022), researchers derived EPSCs from eight-cell mouse embryos and fused them to neural stem cells, with resultant hybrid cells exhibiting pluripotential markers with upregulated EPSC-specific gene expression. The hybrid cells were then found to contribute to extraembryonic and embryonic lineages in vivo and in vitro and show distinct global expression patterns resembling EPSCs without parental expression of NSC markers.

2.40. The study by (Dong *et al.*, 2022), elucidated that Ying Yang 1 (YY1) transcriptional repressor protein binds to specific open chromatin regions in EPSCs. Depletion of YY1 leads to a gene expression pattern similar to that of embryonic stem cells than control EPSC, triggering a series of epigenetic crosstalk activities, including changes in DNA methylation, histone modifications and high-order chromatin structures. This indicates that YY1 functions as a key regulator of multidimensional epigenetic crosstalk associated with extended pluripotency.

2.41. The study by (Chen *et al.*, 2023b), identified the membrane protein podocalyxin-like protein 1 (PODXL) as being essential for extended and primed pluripotency. Alteration of PODXL expression levels affects self-renewal, protein expression of c-MYC and telomerase, and iPSC and EPSC colony formation. The addition of exogenous cholesterol fully restores PODXL knockdown-mediated loss of pluripotency, leading authors to conclude that cholesterol regulation via PODXL signalling is critical for embryonic stem cell and EPSC state.

2.42. The study by (Malik *et al.*, 2022) compared the transcriptome, chromatin accessibility, active histone modification marks, and relative proteomes of embryonic stem cells and two well-established EPSC lines to probe the molecular foundation underlying EPSC developmental potential. By defining sets of molecular signatures that distinguish EPSCs from embryonic stem cells in transcriptional and translational regulation as well as metabolic control, authors demonstrated that EPSCs show similar reliance on pluripotency factors Oct4, Sox2, and Nanog for self-renewal as embryonic stem cells.

2.43. Characterisation of X chromosome status in human EPSC was described by x. By deriving EPSCs from primed human embryonic stem cells with defined (Wang *et al.*, 2023) chromosome status authors showed that EPSCs derived using both methods had highly similar transcription profiles and X chromosome status. However, the X chromosome status of EPSCs was found to be largely determined by the primed ESCs from which they were derived, suggesting a lack of complete reprogramming of X chromosome during primed to EPSC conversion. In addition, X chromosome status of EPSCs affected their ability to differentiate into embryonic or extraembryonic lineage cells.

2.44. The study by (Ruan *et al.*, 2024) presents an optimised culture system for efficient derivation of expanded potential stem cells from preimplantation embryos or by reprogramming somatic cells of multiple mammalian species. New EPSC lines were found to proliferate robustly over long-term passaging and were amenable to both simple indels and precision genome editing with up to 100% targeting efficiency. The EPSCs differentiated into embryonic cell lineages in vitro and teratomas in vivo, and into porcine trophoblast stem cells in human trophoblast stem cell medium.

2.45. The study by (González-Martínez and Malumbres, 2021) describes a novel technique for the expansion of differentiation potency from pluripotent stem cells using the transient expression of a single microRNA molecule. This method requires no genetic modification of pluripotent stem cells and achieves stable improvement of the differentiation potential of these cells through several cell passages both in vitro and in vivo.

2.46. A detailed protocol for the differentiation of human EPSC into human trophoblast stem cells is presented by (Xu *et al.*, 2023). In doing so, researchers demonstrate that human EPSC-dervied TSC lines can be continuously passaged and are functional in further differentiation into syncytiotrophoblasts and extravillous trophoblasts, offering a valuable cell source for studying human trophoblast development.

2.47. The development of totipotent stem cells, which exhibit molecular similarities to two- and four-stage blastomeres, have additionally been established from murine cell lines: The study by (Shen *et al.*, 2021) reported that spliceosomal repression, mediated by splicing inhibitor pladienolide B (PlaB), achieved a stable in vitro culture of mouse totipotent stem cells which were comparable to 2- and 4-cell blastomeres with bidirectional embryonic and extraembryonic differentiation potential. Induction and maintenance of totipotent stem cells was also achieved by (Hu *et al.*, 2023) using a combination of three small molecules: the retinoic acid analogue TTNPB, 1-azakenpaullone and the kinase blocker WS6. The derived cells also exhibited bidirectional developmental potentials and were able to produce both embryonic and extraembryonic cells in vitro.

