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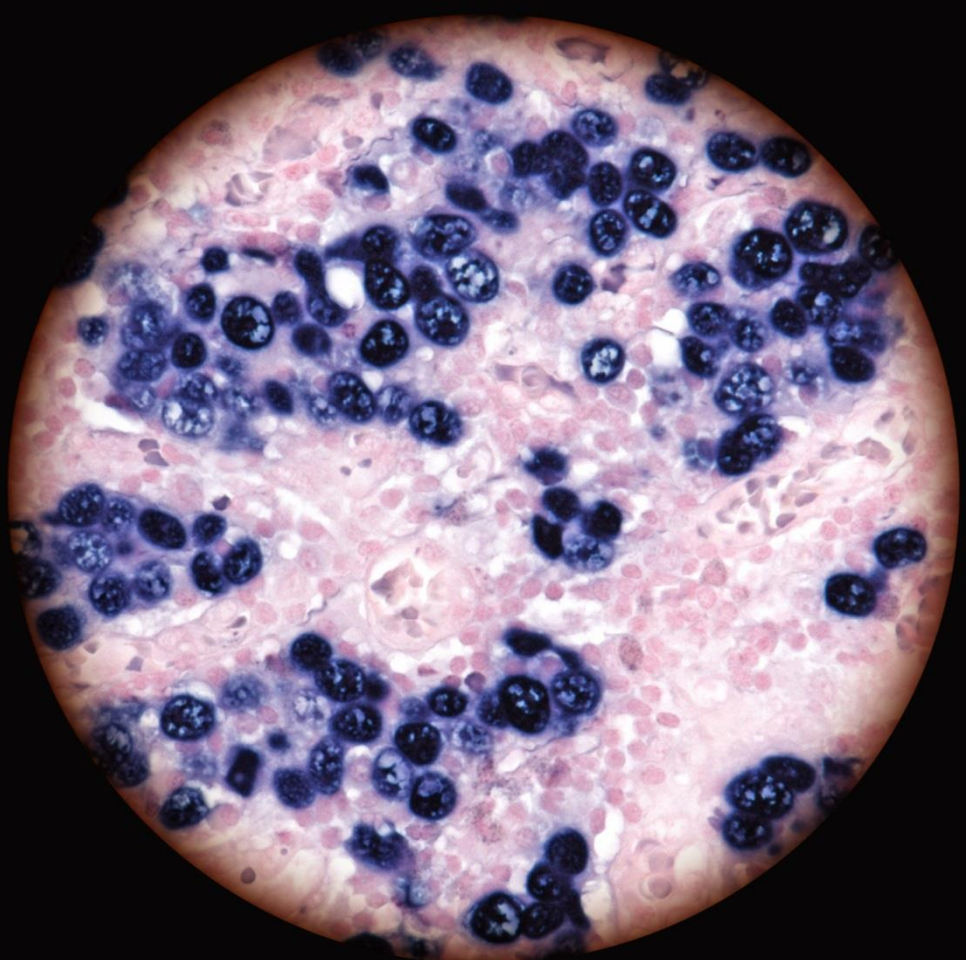

Table of Contents

Editorial

This is the first issue of our new Journal, the Journal of Surgical and Molecular Pathology (JSMP). Our Journal is an international, open access, peer-reviewed and refereed online, free of charge, scientific journal published by the Hellenic Society of Pathology, HSP (www.pathology.gr), guaranteeing high quality of accepted papers and rapid publication. Our main objective is to integrate morphology, molecular pathology (particularly the tissue-based one), clinical laboratory medicine and clinical medical specialties, especially oncology and its subspecialties. We intend to promote interdisciplinary scientific studies and thus, we encourage the submission of interdisciplinary articles. Systematic review articles are also welcome and they will be of priority in publishing.

We invite for submission of original, not previously published articles, all pathologists specializing in anatomical pathology, molecular pathology, and cytology, as well as all researchers in related (in a broad sense) fields. We encourage other scientists (such as clinical oncologists, surgical oncologists and radiation oncologists, involving anatomical pathology in their research) to publish in our Journal clinical protocols involving pathology. Articles on health policies and educational articles, as well as articles on bioethics are welcome.

