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Models for measuring metabolic chemical changes in the metastasis of high grade serous ovarian cancer: fallopian tube, ovary, and omentum

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Ovarian cancer (OC) is the most lethal gynecologic malignancy and high grade serous ovarian cancer (HGSOC) is the most common and deadly subtype, accounting for 70–80% of OC deaths. HGSOC has a distinct pattern of metastasis as many believe it originates in the fallopian tube and then it metastasizes first to the ovary, and later to the adipose-rich omentum. Metabolomics has been heavily utilized to investigate metabolite changes in HGSOC tumors and metastasis. Generally, metabolomics studies have traditionally been applied to biospecimens from patients or animal models; a number of recent studies have combined metabolomics with innovative cell-culture techniques to model the HGSOC metastatic microenvironment for the investigation of cell-to-cell communication. The purpose of this review is to serve as a tool for researchers aiming to model the metastasis of HGSOC for metabolomics analyses. It will provide a comprehensive overview of current knowledge on the origin and pattern of metastasis of HGSOC and discuss the advantages and limitations of different model systems to help investigators choose the best model for their research goals, with a special emphasis on compatibility with different metabolomics modalities. It will also examine what is presently known about the role of small molecules in the origin and metastasis of HGSOC.

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Introduction

Ovarian cancer (OC) is the deadliest form of gynecological malignancy representing the sixth leading cause of cancer-related deaths among women worldwide.[1] The Global Cancer Observatory estimates that in 2020 about 313 959 women will

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be newly diagnosed with OC, and 207 252 will die from the disease.[2] High-grade serous ovarian cancer (HGSOC) is the most common and lethal OC subtype, responsible for 70–80% of these deaths.[3,4] HGSOC can be characterized by a far more aggressive pattern of disease behavior than other OC subtypes and is known to have a very distinct pattern of metastasis; originating in the fallopian tube with the transformation of fallopian tube epithelial (FTE) cells which metastasize first to the ovary, then to the omentum.[5–7] Due to nonspecific symptoms and a lack of early detection strategies, the majority of women with HGSOC are diagnosed at a late stage when the five-year survival rate can be as low as 17%.[8] While outcomes have improved in recent years, this five-year survival rate remains dismally low, highlighting the urgent need to investigate the molecular events underlying HGSOC pathogenesis to facilitate the identification of novel diagnostic biomarkers and therapeutic targets for treatment and prevention.[9,10]

Metabolomics is a rapidly evolving field focused on measuring the complete set of metabolites in biological samples. Metabolomics is a promising tool for human health research because it offers a unique perspective where changes in the expression of an enzyme do not necessarily lead to proportional alterations in metabolism. Genomic based techniques identify what may be happening in a biological system whereas metabolomics represents the measure of the final protein-modified products in the system.[11] As such, metabolomics is an extremely useful tool for exploring the molecular changes underlying many disease states including HGSOC. To date, mass spectrometry (MS) and nuclear magnetic resonance (NMR)-based metabolomics techniques have been utilized to characterize the overall metabolic changes in HGSOC tissues and fluids and several studies have reproducibly noted changes in glycolysis, fatty acid oxidation, and oxidative stress response as well as increases in specific metabolites including tocopherols and glutathione.[12–18] These known metabolomic changes are important to note because they enhance our understanding of the OC metastatic microenvironment, and, when combined with other approaches, can be utilized to ascertain the identities and functional roles of specific molecules within that microenvironment. For example, in a 2018 study, Nazari *et al.* used quantitative mass spectrometry imaging to analyze the metabolite content in healthy and cancerous hen ovarian tissue sections and found a ~2-fold increase in glutathione, which has previously been implicated in resistance to platinum-based chemotherapies.[13,14,19] In time, this finding could lead to the development of new treatment strategies which combine the use of platinum-based drugs with small-molecules that target glutathione synthesis to prevent the development of chemoresistance in HGSOC and improve clinical outcomes. The use of untargeted metabolomics techniques to search for HGSOC biomarkers has also been very popular, as the main reason for the poor prognosis related to the disease is late diagnosis. Currently, there are no routine screening methods in women's health exams for the early detection of HGSOC. The FDA-approved diagnostic test, which relies on cancer antigen (CA)125 combined with pelvic ultrasound, has high false-positive/negative rates and clinical applications are limited to the differential diagnosis of OC tumors and malignancy risk-assessment.[20–22] A systematic review of the literature in 2016 observed that serum and plasma were the most common type of biospecimen utilized for OC biomarker discovery and noted that metabolites related to cellular respiration, carbohydrate, lipid, protein and nucleotide metabolism were often found to be significantly altered.[23] A recent study by Huang *et al.* performed a comprehensive survey of serum metabolic alterations across the entire spectrum of the disease by analyzing the serum metabolome of a triple-knockout mouse model (which spontaneously develops HGSOC) at premalignant, early, and advanced stages. This longitudinal murine model revealed a panel of 29 metabolites which distinguished mice with early-stage HGSOC from mice with advanced-stage and controls with >90% accuracy.[12] These findings have yet to be translated to clinical samples, but it supports the notion that further investigation of the serum metabolome across the spectrum of HGSOC models could lead to the development of new diagnostic techniques which could ultimately lead to early diagnosis and improved outcomes.

While the majority of metabolomics studies on HGSOC have focused on characterizing overall changes in metabolism and searching for potential biomarkers from biospecimens or 2D cell culture, a few studies have challenged this paradigm by combining mass spectrometry-based metabolomics with cutting edge cell-culture techniques to model the HGSOC metastatic microenvironment for the investigation of cell-to-cell communication. In one such study aimed at modeling primary metastasis, Zink *et al.* developed an imaging mass spectrometry (IMS) protocol for analyzing co-cultures of healthy tissues (organoids) and a 3D-mammalian cell culture in an agarose matrix. This IMS co-culture model was used to probe the small-molecule exchange between tumorigenic FTE cells co-cultured with healthy murine ovaries. They found tumorigenic FTE cells, but not FTE or murine surface epithelial cells, repeatedly induced a signal from the ovary at m/z 170, which was identified as norepinephrine and confirmed to stimulate the invasion of tumorigenic FTE cells.[24] In another study aimed at modeling secondary metastasis, Mukherjee *et al.* profiled the metabolome

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and proteome of cancer cells co-cultured with primary human omental adipocytes. They found significant alterations of the lipidome with the corresponding upregulation of proteins involved in lipid metabolism. Through this study a lipid chaperone protein, FABP4, was identified as a key regulator of lipid responses and a potential therapeutic target.[25]

The purpose of this review is to serve as a tool for researchers aiming to model the metastasis of HGSOC for metabolomics analyses. It will provide a comprehensive overview of current knowledge on the origin and pattern of metastasis of HGSOC and discuss the advantages and limitations of different HGSOC model systems to help investigators choose the best model for their research goals, with compatibility with different metabolomics modalities as a special emphasis. It will also examine what is presently known about the roles of small-molecules in the origin and metastasis of HGSOC.