Eight-cell like cells

2.48. Similarly, cells resembling eight-cell stage human blastomeres, eight-cell like cells (8CLC) have since been derived from human pluripotent stem cells having the ability to generate blastoids comprising trophectoderm, epiblast, and hypoblast-like cells (Taubenschmid-Stowers and Reik, 2023). These cells represent an improved model system to study embryonic genome activation in humans.

2.49. The study by (Taubenschmid-Stowers *et al.*, 2022) reports on the discovery of human 8CLC among human naïve embryonic stem cells, which transcriptionally resemble the 8-cell human embryo expressing markers such as ZSCAN4 and LEUTX, and transposable elements, HERVL and MLT2A1. As such, these cells represent a unique model to characterise human zygotic genome activation-like transcription and reveal critical insights into early embryogenesis in humans. Discovery and characterisation of 8CLC in populations of naïve human iPSC was also reported in the study by (Moya-Jódar *et al.*, 2023).

2.50. The paper by (Mazid *et al.*, 2022) describes a transgene-free, rapid and controllable method for producing 8CLCs from human pluripotent stem cells, identifying fundamental roles of DPPA3 and

TPRX1 in the 8CLC conversion process. In vitro and in vivo production of blastoids and complex teratomas confirm the capacity of 8CLC to produce embryonic and extraembryonic lineages.

2.51. A short-pulsed expression of DUX4 in primed human embryonic stem cells was also shown to induce 8CLC by (Yoshihara *et al.*, 2022), cells demonstrated a marked reduction of POU5F1 protein, as previously observed in mouse two-cell-like cells, and were successfully enriched using an antibody against NaPi2b (SLC34A2), which is expressed in human blastomeres.

2.52. A human specific report to isolate 8CLC from preimplantation epiblast-like stem cell populations has since been developed by (Yu *et al.*, 2022). Using the non-canonical promoter of LEUTX that regulates human zygotic genome activation, researchers were able to isolate 8CLC, further optimising the chemical-based culture condition to increase and maintain 8CLC populations.

2.53. Transcriptomic differences between 8CLC reprogrammed using different methods have since been investigated by (Yoshihara and Kere, 2023). Observations will allow for comparison and validation of models and stimulate further in-depth research to characterise genes involved in human embryonic genome activation and preimplantation development, facilitating further studies on human embryogenesis.

Deriving extraembryonic cell lineages

2.54. In addition to the potential of 8CLC, ongoing efforts are being made to generate human extraembryonic stem cells representing the trophectoderm and hypoblast lineages from primed and naïve human pluripotent stem cells. A number of protocols have been described for the induction of these cells (Dong and Theunissen, 2022; Wei *et al.*, 2022):

2.55. The study by (Viukov *et al.*, 2022) reports that inhibition of the transforming growth factor β (TGFB) pathway and avoidance of WNT stimulation leads to conversion of primed human pluripotent stem cells into trophoblast stem cells. Resulting primed pluripotent stem cell-derived trophoblast stem cells exhibit self-renewal, can differentiate into the main trophoblast lineages, and present RNA and epigenetic profiles that are indistinguishable from trophoblast stem cell lines derived from human placenta, blastocysts, or isogenic human naïve pluripotent stem cells.

2.56. Conversion of human primed pluripotent stem cells to trophoblast stem cell-like cells via short-term treatment with bone morphogenetic protein 4 (BMP4) and trophoblast stem cell culture medium (TSCM) is described by (Jang *et al.*, 2022). Resultant trophoblast cells show morphology and global gene expression profiles comparable to bona fide human trophoblast stem cells alongside long-term self-renewal capacity with bipotency that allows the cells to differentiate into functional extravillous trophoblasts and syncytiotrophoblasts.

2.57. Refining a previous BMP4-based protocol, (Soncin *et al.*, 2022) applied a culture medium developed for maintenance of induced trophoblast cells reprogrammed from cytotrophoblast cells to improve conversion of primed human pluripotent stem cells to self-renewing trophoblast stem cells. Resultant cells resemble placenta and naïve derived trophoblast stem cells, sharing function and transcriptome with primary human trophoblast cells.

2.58. Chemical conversion of human conventional pluripotent stem cells to trophoblast stem cells is described by (Zorzan *et al.*, 2023). Using a modified chemical resettling protocol, authors found that chemical resetting generates a plastic intermediate state characterised by co-expression of naive and TSC markers, following which cells respond to the signalling environment.

2.59. The study by (Wang *et al.*, 2022a) describes the establishment of human trophoblast stem cells from human blastocysts, characterising nuclear enlargement in syncytiotrophoblast differentiated from human trophoblast stem cells. Specifically, authors reveal that CRISPR/Cas9-mediated LMNA disruption perturbated nuclear volume during human trophoblast stem cells syncytialisation.