Analytically, the Journal of Surgical and Molecular Pathology (JSMP)'s aim is the publication of scientific articles of basic research and laboratory-clinical investigation in the following fields:

- Traditional Morphology and Histochemistry, Comparative Pathology, Autopsy Pathology, Cytopathology and Clinical Cytology
- Antibodies production for diagnosis, prediction, prognosis and targeted therapy
- Immunohistochemistry and Immunocytochemistry
- Molecular Pathology, Molecular Genetics, Proteomics, high-throughput technologies
- Translational research
- Circulating Tumor Cells and Liquid Biopsy
- Biomarkers, predictive and/or prognostic
- Impact of biomarkers and new technologies on targeted therapies
- Cryogenics, especially related to biological sciences
- Morphometry and fractals applications in pathology
- Human Biology and related sciences
- Biomaterials and Biotechnology, mostly applied
- Bioinformatics
- Bioethics
- Pathology-related Education
- Mechanisms of disease and novel diagnostic and therapeutic strategies involving pathology

Our interests are not limited to the above fields. We consider the publications in related fields leading to the future pathology, the integrated morphomolecular science, both research and diagnostic, as well as their scientific historical and philosophical background. We aim to be a liaison between pathology and other sciences (such as clinical, surgical and radiation oncology), to unify fields and spherically face diagnostic and research challenges.

We are seeking papers for the upcoming issues and we invite you all sharing our interests to submit original articles.

For more information, please visit the official website of the Journal.

Enjoy this first issue.

E. N. Stathopoulos, MD, PhD, Professor Emeritus, Pathology, University of Crete, Greece

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Carcinomas with Lymphoid Stroma within the Gastrointestinal Tract: Histology and Molecular Pathology

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Abstract

Carcinomas with lymphoid stroma represent a distinct morphological subtype of gastrointestinal cancer. They occur most often in the stomach or in the colon. On the morphological level, they may be separated into medullary and lymphoepithelioma-like cancers. The former are characterized by predominantly syncytial growth and dense lymphocytic infiltration that prevails at the tumour periphery. Lymphoepithelioma-like cancers are made up of small clusters and aggregates of tumour that are broken up by large numbers of intratumoural lymphocytes. Differential diagnosis may be challenging and often requires immunohistochemistry. Several markers are often necessary to separate medullary colon carcinoma from poorly differentiated non-medullary carcinoma, as the immunophenotypes are overlapping. Diagnosis of gastrointestinal carcinoma with lymphoid stroma should always prompt further investigations, aiming at the detection of Epstein-Barr virus (EBV) infection and high level microsatellite instability (MSI-H). The majority of carcinomas with lymphoid stroma occurring in the stomach are lymphoepithelioma-like cancers. On the molecular level, these cancers are positive for EBV. In the colon medullary cancers prevail. They occur most often as right-sided lesions. These tumours almost invariably show MSI-H due to sporadic epigenetic silencing of the MLH-1 gene.

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Introduction

The tumour microenvironment is a collective term that includes the tumour's surrounding and supportive stroma, including cancer-associated fibroblasts and pericytes, the different effectors of the immune system, blood platelets, hormones and other humoral factors [1]. Most of the immune cell populations interplay with the stromal factors, with distinct impact on tumour growth capacities, that is, proliferation, invasion and potential for spread [2].

The role played by lymphocytes is complex. The key tumoricidal lymphocyte is the natural killer (NK) cell, which is positive for CD56 and also CD8 (up to 80%) upon immunohistochemistry. Unlike other cytotoxic lymphocytes, NK cells kill neoplastic cells independent of their MHC protein, through their strong perforin. Furthermore, NK cells are also regulatory cells engaged in reciprocal interactions with dendritic cells, macrophages, distinct T cell subsets and endothelial cells [1].

The T_H (Helper) population bearing the marker CD3+ and CD4+ performs a dual function, based upon the subsets and the ratio of their populations. T_H1 cells mediate a tumour suppressor inflammatory reaction, whereas T_H2 cells are players in cancer-associated inflammation, which are well recognized for their tumour-promoting capabilities [3]. B-lymphocytes mediating humoral immunity can promote cancer progression by altering the T_H1/T_H2 ratio [1]. Cytotoxic T lymphocytes bearing the marker CD8+ can identify and destroy cancer cells through their MHC recognition when recruited to the tumour milieu [4].

Regulatory T cells (Tregs) are a specialized subset of CD4 T cells that have an indispensable role in maintaining immune homeostasis and tolerance. Increased Treg numbers and/or function may exhibit tumour progression by interfering with immune surveillance. Conversely, in cancers with an inflammatory component, such as colorectal, Tregs can inhibit cancer progression by dampening inflammation [5].

In the gastrointestinal tract, tumours with prominent inflammatory component have been described under different terms, such as lymphoepithelioma-like and medullary carcinoma. In this review, we will summarize the histology and molecular pathology of gastrointestinal adenocarcinomas with prominent lymphoid stroma and will also refer to their clinical significance.

Histological variants

Traditionally, tumours with a dense lymphoid component can be classified into lymphoepithelioma-like and medullary carcinomas, but many authors do not regard these morphological variants as distinct entities and apply the two terms synonymously [6]. The more descriptive term “adenocarcinoma with lymphoid stroma” may alternatively be used for these lesions following the recommendations of the most recent World Health Organization (WHO) classification for tumours of the digestive system [7].