HGSOC origin and pattern of metastasis

While the term "ovarian cancer" implies a unitary disease, there are many histologically distinct subtypes of OC which are broadly classified as either epithelial or non-epithelial based on the cell of origin. Epithelial OCs, which account for 90% of all cases, are subdivided into two types: low-grade (type I) and high-grade (type 2) differing in both origin and disease behavior. Low-grade carcinomas, are typically slow growing and less aggressive.[3,26,27] High-grade serous carcinomas (HGSCs) on the other hand are highly aggressive, characterized by mutations in p53 and genomic instability due to defects in DNA repair pathways.[27] HGSCs were initially thought to originate from the ovarian surface epithelium, but a growing wealth of evidence has indicated that the majority originate in the fallopian tube with the accumulation of deleterious mutations in fallopian tube epithelial (FTE) cells, leading to the development of a p53-signature.[27–35] FTE cells may form a premalignant lesion called a serous tubal intraepithelial carcinoma (STIC), and metastasize to the ovary (Fig. 1A).[31,36–38] Multiple studies suggest a critical role for the ovary in promoting ovarian cancer development and metastasis.[39–41] For example, Perets *et al.* revealed that in a murine ovarian cancer model arising in the fallopian tube, removal of the ovary significantly restricts metastasis.[42] Further, in a vascular model of ovarian cancer metastasis, oophorectomy resulted in a complete loss of metastasis and in another study tumorigenic murine oviductal epithelial cells allografted on ovarian bursa resulted in aggressive tumors, while intraperitoneal xenografting the same number of cells did not.[43,44]

HGSOC has a distinct pattern of disease progression.[5–7] While the molecular mechanisms underlying this predilection are unclear, the metastatic behavior of HGSOC suggests the intraperitoneal microenvironment centered on the omentum is a privileged metastatic location (Fig. 1A–D).[45–47] Several studies provide evidence for an "activated" phenotype of the peritoneal microenvironment associated with OC, suggesting chemical messengers released from the tumor prone omental tissues for

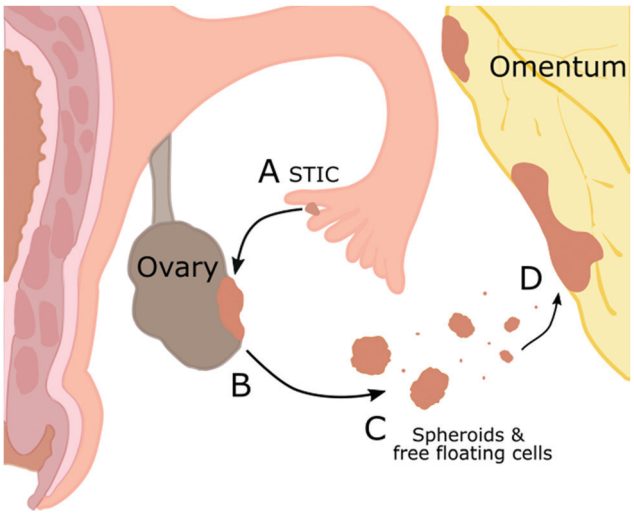


Fig. 1 HGSOC has a distinct pattern of disease progression; (A) it originates with the transformation of FTE cells which may form a premalignant lesion called a STIC before metastasizing to the ovary. (B) After colonizing the ovary, it spreads by direct extension to the peritoneal cavity through the detachment of cells from the primary tumor. (C) These cells often survive by forming multicellular spheroids which float in the ascitic fluid and metastasize to organs in direct contact with the peritoneal cavity. (D) Although many organs may be involved in secondary dissemination, the main site of secondary metastasis is the omentum, a large apron-like expanse of visceral adipose tissue that covers the spleen, stomach, pancreas, and colon (Fig. 1D).[5–7]

metastasis. In support of this theory, the omentum harbors a variety of stromal cell types, including adipocytes, mesenchymal stem cells, fibroblasts, and macrophages, which can be dynamically converted to "cancer-associated" cells known to play crucial roles in metastasis. For example, cancer-associated adipocytes have been shown to transfer lipids to OC cells, providing them with energy for rapid metastatic growth.[47–49] While the most common mechanism of HGSOC metastasis is thought to be peritoneal dissemination, hematogenous spread does occur and studies have shown that in vascular models of ovarian cancer metastasis preferential spread to both the ovary and the omentum is observed. Pradeep *et al.* used a parabiosis model (two mice, one with ovarian cancer and one cancer free who share a blood supply) to investigate the hematogenous spread of HGSOC and observed preferential spread to the omentum.[50] In another study Coffman *et al.* developed three *in vivo* models of ovarian cancer resulting in metastatic disease *via* hematogenous spread. Strikingly, all three models demonstrated the development of intra-ovarian metastatic disease and ascites, supporting a tropism for the ovary and a role for the vascular spread of HGSOC.[43] Given the multi-organ involvement in disease progression, it is important to take these considerations into account when selecting a model from which to design a metabolomics experiment.