2.60. The study by (Ohgushi *et al.*, 2022) reports that chemical blockage of ACTIVIN/NODAL and FGF signals is sufficient to steer human primed embryonic stem cells into GATA3-expressing cells that give rise to placental hormone-producing syncytia analogous to syncytiotrophoblasts of the post-implantation stage of the human embryo. These syncytia arise from the non-trophoblastic differentiation trajectory that recapitulates amniogenesis, providing an insight into possible unique extraembryonic differentiation pathways in primate embryogenesis.

2.61. The study by (Zijlmans *et al.*, 2022) identified strong enrichment of polycomb repressive complex 2 (PRC2)-associated H3K27me3 in the chromatin of naïve pluripotent stem cells and H3K27me3 enrichment at promoters of lineage-determining genes, including trophoblast regulators. PRC2 activity acts as a chromatin barrier restricting the differentiation of naive cells towards the trophoblast lineage, whereas inhibition of PRC2 promotes trophoblast fate induction. This indicates that human naïve pluripotent stem cells are not epigenetically unrestricted, but instead possess chromatin mechanisms that oppose the induction of alternative cell fates.

2.62. The study by (Pham *et al.*, 2022) demonstrates that naïve human pluripotent stem cells are competent to differentiate into extraembryonic mesoderm cells. Indicating that extraembryonic mesoderm cells arising via primate-specific specification between implantation and gastrulation can be modelled in vitro.

2.63. Cell fate dynamics in human primed to naïve transition have been reported by (Bi *et al.*, 2022), who used a dual fluorescent reporter system to describe fate dynamics from primed state toward naive pluripotency with ALPG activation followed by the activation of OCT4-distal enhancer. Integration of transcription profiles and chromatin accessibility landscape revealed the appearance of primitive endoderm and trophectoderm signatures in transitioning subpopulations with the capacities for derivation of extra-embryonic endoderm and trophoblast stem cell lines.

2.64. The study by (Ogawa *et al.*, 2022) describe triploblastic differentiation from human amniotic membrane mesenchymal stem cells and multilineage-differentiating stress-enduring (Muse) cells. Similarly human amniotic membrane mesenchymal stem cells differentiated into triploblastic cells from a single cell, self-renewed, and exhibited non-tumorigenicity; However, they exhibited higher expression of genes related to germline- and extraembryonic cell-lineages compared with those in Muse cells and enhanced expression of markers relevant to germline- and extraembryonic cell- lineages, suggesting a broader differentiation potential similar to naïve pluripotent stem cells.

2.65. The study by (Kong *et al.*, 2022) established a triploid human trophoblast stem cell line from tripronuclear embryos, which were clinically discarded but readily available, for potential applications in basic placental research and disease modelling. Karyotyping analysis showed that cell lines contained three sets of chromosomes and exhibited typical features of human trophoblast stem cells, with the ability to differentiate into two trophoblast lineages: extravillous cytotrophoblasts and syncytiotrophoblasts.

2.66. Castel and David (2022) discuss the lack of a cellular models for studying placental development. To address this, authors describe an optimized protocol for reprogramming somatic cells into human induced trophoblast stem cells and converting pluripotent stem cells into human converted trophoblast stem cells. This protocol facilitates genome-specific placental disease modelling. They also outline differentiation protocols for extravillous trophoblast and syncytiotrophoblast from these induced cell lines. The protocol spans 4 months and requires advanced cell culture skills similar to those needed for reprogramming somatic cells into human induced pluripotent stem cells.

2.67. The study by (Tan *et al.*, 2022) establishes a protocol for the generation of human induced trophoblast stem cells via reprogramming of adult fibroblast cells, highlighting a method by which researchers may generate patient-specific cell lines to understand trophoblast biology and study interactions with embryonic cells when modelling disease. The protocol overviews (1) recovery of cryopreserved human dermal fibroblasts, (2) somatic cell reprogramming, (3) passaging of reprogramming intermediates and (4) the derivation of induced trophoblast cell cultures followed by routine maintenance of induced trophoblast cells.

2.68. The study by (Naama *et al.*, 2023) explores whether the human trophoblast stem cell state can be induced independently of pluripotency and the mechanisms underlying its acquisition, identifying GATA3, OCT4, KLF4 and MYC (termed GOKM) as a combination of factors that can efficiently generate functional human induced trophoblast stem cells from fibroblasts.