Historically, the term “medullary carcinoma” was introduced to depict a distinct variant of breast carcinoma with microscopic resemblance to the normal medulla oblongata. On low power, these lesions show an inner pale, solid area (corresponding to the cancer) and a surrounding darker zone (corresponding to the lymphoid infiltrate) [6]. On high power, true medullary carcinomas have well defined peripheral margins. It is important to assess the leading edge of invasion, which is non-infiltrative, as the outer border of the inflammatory component may be

slightly irregular [6]. The tumour cells are usually disposed in syncytial sheets, the overall appearance of the tumours being organoid or solid. The nuclei are vesicular, with prominent nucleoli. Tumours exhibit a dense lymphocytic response, which consists mainly of mature lymphocytes. The preponderance of inflammation is peritumoural (Figure 1).

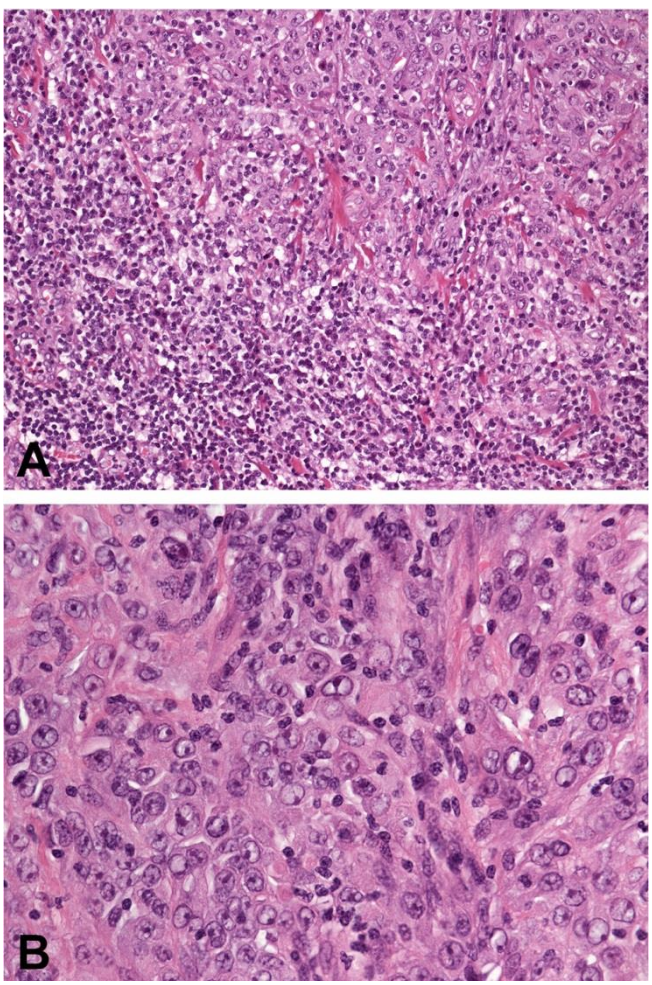

Figure 1. Medullary carcinomas have well defined peripheral margins with prominent peritumoural inflammation (A). The tumour cells are disposed in syncytial sheets, the overall appearance of the tumours being organoid or solid. The nuclei are vesicular, with prominent nucleoli. Admixed with the tumour cells mature lymphocytes and also plasma cells can be identified (B).

This is in contrast to the so-called "lymphoepithelioma-like carcinomas". In these tumours the lymphoid infiltrate tends to be more intratumoural than peritumoural. Lymphoepithelioma-like carcinomas were first described in the nasopharynx [8, 9], but later discovered also in other anatomic sites. On low power, these tumours are likewise well circumscribed, while on high power they do not display continuous sheets of tumour but are instead made up of small clusters and aggregates of tumour that are broken up by large numbers of intratumoural lymphocytes (Figure 2) [6].

Differential diagnosis mainly includes malignant lymphomas, in particular diffuse large B-cell lymphoma (DLBCL). With the help of immunohistochemistry, however, malignant lymphomas can easily be excluded.

Undoubtedly, there are morphological features that allow for their separation, but the distinction between medullary and lymphoepithelioma-like carcinomas may still be challenging, if not impossible in certain cases. Facing the rapidly evolving integration of molecular testing in the routine pathological work-up of cancer specimens, a distinction based upon routine haematoxylin and eosin evaluation may no longer be crucial. Instead, for all gastrointestinal adenocarcinomas characterized by a dense lymphoid stroma, the molecular basis of disease should be sought for, which may be either Epstein-Barr virus (EBV) infection or microsatellite instability (MSI).