Modeling HGSOC for metabolomic analysis

Metabolism is crucial for fully understanding important biological phenomena, including HGSOC. Metabolomics, or the analysis of

the complete set of small-molecule metabolites (50–3000 Da) in biological samples, is an important tool for uncovering metabolite changes. Comprehensive metabolomics investigations can be an analytical challenge as they require special considerations for sample preparation and separation/purification for mass spectrometry (MS) or nuclear magnetic resonance (NMR) analyses. When designing a metabolomics experiment to investigate HGSOC it is important to consider: (1) the biological question, (2) the stage of disease being investigated, and (3) the instrumentation available (Table 1). Special care must be taken to ensure that the OC microenvironment is replicated as closely as possible and that the sample is compatible with the instrument being used; this includes the separation modality, ionization source (for MS), and analyzer. Though there have been several NMR-based metabolomics studies on HGSOC,[51–55] many of the models discussed in this review are going to be inherently incompatible with NMR due to sample constraints. Thus, below is a summary of the advantages and disadvantages of different cell culture techniques and biospecimens that have been used to model HGSOC, as well as a perspective on modeling for different MS-based metabolomics modalities.

2-Dimensional (2D) cell culture

2D mammalian cell culture has been in use since the early 1990's as a model for human HGSOC. The first method for the non-polarized 2D cell culture of human FTE cells was developed in 1990 by Heinrikson *et al.*[56] This method was expanded on in 1994 by Kervancioglu *et al.* who developed a technique for the polarized cell culture of human FTE cells by incorporating a commercially available extracellular matrix (ECM) on a permeable filter (Fig. 2A). Polarized cell culture has distinct advantages over non-polarized as it essentially doubles the lifespan of FTE cells and allows for the establishment of subcultures; polarized cells also more closely mimic the *in vivo* morphology of human FTE cells, making them preferable for functional studies.[57] That being said, even polarized 2D cultures suffer from rapid dedifferentiation and loss of polarization over time, issues that are less prevalent with 3D methods.

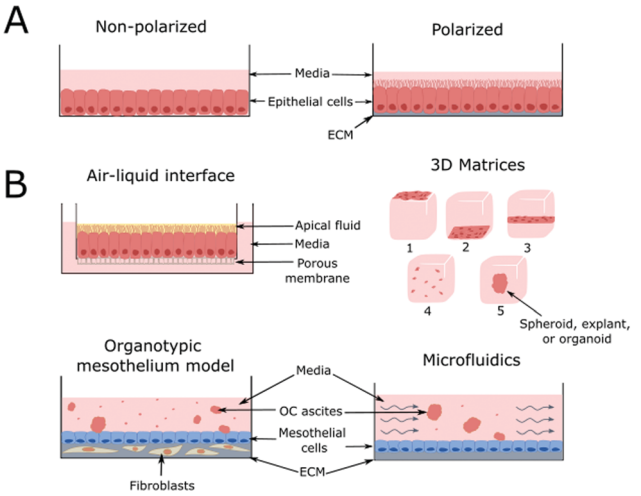


Fig. 2 Schematic of 3D cell culture models. (A) Non-polarized *vs.* polarized 2D cell culture. (B) Air–liquid interface cell culture (top left). Cell culture in 3D matrices (top right) with (1) cells seeded on top (2) cells seeded on bottom (3) cells seeded in a layer in the middle (4) cells seeded throughout and (5) cells seeded in the middle or spheroid/organoid/explant cultured within. Organotypic mesothelium model[74] (bottom left), and microfluidics (bottom right).

Despite these limitations 2D cell culture has been heavily employed for genetic and biochemical studies on HGSOC, though it has been comparatively less popular for metabolomics studies.

A few groups have successfully employed 2D cell culture to identify important metabolite changes in HGSOC. In one study, Halama *et al.* characterized the metabolome of two ovarian cancer cell lines (OVCAR3 and SKOV3) using untargeted MS-based metabolomics.[58] The resulting metabolite profile showed increases in the TCA cycle, lipid metabolism, and β-oxidation, a finding that has been supported by subsequent studies.[15–17,58] In a later study the same group used 2D co-culture to unearth metabolite changes in OC cells in response to direct contact with fibroblasts. They found that

Table 1 Advantages and limitations of the different cell culture methods as they apply to different metabolomics workflows

Cell-culture ?thyc?> model	Advantages	Disadvantages	Ref.
2D culture	– Individual cell types or pairwise interactions can be assessed	– Original histology and paracrine influences are lost	56 and 57
	– Inexpensive	– Cells can lose polarization normally present in intact tissues	
	– Well established	– Premature senescence or de-differentiation may occur	
	– Easy observation/measurement	– Not compatible with IMS	
	– Lots of comparative literature	– Not as physiologically relevant as polarized 2D cell culture	
3D culture	– Organotypic models are possible	– Not as well established as 2D culture techniques	60–78
	– Dynamic range of complexity	– Not all models are compatible with IMS	
	– Easily adaptable to the research question	– Less comparative literature due to dynamic range of complexity	
	– Can be compatible with IMS		
Biospecimens	– The most physiologically relevant	– Human donor-to-donor variability	79 and 80
	– Original histology and paracrine influences are maintained	– Human samples can be hard to come by	
	– Tissue samples are compatible with IMS		
	– Well established		
	– Lots of comparative literature		

fibroblasts induced significant changes in fatty acids, glycerophospholipids, and carbohydrates in OC cells over time.[59] In another such study, Dahl *et al.* compared the metabolite profiles of normal and tumorigenic FTE cells grown in 2D culture and found that cancer cells preferentially utilize the TCA cycle. This ultimately led the researchers to investigate TCA cycle enzymes and to identify isocitrate dehydrogenase 1 (IDH1) as a potential therapeutic target.[18] These studies demonstrate that, while 2D cell culture has many limitations compared to other methods, it can be very useful. In cases where the research interest is simply the difference in metabolism between two individual cell types, or a pairwise interaction, 2D cell culture is a simple, inexpensive, and well-established technique that can be used to garner meaningful results.

3-Dimensional (3D) cell culture

Ovarian tumors are not purely composed of tumorigenic epithelial cells, but rather a heterogenous mixture of epithelial, stromal, immune and endothelial cells.[5] The tumor microenvironment (TME) is grossly affected by the interplay between these different cell types which have an influence on tumor histology, growth potential, invasiveness, and the development of chemoresistance. The TME is made up of (1) a primary tumor with associated stromal and inflammatory cells, (2) non-adherent cells and spheroids suspended in ascites in the peritoneal cavity, and (3) intra-peritoneal metastasis involving adherence to mesothelial cells, adipocytes, and fibroblasts at the metastatic site.[5] Different 3D cell culture models have been developed to simulate each domain of the TME. These 3D models are crucial for studying HGOC metastasis in detail as they have a reduced complexity when compared to *in vivo* systems, allowing specific interactions to be investigated without the influence of confounding variables present in biospecimens and *in vivo* models. Below is an overview of the different 3D cell culture models used to study HGSOC.