2.69. The study by (Tietze *et al.*, 2024) researchers outline a novel strategy, referred to as the "TS condition", for the differentiation of primed pluripotent stem cells into trophoblast stem cells (TSC). By selectively activating Epidermal Growth Factor (EGW) and Wingless-related integration site (WNT) pathways, and inhibiting tumour growth factor beta (TGFβ), histone deacetylases (HDAC), and Rho-associated protein kinase (ROCK) signalling, researchers were able to generate stable, proliferative cells resembling first-trimester placental cytotrophoblast – all without the addition of exogenous Bone morphogenetic protein 4 (BMP4)-a. The ability to differentiate into TSC populations was observed across multiple cell lines, including iPSC, demonstrating that primed pluripotent stem cells have the capacity to differentiate directly into TSC without progressing through a naïve state.

2.70. The study by (Inohaya *et al.*, 2024) demonstrates that exposure to shear stress significantly promotes the differentiation of naive human PSC-derived cytotrophoblast stem cells into syncytiotrophoblasts. After 72 hours of 10 dyn/cm² shear stress, PSC-derived cytotrophoblast stem cells exhibited increased fusion, upregulation of syncytiotrophoblast markers, enhanced hormone secretion, and notable changes in cellular morphology and gene expression indicative of differentiation.

2.71. The study by (Morey *et al.*, 2024) converted primed iPSC to naïve iPSC in order to derive trophoblast stem cells and extravillous trophoblast (EVTs) cells to evaluate the molecular mechanisms underlying preeclampsia. It was found that, unlike their naive counterparts, primed iPSC-derived PE-EVTs exhibited reduced surface HLA-G, impaired invasiveness, and altered gene expression, correlating with promoter hypermethylation in the epithelial-mesenchymal transition pathway. This indicates that abnormal epigenetic regulation may contribute to preeclampsia pathogenesis.

2.72. Trophoblast stem cells isolated from naïve human pluripotent stem cell cultures have also been reported to efficiently self-organise into three-dimensional stem-cell derived trophoblast organoids producing different trophoblast subtypes, including cytotrophoblasts, syncytiotrophoblasts, and invasive extravillous trophoblasts (Cui *et al.*, 2022b, 2022a; Deng *et al.*, 2022; Huang *et al.*, 2022; Karvas *et al.*, 2022; Tu *et al.*, 2023). Such generation of trophoblast organoids from pluripotent stem cell lines provide accessible 3D model systems of the developing placenta and its susceptibility to emerging pathogens.

3. Applications of human pluripotent stem cells as therapeutics and for drug and disease modelling

3.1. Exposure of cadmium to human trophoblast stem cell lines was found to inhibit differentiation into extravillous cytotrophoblasts by (Ogushi *et al.*, 2023), suggesting that cadmium inhibits placental formation by supressing differentiation. This suppression may underlie the increased risk of gestational hypertension in women with high whole-blood Cd levels and demonstrates a useful application of trophoblast stem cell models for elucidating disease mechanisms in research.

3.2. The study by (Smela *et al.*, 2023) reports that simultaneous overexpression of two transcription factors NR5A1 (Nuclear Receptor Subfamily 5 Group A Member 1) and Runt-domain transcription factors RUNX1 or RUNX2 can direct the differentiation of human iPSCs to granulosa-like cells. Transcriptomes were found to be similar to human fetal ovarian cells and recapitulate key ovarian phenotypes including follicle formation and steroidogenesis.

3.3. The study by (Wang *et al.*, 2022b) reports that iPSC reprogrammed from $CD62L^+$ naïve and memory T cells followed by CD19-chimeric antigen receptor (CAR) engineering and 3D-organoid system differentiation can produce products with conventional CD8αβ-positive CAR T cell characteristics. Expanded iPSC CD19-CAR T cells showed comparable antigen-specific activation, degranulation, cytotoxicity, and cytokine secretion compared with conventional CD19-CAR T cells and maintained homogeneous expression of the TCR derived from the initial clone. iPSC CD19-CAR T cells also mediated potent antitumor activity in vivo, prolonging survival of mice with $CD19^+$ human tumor xenografts, establishing a feasible methodology to generate highly function al CAR T cells from iPSC to support development of manufacturing strategies.

3.4. A two-week protocol for efficient and scalable production of Pax7-positive myogenic progenitors from human iPSC using 3D suspension-based cultures that mimic the natural embryonic environment is described by (Mashinchian *et al.*, 2022). When transplanted into a mouse model of Duchenne muscular dystrophy the iPSC-derived myogenic progenitors integrated into the muscle stem niche, reactivated after repeated injury, and lead to increased muscle function, demonstrating this approach has promising therapeutic potential for treating the disease in humans.