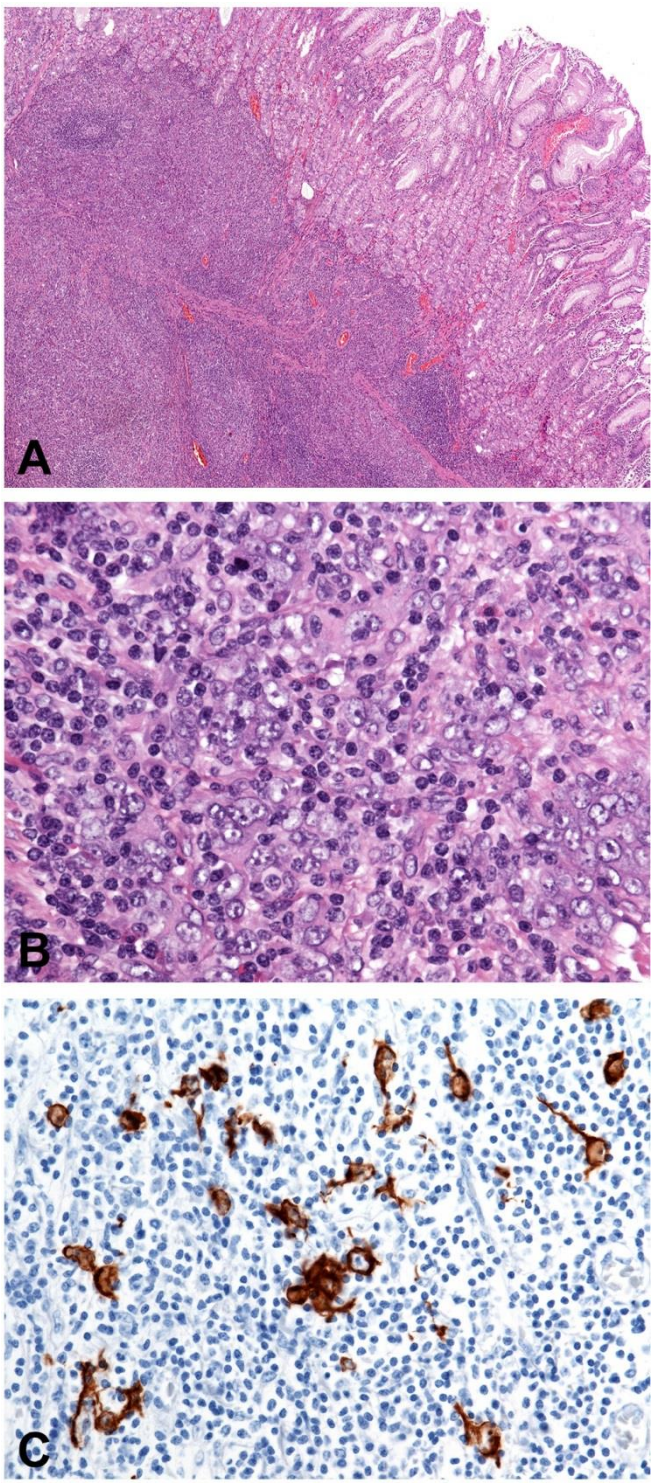

*Figure 2. Lymphoepithelioma-like carcinomas are well circumscribed tumours (**A**). On high power they do not display continuous sheets of tumour, but are instead made up of small clusters and aggregates of tumour that are broken up by large numbers of intratumoural lymphocytes (**B**), which can be highlighted by keratin immunostaining (**C**).*

Stomach

Gastric carcinoma with lymphoid stroma was recognized as a distinct variant of gastric cancer almost forty years ago [10]. Grossly, this carcinoma was characterized by clear circumscription, usually with a central ulceration. A histological feature distinguishing this carcinoma was the presence of a non-desmoplastic stroma infiltrated uniformly with an abundance of mature lymphocytes and plasma cells throughout the entire area of the tumour. Carcinoma of this type was found in 4% of a total of 1041 cases of gastric carcinoma removed surgically [10]. Intratumoural lymphocytic infiltration occurs in association with reactive hyperplasia of the regional lymph nodes, particularly paracortical hyperplasia, and in such cases, there is a favourable prognosis, regardless of the presence of lymph node metastasis [11].

In 1990, Burke et al. [12] first described a "lymphoepithelial gastric carcinoma" that was associated with EBV. Three additional cases that were likewise positive for EBV were reported in the subsequent year by another group [13]. It is of note, however, that not all EBV-associated gastric cancers bear a dense lymphocytic component. Thus, EBV-infection may be detected in "classical" tubular or tubulopapillary adenocarcinomas that lack a dense inflammatory infiltrate. In the study by Chang et al. [14], EBV infection was detected in 30 out of 45 (67%) gastric cancers with lymphoid stroma, but also in 10 out of 292 (3.4%) consecutive cases of gastric carcinomas without lymphoid stroma ($p < 0.05$). In another study, four out of 52 (8%) conventional carcinomas were EBV positive [15].