(1) Air–liquid interface (ALI) cell culture. Air–liquid interface cell culture was originally developed to culture respiratory tract and epidermal epithelia *in vitro* but has also been shown to support the differentiation of epithelial cells that are not exposed to ambient air *in vivo*, including female reproductive tract epithelia.[60] Epithelial cells are initially seeded in compartmentalized culture systems with porous filter supports or gel substrata and are grown, submerged in media, for an initial propagation period. After the initial propagation period media is removed from the apical side, exposing the top of cells to ambient air while media/nutrients are supplied from the bottom (Fig. 2B).[60] Levonon *et al.* were the first to report the use of this cell culture technique to study cancer in primary human FTE cells; they noted several advantages over previously reported polarized 2D cell culture methods, including *in vivo* like apical secretions and the maintained polarization/differentiation of FTE cells which resulted in the first true co-culture of primary ciliated and secretory FTE cells.[61]

(2) Cells cultured in 3D matrices. Initial strategies for the culture of OC cells in 3D matrices involved mixing OC cells with different forms of ECM consisting of purified proteins, such as collagen, or a more complex mixture such as Matrigel® (Fig. 2B).[62] OC cells cultured in 3D matrices have been used to model various events in the progression of HGSOC, but have proven particularly useful for the study of adhesion and invasion. To model adhesion OC cells are cultured on top of 3D gels, and to model cancer cell survival and proliferation within a mechanically constrained environment (*i.e.* invasion) cells can be seeded inside of or throughout 3D gels (Fig. 2B).[63,64] Additionally a 3D matrix can be incorporated to make cell cultures compatible with imaging mass spectrometry (IMS), a metabolomics modality that will be discussed later in this review.[24,65]

(3) Explants. The 3D culture of *ex vivo* human or mouse organ explants can be useful for uncovering important interactions between ovarian cancer cells and intact tissues (Fig. 2B). In a 2018 study, Zink *et al.* used this technique to co-culture murine ovarian explants with tumorigenic FTE cells as a model for studying the primary metastasis of HGSOC and found that tumorigenic cells, but not normal cells, induced a signal at m/z 170 which was identified as norepinephrine and confirmed to stimulate the invasion of OC cells.[24,65] Additionally, Kahn *et al.* used explant cell culture to model secondary metastasis by co-culturing OC cells with healthy omental tissue and found that OC cells preferentially adhere to clusters of immune cells on the omentum called milky spots.[66] While organ explants are useful for assessing interactions between cancer cells and healthy tissues, they are limited by a lack of vasculature and extracellular components normally present *in vivo* and can only provide reproducible conditions for a short period of time. This gives them distinct disadvantages compared to biospecimens from patients or animal models that were not cultured *ex vivo*.

(4) Spheroids/organoids/organotypic models. Spheroids, organoids, and organotypic models are all multicellular models which can incorporate multiple cell types to mimic a tumor or tissue within the TME. The terms are used somewhat interchangeably. In general, OC spheroids are multicellular aggregates comprised of OC cells which range from 30–200 μm in diameter.[67] Spheroids can be cultured *in vitro* on non-adherent plates, in spinner flasks, in 3D matrices, or using the hanging-drop culture method (Fig. 2B).[68–70] They can also be cultured in microfluidics chambers which will be discussed later. In a 2013 study, Lawrenston *et al.* established and characterized the first 3D spheroid culture model of primary FTE cells and found that 3D spheroid culturing drastically altered the molecular characteristics of FTE cells when compared to 2D cultures of the same cells.[71] These molecular changes are important to note as the formation of spheroids in the peritoneal cavity is an important prerequisite to the adhesion of OC cells to healthy tissues at the metastatic site.[70] Organoids are also multicellular aggregates, but do not necessarily utilize cancer cells. In 2015, Kessler *et al.* reported the growth of fallopian tube organoids from human FTE stem cells using a re-constructed milieu consisting of growth factors and Matrigel. Through this study, the Notch and Wnt pathways were identified as key regulators of stemness and differentiation in human FTE organoids.[72] From the modeling perspective, organoids and spheroids can be advantageous as in the sense that they can easily be incorporated into organotypic systems to study interactions with

other components of the TME. Reported HGSOC organotypic models incorporate multiple cell types, cultured in layers, to simulate the complex interactions seen within a tissue in the OC TME. In 1985, Niedbala *et al.* developed the first organotypic model of the OC TME by growing human primary mesothelial cells in a monolayer on ECM and seeding ovarian cancer cells derived from patient ascites on top.[73] This was expanded on in 2007 by Kenny *et al.* who incorporated a second stromal cell type, fibroblasts, to more closely mimic the TME.[74] In this model, termed the organotypic mesothelium model, primary human omental fibroblasts were embedded in ECM and overlaid with human primary mesothelial cells in a 1 : 5 ratio before the seeding of OC cells (Fig. 2B). This study revealed that both stromal cell types play key roles in OC cell adhesion and invasion, and the model developed for it has since been used in numerous studies which have further illuminated the mechanisms of HGSOC metastasis.[74,75]