3.5. The study by (Mavrommatis *et al.*, 2023) provides a robust 3D in vitro developmental system for investigating muscle tissue morphogenesis and homeostasis through the production of human skeletal muscle organoids from iPSC lines. Challenges and considerations for clinical translation of iPSC-based myogenic cell therapies are discussed by (Sun *et al.*, 2024) in their follow up review.

3.6. The study by (Mouka *et al.*, 2022) reports on production of primordial germ-like cells from naïve-iPSC derived from the erythroblasts of two infertile patients with azoospermia and XX male syndrome. Through iPSC derived models, authors indirectly showed that infertile men harbouring complex genetic abnormalities could produce PGC-LCs. This result acts as a useful model to help identify causative factors leading to early germ cells development failure, with the potential to identify novel therapeutic strategies.

3.7. The study by (Menon *et al.*, 2023) presents a strategy to derive pericytes from human induced pluripotent stem cells, which may be utilised as a more versatile and uniform tool to study direct lineage reprogramming into induced neurones. The potential for generating pluripotent stem cell spheroids, which have the capacity to differentiate into cells with electrical activity similar to neurones, from umbilical cord derived cells was also explored by (Maassen *et al.*, 2023). Indicating the applications of iPSC as a tool to study neuronal cells and replacement therapy.

3.8. A number of review articles review the application of iPSC in relation to specific disease models and therapeutic applications, including: amyotrophic lateral sclerosis (ALS), age-related macular degeneration (AMD), neurodegenerative and cardiovascular diseases, brain stroke, cancer, diabetes, and osteoarthritis (Thanaskody *et al.*, 2022; Du *et al.*, 2023; Esteves *et al.*, 2023; Okano *et al.*, 2023; Pohjolainen *et al.*, 2024).

3.9. As a result of iPSC-based drug evaluation/screening, clinical trials testing candidate drugs for ALS have been performed (Ito *et al.*, 2023). These trials have confirmed the reliability and application of stem-cell based models in drug discovery.

3.10. Naïve pluripotent and trophoblastic stem cell lines have also been used as a model for detecting missing proteins in the context of the Chromosome-Centric Human Proteome Project, as described by (Girard *et al.*, 2023). Researchers analysed naive pluripotent and trophoblastic stem cells and discovered four new missing proteins that may play a crucial role in setting up the molecular events underlying early embryonic development.

3.11. Application of iPSC in the context of tissue engineering is reviewed by (Shukla *et al.*, 2022), who discuss developments the use of iPSC in conjunction with 3D bioprinting technology for the construction of functionalised tissue and organ analogues, for example 3D disease models of cardiac and Alzheimer's disease.

3.12. Further applications of pluripotent stem cells include the generation of stem cell-based embryo models (SCBEM) and in vitro derived gametes, as reviewed by (Terhune *et al.*, 2022). These are considered by the SCAAC as separate topics so are not detailed here.

4. Conclusions

4.1. SCAAC last considered research in this area in January 2022. At the time it was concluded that the cells being created were an insufficient replacement for embryonic derived stem cells and that further research on hESC-like cells is needed. The committee were satisfied that UK regulations pertaining to the use of embryonic cells in research were strong, and that the International Society for Stem Cell Research guidelines consider countries which do not permit research on embryos. During the discussions there was an emphasis on not describing the use of iPSC in research as raising fewer ethical concerns than the use of embryonic stem cells.

4.2. Novel methods of generating human trophoblast stem cells from pluripotent and somatic cells highlights new applications of hESC and hESC-like cells in the study of human placental development and the etiology of pregnancy-related diseases.

4.3. While the hESC-like cells can be reprogrammed to exhibit properties similar to human embryonic stem cells, they are not yet able to replace embryonic stem cells in their entirety. Ongoing research and technological advancements are needed to overcome challenges with epigenetic memory and genetic stability, differentiation control and functional equivalence, reprogramming efficiency, and scalable production.

5. Recommendations

5.1. Members are asked to:

- consider the progress of research into alternative methods to derive embryonic or embryonic-like stem cells;
- advise the Executive if they are aware of any other recent developments; and
- review whether any outputs from the HFEA are required.

5.2. A summary of developments presented by this paper and the recommendations given by SCAAC in response will be used to update the paper 'Alternative methods to derive stem cells' used by the HFEA's Licence Committee when considering research licence applications involving the use of viable embryos for research purposes.

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