In general, EBV-associated tumours account for about 10% of gastric carcinomas worldwide, and > 80,000 patients are estimated to develop EBV-associated gastric cancer annually [16, 17]. The tumours occur predominantly in males, often in the proximal stomach, and for reasons that are largely unclear with higher frequency as gastric stump carcinomas (Table 1) [18]. Thus, in the study by Baas et al. [19] EBV was detected in 9 out of 26 (35%) stump carcinomas compared to 2 out of 24 (8%) carcinomas originating from non-operated stomachs.

The prognosis of EBV-associated gastric is favourable [20]. It is largely unclear, however, how EBV infection contributes to the survival benefit. Most probably, the link is the inherent lymphocytic infiltration, which proved to be a predictor of favourable outcome in several studies [21, 22]. Very recently, Kim et al. [23] performed systematic gene expression profile analysis to compare tumour and non-tumour gastric tissues from 12 patients with EBV-associated gastric cancer and 14 patients with gastric cancer not related to EBV. Based upon Pearson correlation matrix analysis, EBV-associated cancers had a significantly higher degree of homogeneity than gastric cancers not related to EBV. Notably, most changes in EBV-associated cancers occurred in immune response genes. These changes might allow EBV-associated cancers to recruit reactive immune cells, which might contribute to the better outcomes of these patients, compared to those with cancers not related to EBV.

In the colon, dense intra- and peritumoural inflammatory reaction as well as the presence of Crohn's-like lymphoid aggregates have been associated with high level microsatellite instability (MSI-H), which is the hallmark of cancer arising in the setting of Lynch Syndrome (hereditary non-polyposis colorectal carcinomas, HNPCC) [24-26]. In the stomach, adenocarcinomas with lymphoid stroma have likewise been associated with MSI-H status in a considerable number of cases, the majority of which occurring in a sporadic setting [27]. MSI-H gastric cancers share a significantly better prognosis than microsatellite stable cancers [21].

Grogg et al. [28] explored the relationship between EBV infection and MSI-H status in the setting of lymphocyte-rich gastric cancers. An interesting conclusion was that EBV and MSI were mutually exclusive. Thus, none of the tumours that were EBV-positive were MSI-H. Similar findings were obtained in the study by Leung et al. [29]. These studies support the notion that there are two separate pathways involved in the development of adenocarcinomas with lymphoid stroma. In the stomach it appears safe to assume that not all gastric cancers with a lymphoid stroma are medullary carcinomas. Indeed, if such a cancer is not MSI-H, it will more than likely be EBV-positive, and vice versa [6].

The role of EBV infection in the molecular pathogenesis of gastric cancer is only partly understood. In the study by Matsunou et al. [30] 85% of gastric carcinomas with lymphoid stroma were related to EBV. The authors detected EBV in nine of ten cancerous lesions in four cases of synchronous multiple cancers and in all five cancerous lesions in two cases of metachronous multiple cancers, suggesting that EBV infection may be an early event during carcinogenesis. Molecular evidence comes from an earlier study, which detected a single episomal form of EBV that was present in all tumour cells [31]. This finding strongly suggests that EBV infection occurs before transformation and latent infection of EBV is related to oncogenesis of EBV-associated gastric carcinomas.

Table 1. Characteristic clinicopathological features of carcinomas with lymphoid stroma in stomach and colon

	Stomach	**Colon**
Age *	similar	similar
Gender	male > female	female > male
Prognosis *	better	better
Localization	proximal > distal	right > left
Histology lymphoepithelioma-like medullary	 +++ +	 + +++
EBV association	+++	very rare
MSI-H	+	+++

* Age and prognosis are compared to "usual" types of gastric and colonic cancer, respectively.
Abbreviations: EBV Epstein-Barr virus
MSI-H High level microsatellite instability

The difficulties encountered in infecting and transforming primary epithelial cells in experimental systems suggest that the role of EBV is complex and multi-factorial in nature [32]. Genetic alterations in the premalignant epithelium may support the establishment of latent EBV infection, which is believed to be an initiation event. Oncogenic properties have been reported in multiple EBV latent genes. The BamH1 A rightwards transcripts (BARTs) and the BART encoded microRNAs (miR-BARTs) are highly expressed in EBV-associated epithelial malignancies and may induce malignant transformation [32]. However, enhanced proliferation may not be the crucial function of EBV infection in epithelial malignancies, at least in the early stages of cancer development. EBV-encoded gene products may confer anti-apoptotic properties and promote the survival of infected premalignant epithelial cells harbouring genetic alterations. Multiple EBV-encoded microRNAs have been reported to have immune evasion functions. Genetic alterations in host cells as well as inflammatory stroma could modulate expression of EBV gene expression and alter the growth properties of infected premalignant epithelial cells encouraging their selection during carcinogenesis [32].