(5) Microfluidics. The peritoneal dissemination of OC spheroids and free-floating cells is not a static process, it is governed by hydrodynamic forces generated by the increased production of fluid in the peritoneal cavity (ascites). To model this aspect of the peritoneal microenvironment, Li *et al.* developed a 3D-microfluidic platform which mimics the hydrodynamic forces OC cells experience in the peritoneal cavity. For this model, mesothelial cells are plated on ECM in micrometer sized chambers, then OC spheroids are added and co-cultured under continuous flow conditions to simulate the flow of peritoneal fluid induced by OC (Fig. 2B).[76] This model more closely simulates the *in vivo* peritoneal TME than other cell culture models discussed in this review. Additionally, it allows for acute control over the microenvironment within the chamber through the continuous supply of nutrients and growth factors. In 2018, Carroll *et al.* added another layer of complexity to this model by incorporating alternatively activated macrophages (AAMs) to investigate their impact on OC metastasis. They found that AAM-secreted macrophage inflammatory protein-1 increased the expression of P-selectin in mesothelial cells and this enhanced the adhesion of OC cells.[77] In another study, Xiao *et al.* developed a microfluidic culture system that supports the production of the hormone profile of the human 28 days menstrual cycle by murine ovarian follicles, termed EVATAR. In this system endocrine loops between multiple organs can be simulated in multiple unit platforms where interconnected chambers house organs (ovary, fallopian tube, uterus, cervix, *etc.*) and a circulating flow between tissues is maintained.[78] This model could be useful for studying the origin of HGSOC, as ovulation is a known risk factor.[36,37]

While 3D models undoubtedly more closely mimic the complexities of the TME than 2D, they also have some key disadvantages. In general, 3D models can be more costly and difficult to employ. Also, they are not as well established as 2D and there is less comparative literature due to the dynamic range of complexity seen with 3D models. Despite these disadvantages, 3D cell culture models offer an innovative platform for investigating metabolite changes across the progression of HGSOC without the influence of confounding factors (diet, genetics, *etc.*) that may be present in biospecimens from animal models or patients.

Biospecimens

Biospecimens, such as tissue, blood, serum, urine, *etc.*, collected from animal models or patients are by far the most popular and arguably the most clinically relevant models for metabolomics studies on HGSOC; as they provide the most accurate representation of metabolic changes in response to the *in vivo* TME. Xenograft mouse models, where OC cells are injected into mice and tumor growth is monitored, have been used since the early 1980's and can recapitulate some aspects of OC progression and metastasis, although they cannot be used to study the transforming events leading to tumorigenesis.[79] Several genetically engineered mouse models and murine cell lines have been developed with key mutations to model HGSOC from the fallopian tube. It is important to note these genetic changes, as they are relevant to the stage of disease being modeled; certain mutations arise early on whereas others are only seen in metastatic disease. Genetic mouse models that result in spontaneous disease, or longitudinal models, such as the model used by Huang *et al.* discussed above, are advantageous for some studies as they enable the analysis of metabolic changes across the entire disease spectrum, including the early events of tumorigenesis; this is particularly strategic for the investigation of diagnostic biomarkers for early detection.[12]

One key disadvantage of using mice is they require genetic manipulation to develop OC. Laying hens, on the other hand, spontaneously develop OC with many features in common with human disease, including OC heterogeneity with at least four distinct histological subtypes. The clinical presentation of OC in hens is similar to that in women; they develop substantial volumes of ascites fluid and show extensive peritoneal metastasis.[80] Despite these advantages relatively few studies have used hens to investigate the OC metabolome. In one such study Nazari *et al.* analyzed the metabolome of healthy and cancerous hen ovarian tissue sections using polarity switching IMS and found significant metabolic alterations, including a $\sim$2 fold increase in glutathione in cancerous hen ovarian tissue compared to healthy tissue;[13,14] glutathione has previously been implicated in OC resistance to platinum-based chemotherapies.[19,81] Ultimately the information gained using animal models must be translatable to humans to yield clinically useful drug targets and/or diagnostic biomarkers. As such, biospecimens taken from patients in clinics are indispensable; unfortunately, they can be difficult to obtain and expensive to store/ship making it advantageous, in many cases, to use animals. Clinical samples also suffer from a lack of control over variables, including diet and genetics, that can be controlled for (to some extent) with laboratory animals.

Modeling for different MS-based metabolomics modalities

When selecting a HGSOC model for metabolomics analyses the most important consideration is whether or not there is a spatial component to the biological question at hand. We define spatial components to mean whether local microenvironments are

required to be intact in order for chemical processes to occur through space in these microenvironments. If there is a spatial component, then the sample must be adapted for compatibility with imaging mass spectrometry (IMS), a metabolomics technique that allows for the acquisition of both spatial and spectral information from a single biological sample. If there is no spatial component, liquid chromatography-MS(/MS) or NMR analysis have been a mainstay. One of the most critical steps for the success of a metabolomics experiment is sample preparation. As many of the models discussed above are incompatible with NMR, our analysis will focus on MS-based techniques. A comprehensive review of NMR-based metabolomics is covered in Emwas *et al.* Below is a summary of sample considerations for imaging and non-imaging MS-based metabolomics modalities.

(1) Imaging mass spectrometry (IMS). In IMS, ions are detected across a solid biological sample yielding a complete mass spectrum for each x, y coordinate analyzed.[82] These sampling positions become ''pixels'' that are compiled to create images representing the spatial distributions of ions across the sample. Some forms of IMS rely on ablation of the sample and can create voxels as in the example of the hen tissue which was analyzed with infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI), *vide supra*. The relative intensity of an ion can also be visualized as a heat map, allowing for relative quantitation of an ion within a single analysis. Several ionization techniques are compatible with IMS and each has its own sample requirements; one major determinant of those requirements is whether or not ionization occurs under vacuum pressure. Ambient ionization methods have a number of advantages, as keeping the samples under atmospheric pressure allows for direct analysis of samples without desiccation or cryopreservation to remove moisture. Despite these advantages, matrix-assisted laser desorption/ionization (MALDI) based analyses are the most common application for IMS; MALDI occurs under vacuum and requires the sample be flat and completely dry. It also requires the application of a matrix, which aids in ionization and can be optimized for specific classes of compounds. Other IMS compatible ionization methods, including desorption electrospray ionization (DESI) and liquid extraction surface analysis (LESA), use a solvent mixture to aid in ionization so they do not require application of a matrix, this can be advantageous if the sample is desired for subsequent analysis. These various ionization sources and their limitations and advantages are comprehensively covered in Spraker *et al.*[82]