Though *in vitro* evidence is compelling, EBV has never been convincingly shown to be present in preneoplastic gastric lesions (intestinal metaplasia and dysplasia). In the most extensive study on this topic, zur Hausen et al. [33]

systematically investigated 19 patients with EBV-associated gastric cancer. In all of them, EBV-positivity was restricted to the cancer cells, but were absent in the preneoplatic lesions.

Very recently, the Cancer Genome Atlas Research Network published a comprehensive molecular characterization of gastric adenocarcinomas [34]. The authors proposed a molecular classification dividing gastric cancer into four subtypes: tumours positive for EBV, which display recurrent PIK3CA mutations, extreme DNA hypermethylation (CpG island methylator phenotype; CIMP), and amplification of JAK2, CD274 (also known as PD-L1) and PDCD1LG2 (also known as PD-L2); microsatellite unstable tumours, which show elevated mutation rates, including mutations of genes encoding targetable oncogenic signalling proteins; genomically stable tumours, which are enriched for the diffuse histological variant and mutations of RHOA or fusions involving RHO-family GTPase-activating proteins; and tumours with chromosomal instability, which show marked aneuploidy and focal amplification of receptor tyrosine kinases.

Detection of EBV in tumour tissue is nowadays mainly done by *in situ* hybridization (ISH) (Figure 3). EBV encodes two small nuclear RNAs named EBER-1 and EBER-2, which are transcribed in latently EBV-infected cells in high concentration (up to 107 copies per cells) enabling the detection of EBV in the tumour cells (EBER-ISH) [31]. Some rare cases may show strong EBER-ISH staining in occasional surrounding lymphocytes but not in the tumour cells [35]. Therefore, if polymerase chain reaction (PCR) is used for virus detection, this needs to be done by quantitative real time PCR, not by qualitative PCR to avoid false-positive reporting.

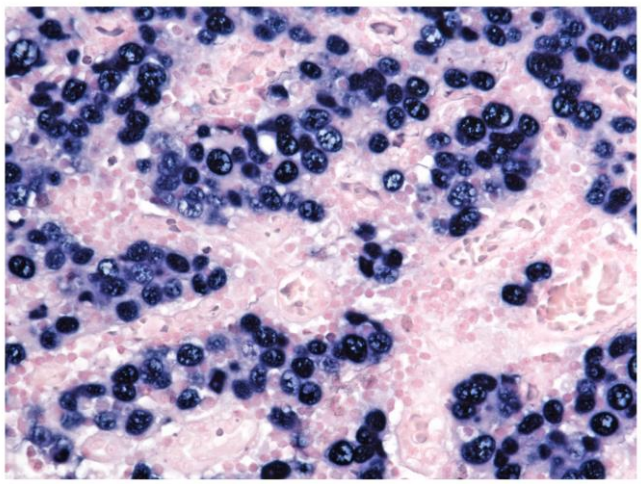

Figure 3. EBV in tumour tissue is nowadays mainly done by in situ hybridization (ISH). EBV encodes two small nuclear RNAs named EBER-1 and EBER-2, which are transcribed in latently EBV-infected cells in high concentration enabling the detection of EBV in the tumour cells (EBER-ISH).

Colon

In the lower gastrointestinal tract, cancers with lymphoid stroma have been ignored for a long time. In 1977, Gibbs [36] reported eight cases of undifferentiated carcinoma of the large intestine, which had grown to a large size before symptoms were produced and which, despite the undifferentiated histology, had a favourable prognosis when locally resectable. Five patients survived between 6 and 28 years, one was well 6 months after operation and two cases where local removal could not be achieved, died within one year. Twenty years later Rüschoff et al. [37] reported a series of poorly differentiated adenocarcinomas with minimal or no glandular differentiation, combined with an expansive growth pattern and a significant dense lymphoid infiltration, comparable with solid or medullary gastric carcinomas. The tumours were MSI-H and demonstrated a generally favourable outcome.

In 1999, Jessurun et al. [38] reported a series of eleven cases of medullary adenocarcinoma of the colon with nests or trabeculae of regular small to medium-sized cells with moderate amounts of eosinophilic cytoplasm. All patients were female and their tumours were located in the caecum or proximal colon. Endocrine markers were negative. The authors stressed the importance to distinguish this tumour type from other more aggressive, nonglandular lesions. In the same year, Lanza et al. [39] confirmed Rüschoff's observations [37] by proving that the majority of medullary cancers are MSI-H, diploid, and negative for p53.

The next major study was undertaken by Wick et al. [40]. These authors compared medullary carcinomas with poorly differentiated conventional enteric-type adenocarcinomas and neuroendocrine carcinomas. Medullary carcinomas were significantly more common in the right colon, particularly the ascending colon, compared to enteric-type adenocarcinomas, which occurred mainly in the rectosigmoid. In addition, medullary carcinomas arose in older patients and mainly affected females. It is of note that medullary carcinoma was less likely to manifest with stage III or IV disease. The authors emphasised the similarity to neuroendocrine carcinoma, morphologically and immunophenotypically, but the percentage of neuroendocrine differentiation did not differ meaningfully from that of enteric-type adenocarcinoma.

A large population-based analysis assessing the Surveillance, Epidemiology and End Results (SEER) database confirmed the previous data [41]. Mean age at diagnosis was 69.3 (±12.5) years, with incidence increasing with age. In addition, medullary carcinomas were twice as common in females, who presented at a later age, with a lower stage and a trend towards favourable prognosis (Table 1). Tumours were most common in the proximal colon (74%), and there were no cases reliably identified in the rectum or appendix. Despite general lack of differentiation in tumour tissues, patients commonly presented with stage II disease, only 10% presenting with metastases [41].

According to the most recent edition of the WHO Classification of Tumours of the Digestive System [42], medullary carcinoma is characterized by sheets of malignant cells with vesicular nuclei, prominent nucleoli and abundant eosinophilic cytoplasm exhibiting prominent infiltration by intraepithelial lymphocytes. True lymphoepithelioma-like cancers which account for the majority of carcinomas with lymphoid stroma in the upper gastrointestinal tract are very rare in the colon, and the association with EBV appears to be inconsistent at this site [35]. In fact, EBV-positive lymphoepithelioma-like cancers have only anecdotally been reported [43-45].

Differential diagnosis needs exclusion of poorly differentiated non-medullary colorectal cancer and may include a plethora of different entities, including secondary tumours and malignant lymphomas, in particular high grade large cell lymphomas [46]. In a recent interobserver study including 15 cases initially classified as medullary carcinoma and 30 cases of poorly differentiated adenocarcinomas two pathologists agreed on only 31 of 45 cases (69 %) with kappa = 0.32 [47].

Therefore, the diagnosis of medullary carcinoma often needs immunohistochemical confirmation, which, however, includes several important pitfalls (Figure 4). Though being positive for pankeratin preparations, the majority of medullary cancers lack the expression of keratin 20 [47-49], which represents the predominant keratin in the bowel and which is expressed in the vast majority of non-medullary colorectal cancers [50]. Like conventional adenocarcinomas, some medullary carcinomas may be positive for keratin

7 [47-49, 51]. If keratin 7 positivity is observed in a tumour lacking positivity for keratin 20, this may cause major diagnostic problems and the lesion may easily be mistaken as a secondary tumour.