IMS has predominantly been applied to tissue samples from HGSOC patients or animal models as this sample preparation has become standardized over time.[14,83] This has been very useful for assessing overall metabolite changes in OC tissues, as well as drug penetration in spheroids.[84] This type of analysis allows researchers to generate molecular maps of small molecules, small proteins, tryptic peptides, lipids, and glycans across a tissue sample such as a tumor, spheroid, or organoid. While this approach can yield useful information about biomarkers or downstream pathways for therapeutic intervention, it is not capable of capturing earlier signaling events. Towards this end, some 3D cellular based models, such as organoids or 3D cultures are advantageous as they are better able to capture cross talk prior to tumor formation. Our labs have recently adapted 3D cell culture in agarose coupled with a healthy murine explant to uncover early signalling.[24,65] For this model, an agarose matrix was mixed 1 : 1 with media for 3D cell culture. Excreted metabolites could diffuse through this media mixture, enabling crosstalk between OC cells and healthy tissues to be captured and visualized using IMS. In another such study, Bilandzic *et al.* used a novel *in vitro* invasion assay coupled with MALDI-IMS to take a ''snapshot'' of protein exchange at the spheroid mesothelial interface. For this study, cell–spheroid interface cultures were embedded in agarose and sectioned before proteins were analyzed by IMS. One could envision a similar study adapted for the analysis of small-molecules.

One key advantage of IMS as an approach is that spatial mapping essentially allows for data reduction as a layer of dimensionality. Restricting analyses to areas of interest such as a cell-tissue or subcellular localization can greatly reduce the number of signals of interest, which expedites the verification of highly important signals. In contrast, extraction-based methods allow for the analysis of all ionizable molecules in the sample, which may allow for the identification of molecular changes that could not be observed using IMS. A noted weakness of MALDI based imaging is that the analytes that can be ionized heavily depend on the matrix used and the sample requirements of being a flat surface for DESI or MALDI based imaging. However, the development of different matrices that can select for different biomolecules or specific functional groups has been an active area of innovation and is expanding the utility of imaging for different models. Moreover, there are several IMS compatible ionization techniques to choose from, several of which do not require desiccation/cryopreservation or matrix application prior to analysis.

(2) Non-imaging metabolomics modalities. Non-imaging MS and NMR are both compatible with liquid samples as well as extracted tissue samples. Liquid based extractions have been a mainstay for metabolomics, especially as it pertains to HGSOC as reviewed by Turkoglu *et al.*[23] For liquid based extractions the solvent system can be optimized for specific classes of metabolites, or for a broad range of metabolites depending on the research question at hand. A detailed review on liquid chromatography mass spectrometry based workflows was recently published by Grim *et al.*[85] While targeted metabolomics offers excellent sensitivity, it relies on prior knowledge of the analyte and the availability of isotopically labelled standards, which are drawbacks for those looking to discover novel biomarkers. Untargeted metabolomics, on the other hand, is advantageous for discovery purposes but the results heavily depend on the extraction solvents, liquid chromatography system (reverse phase, normal phase, *etc.*), and the data analysis technique used. To make sense of the wealth of data provided in an untargeted experiment, quality control-based curation steps are necessary to ensure that statistical analyses are performed on analytically robust and potentially identifiable features. Untargeted metabolomics yields a large number of signals that may be important *via* fold

change or significance to a control, but heavily relies on databases to annotate these signals and these databases are constantly expanding. Despite these disadvantages, untargeted MS-based metabolomics remains the preferred technique for biomarker discovery from HGSOC biospecimens.

Clinical based biomarkers for OC have historically failed because they were not specific or sensitive enough; similar to issues seen in serum, blood, and tissue-based metabolomics for HGSOC. For example, the most useful individual biomarker for OC diagnosis to date is CA-125, yet numerous efforts at utilizing it for OC screening purposes have not been fruitful. One popular way to deal with this is to use a multiplexed approach wherein additional biomarkers are sought to complement CA-125. This ''multiplexed approach'' has also been applied to studies aimed at identifying metabolite-based biomarkers from HGSOC biospecimens. In one such study, Gaul *et al.* analyzed serum samples from early-stage OC patients and age matched control women using ultra high-performance LC-MS/MS combined with a customized support vector machine (SVM)-based learning algorithm to identify biomarkers from the OC serum metabolome. This resulted in the identification of a panel of 16 biomarkers which detected OC with 100% accuracy in the cohort tested.[86] In another study by Jones *et al.*, analysis of serum samples from a double knockout mouse model combined with iterative multivariate classification resulted in the identification of a panel of 18 metabolites that yielded 100% accuracy for distinguishing early stage-OC mice from controls.[87] Additionally, several studies by the Li research group have investigated serum, plasma, and urine for HGSOC biomarkers and each of these studies yielded several biomarkers that reliably distinguish OC biospecimens from healthy controls.[88–91] Despite these successes, potential biomarkers or druggable pathways have yet to be translated from many of these studies. This may be due, in part, to the challenges in removing the spatial context from analysis making it difficult to determine which signals to focus on. Technological advancements in terms of both instrumentation and data analysis could alleviate these challenges in the future, allowing for the development of metabolomics-based diagnostic tests that are robust and translatable for use in clinical settings.

Metabolites implicated in the origin and metastatic progression of HGSOC

Small molecules have been documented to be involved in disease progression of HGSOC. For example, steroid hormones in follicular have been implicated in HGSOC development and metastasis. Additionally, catecholamine signaling (specifically norepinephrine signaling) has been implicated in primary metastasis to the ovary and several other metabolites are known to play crucial roles in the intraperitoneal microenvironment contributing to chemoresistance and metastatic progression. Below is a summary of the functional roles of small molecules in the origin and progression of HGSOC (Table 2).

Steroids: estrogen and progesterone

Lifetime ovulation is positively correlated with OC risk and factors which decrease ovulation are associated with a protective effect.[92,93] During ovulation, ovarian follicles release follicular fluid (FF) which bathes surrounding tissues including the ovarian surface epithelium and proximal fallopian tube fimbria. While the connection between ovulation and OC risk remains poorly understood, two components of FF that have been implicated in the origin and progression of HGSOC are the steroid hormones estrogen and progesterone.

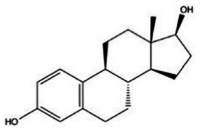
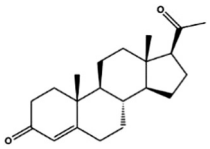
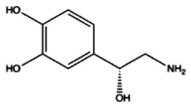
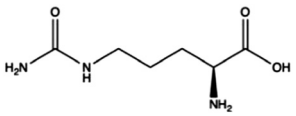
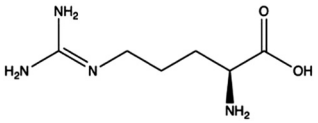
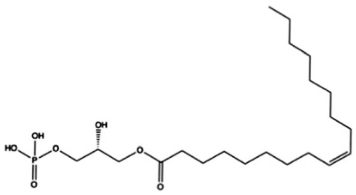
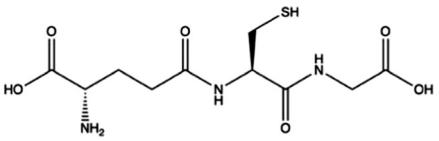
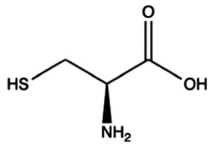
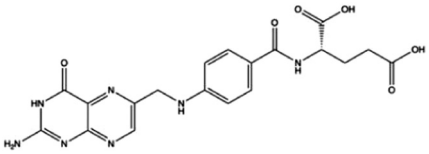
Exposure to estrogens has long been considered a risk factor for the development of OC and estrogen levels in follicular fluid are ~1000-fold that of serum (Fig. 2A).[94–97] While it is generally considered a risk factor, the role of estrogen in the development of HGSOC is somewhat unclear. A study by Moyle-Heyrman *et al.* saw a moderate increase in the expression of proliferation and anti-apoptosis transcripts in murine oviductal epithelial cells with estradiol treatment, although this increase was not consistent between multiple models and estradiol treatment did not induce proliferation or migration.[88] Clinical studies have shown that women taking estrogen only hormone replacement therapy have a higher risk of developing OC compared to women who have never taken hormone replacement therapy or who take estrogen–progesterone combination therapy.[94,95] Taken together, these data indicate that estrogen plays a functional role in the development of HGSOC, though the specifics of that role remain unclear.

Progesterone, another steroid hormone released in FF, is thought to have a protective effect against OC (Fig. 3A). Support for this comes from the observation that women taking oral contraceptives or combined estrogen–progesterone hormone replacement therapy have decreased risk of developing OC.[98–100] As previously mentioned, one of the earliest mutations observed in HGSOC is in *TP53*, and a recent study by Wu *et al.* demonstrated that treatment of p53-null murine oviduct epithelial and p53-deficient human FTE cells with progesterone induced necroptosis (inflammatory cell death). Similar necrotic effects were observed in a p53-null mouse model treated with progesterone and it was found that inhibition of the progesterone receptor led to the accumulation of double stranded breaks.[101] These results suggest activation of the necrosis pathway may underlie the protective effect of progesterone against developing HGSOC.[39]

Small molecules: norepinephrine

The catecholamine norepinephrine is a stress hormone secreted by adrenal glands and stored in many tissues, including the ovary. In a 2018 study, Zink *et al.* probed the small-molecule exchange between tumorigenic FTE cells and healthy murine ovaries using imaging mass spectrometry (IMS). They found tumorigenic cells but not normal cells stimulated norepinephrine secretion by the ovary and enhanced invasion, suggesting norepinephrine may play a role in the primary metastasis of OC (Fig. 3B).[24] Norepinephrine signaling is of interest at a clinical level. In a study of patients with epithelial ovarian cancer, the use of β-adrenergic receptor blockers was found to be associated with a 54%

Table 2 Summary and chemical structures of the small molecules in the origin and progression of HGSOC

Molecule	Formula	Structure	Source	Ref.
Estradiol	$C_{18}H_{24}O_2$		Follicular fluid	94–99
Progesterone	$C_{21}H_{30}O_2$		Follicular fluid	98–101
Norepinephrine	$C_8H_{11}NO_3$		Ovary	24, 102 and 103
L-Citrulline	$C_6H_{13}N_3O_3$		Cancer cells	107
L-Arginine	$C_6H_{14}N_4O_2$		Omentum	107
Lysophosphatidic acid (LPA)	$C_{21}H_{41}O_7P$		Omentum	111–125
Glutathione	$C_{10}H_{17}N_3O_6S$		Fibroblasts	13, 14, 19 and 108–110
Cysteine	$C_6H_{12}N_2O_4S_2$		Fibroblasts	19
Folic acid	$C_{19}H_{19}N_7O_6$		Unknown	126–129

reduction in mortality.[102] This study inspired clinical trials observing the effect of β-blockers on OC patients without hypertension, however, the use of β-blockers as a means of chemoprevention for high risk patients has yet to be investigated.[103] Taken together, this data suggests that norepinephrine signaling is involved in the metastatic progression of HGSOC and β-adrenergic receptors represent viable therapeutic targets for the treatment and prevention of the disease.

Amino acids: arginine and citrulline

The conversion of L-arginine into L-citrulline by nitric oxide synthase (NOS), a protein that has been shown to have high levels of activity in malignant tissue from gynecologic cancers, leads to the production of NO.[104] NO is an intercellular signaling molecule that plays pleiotropic roles in many disease states, including OC, by regulating cellular pH, blood flow, oxygen, and nutrients.[105,106] In a 2015 study, Rizi *et al.* demonstrated that patient-derived omental adipose stromal cells (O-ASCs) regulate NO homeostasis in OC cell lines by secreting arginine (Fig. 3) which is up taken by cancer cells, thereby increasing NO synthesis and promoting OC growth. By modulating NO homeostasis, O-ASCs positively regulate the Warburg effect (switch to anaerobic respiration) in OC cells by increasing glycolysis and reducing

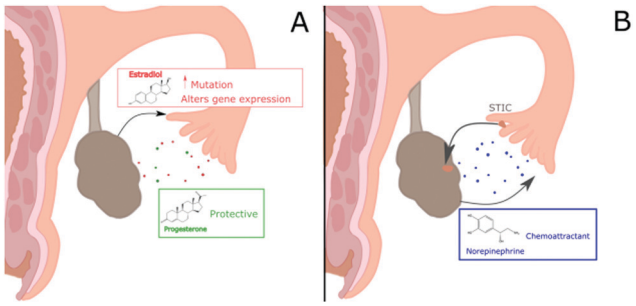


Fig. 3 Small-molecules have been implicated in the origin and primary metastasis of HGSOC. (A) The steroid hormones estradiol and progesterone have been implicated in the origin of HGSOC.[94–101] (B) The catecholamine norepinephrine has been implicated in the primary metastasis of HGSOC.[24,102,103]

mitochondrial ATP generation. In turn, OC cells secrete citrulline (Fig. 4), a byproduct of NOS activity which increases the adipogenesis of O-ASCs. The results of this study also suggest that O-ASCs regulate OC cells response to chemo-drugs through the NO pathway, as inhibition of NO synthesis was found to sensitize cancer cells co-cultured with O-ASCs to paclitaxel.[107] These findings support the notion that metabolic coupling between OC cells and O-ASCs in the tumor microenvironment promotes OC growth and resistance to chemotherapeutics through the modulation of NO synthesis.

Amino acids: glutathione (GSH) and cysteine

GSH is a tripeptide antioxidant composed of glutamic acid, cystine, and glycine that plays a critical role in maintaining cellular homeostasis by scavenging reactive oxygen species (ROS), acting as an intervenient in the metabolism of xenobiotics, and serves as a reservoir of cysteine (a metabolite of the amino acid cystine).[108] In a 2018 study, Nazari *et al.* used quantitative IMS to analyze the metabolite content of healthy and cancerous hen ovarian tissue sections and found a ~2-fold increase in GSH.[13,14] Additionally, several studies have reported an association between high GSH levels or glutathione S-transferase P1 (GSTP1) activity and cisplatin or carboplatin resistance in OC.[81,109,110] Further support for this association comes from a study by Wang *et al.* in 2016, in which CAFs were found to confer platinum resistance to OC cells by releasing GSH and cysteine into the tumor microenvironment (Fig. 4).[19]

Lipids: lysophosphatidic acid (LPA)

Lysophosphatidic acids (LPA's) are a class of bioactive lipids which vary in the length and number of double bonds on the fatty acid side chain esterified to a glycerol backbone. LPAs are potential biomarkers for OC, as numerous blinded and independent studies have reported they are elevated in the blood of OC patients when compared to benign and/or healthy controls.[111–113] However, given how ubiquitous LPA and other lipid molecules are, they would be challenging to develop as biomarkers for clinical diagnostics. OC preferentially metastasizes to the omentum which is known to secrete many chemotactic cytokines and growth factors, including LPA;

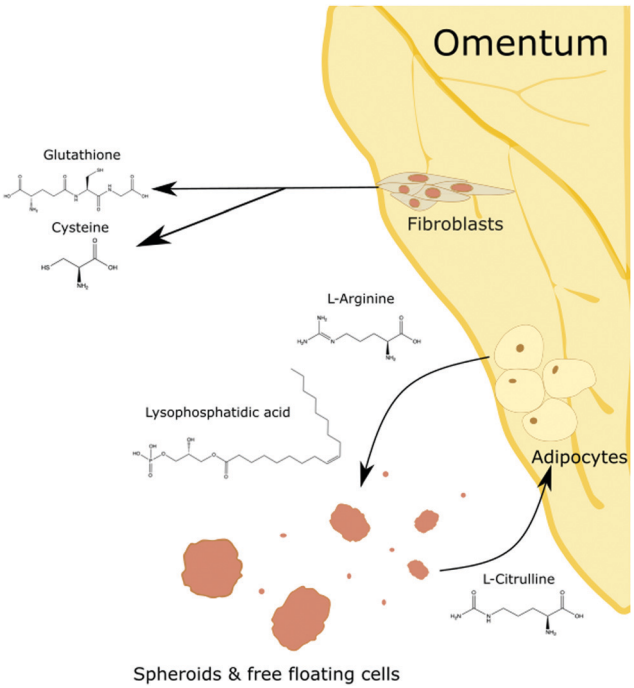


Fig. 4 Small molecules play important functional roles in the HGSOC intraperitoneal microenvironment.

further, about 40% of bodily autotaxin (ATX) is produced by adipocytes which are enriched in the omentum and known to provide energy to cancer cells for rapid tumor growth.[114–117] LPA has been shown to stimulate most tumor promoting activities *in vitro* including cell differentiation or proliferation, prevention of apoptosis, induction of platelet aggregation, stimulation of cell morphology changes, cell migration, adhesion, and invasion. It has also been shown to stimulate tumorigenesis and metastasis *in vivo*.[118–125]

Vitamins: folic acid

Folic acid, also called vitamin B9, is an essential nutrient for normal proliferating cells and is required for the biosynthesis of purine and pyrimidine nucleotides needed for DNA/RNA synthesis as well as epigenetic modification through DNA methylation.[126] Folic acid has previously been implicated in cancer development through DNA methylation and the disruption of DNA integrity causing it to interfere with the expression of proto-oncogenes and tumor suppressor genes, including *TP53* (an early genetic signature of HGSOC).[127,128] The overexpression of folate receptor alpha (FRα) has been observed in OC, and the increased expression of folate binding proteins is positively correlated with OC stage and prognosis.[129]

Future directions/prospective

HGSOC remains a salient public health concern, largely due to a lack of techniques for early diagnosis and effective treatment. Further investigation of the molecular events underlying HGSOC pathogenesis may facilitate the identification of

diagnostic biomarkers and therapeutic targets, and metabolomics is an important tool for investigating these molecular events. Since HGSOC is a progressive disease that involves specific movement to specific organs, we advocate for utilizing models and methodologies that take spatial referencing into account. Given the historical shortcomings of biomarkers for disease detection that did not take the microenvironment into account, specifically the localized reproductive anatomy micro-environment, metabolomics experiments that can capture this localized environment may provide an advantage. There is a wealth of chemical space yet to discover as many metabolomics experiments rely on databases, such as the Human Metabolome Database, yet this may not be comprehensive of the entire chemical space.[130] Models that allow for continued growth may help facilitate the acquisition of enough biomass to identify signals *via* alternative approaches such as NMR. In the coming years, balancing the relevance of the model system and complexity of the female reproductive system coupled with spatial metabolomics will advance our understanding of the chemical exchange that occurs to drive tumorigenic cells to specific tissues.

Conflicts of interest

There are no conflicts to declare.

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