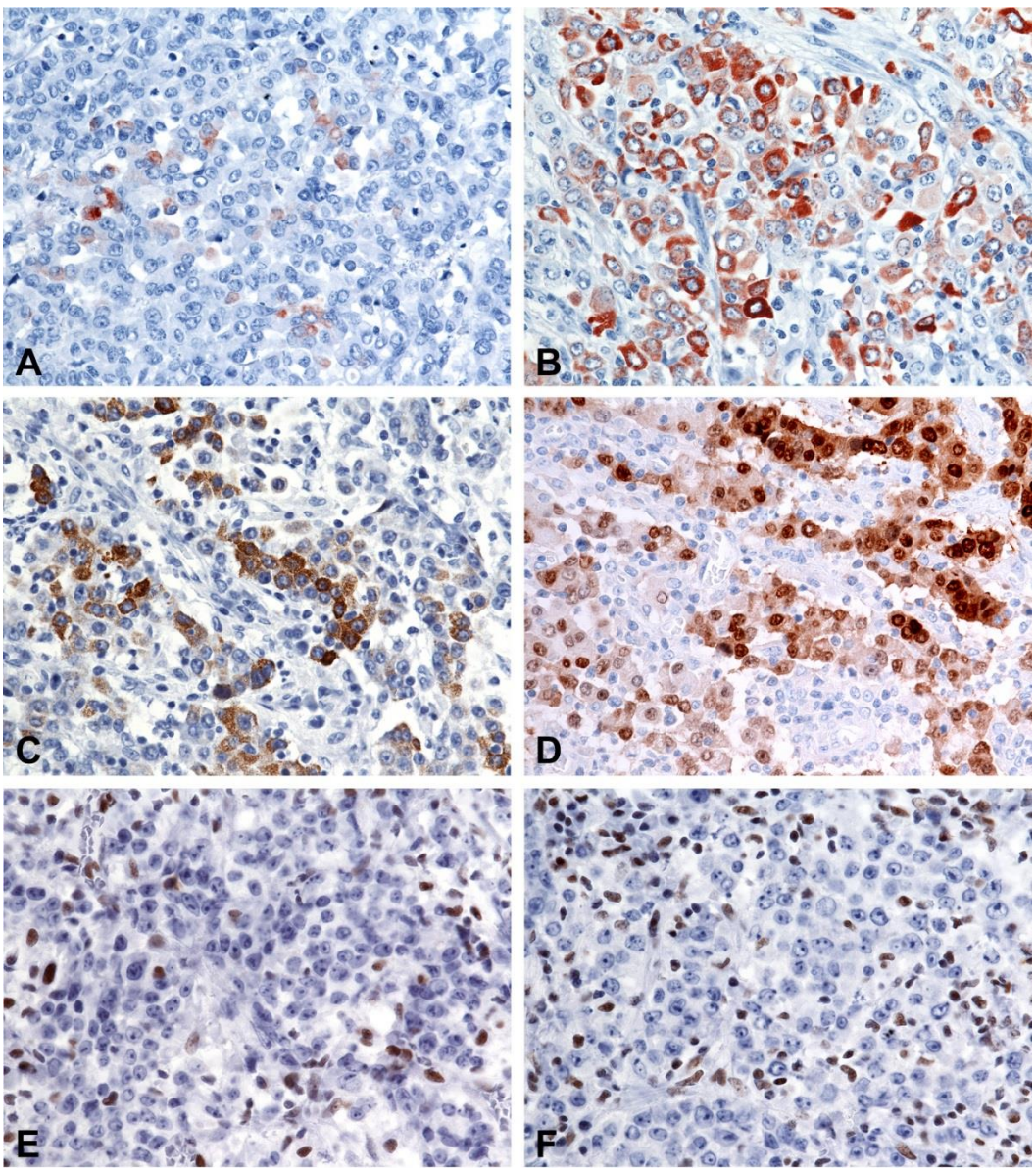

Figure 4. The diagnosis of medullary carcinoma often needs immunohistochemical confirmation, which, however, includes several important pitfalls. Quite often the tumour cells are only weakly of focally positive for keratin 20 (A). When a tumour like that is positive for keratin 7 (B) this may imply major diagnostic problems, especially if biopsy material is evaluated. Positivity for MUC2 (C) and CDX-2 (D) may be of help, but the expression of these markers is variable. As medullary carcinomas almost invariably show high-level microsatellite instability (MSI-H) staining loss of nuclear MLH1 (E) and PMS2 expression (F) may be the key step to accurate diagnosis (note the positive staining of intratumoural inflammatory cells serving as internal positive control).

Staining for the intestinal transcription factor CDX-2 is variable. While some authors reported positivity in the majority of cases, others noted CDX-2 expression in only 19% of cases [47-49]. It is of note that the staining is often focal or weak causing biopsies to be negative due to sampling error. Very recently, Lin et al. [49] suggested SATB2 and cadherin-17 as new diagnostic markers: Expression was noted in 89% of medullary carcinomas, in 97% and 98% of non-medullary colorectal adenocarcinomas, respectively, whereas only 3.6% and 3.3% of non-gastrointestinal tumours were positive, respectively.

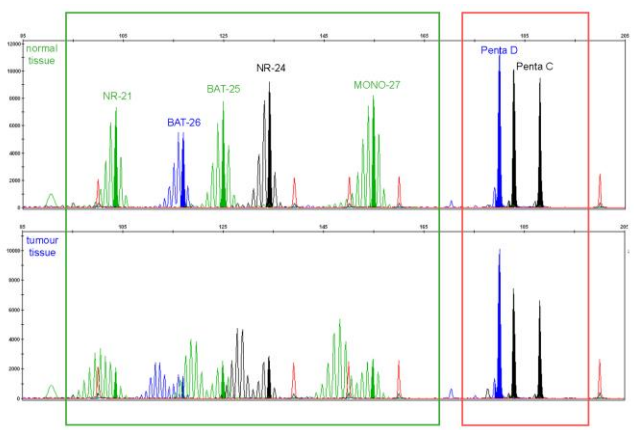

Figure 5. Example of a medullary colon cancer with high-level microsatellite instability (MSI-H). The MSI profile assessed by a panel of five nearly monomorphic mononucleotide repeats (pentaplex panel) illustrates instability for all markers, as shown by additional alleles (allelic shifts). Two polymorphic pentanucleotide repeats (Penta C and Penta D) are included for sample identification.

As medullary carcinomas are almost invariably MSI-H (Figure 5), it is suggested to perform staining of the mismatch repair proteins MLH-1, MSH-2, MSH-6 and PMS-2 not only to confirm MSI, but also for diagnostic reasons. Loss of MLH-1 expression (and consequently its minor partner PMS-2) due to epigenetic silencing of the MLH-1 gene represents the most common phenotype [49]. In the study by Fiehn et al. [47] loss of MLH-1 (and PMS-2) was present in 8 out of 9 (89%) of medullary carcinomas as opposed to 10 out of 22 (45%) poorly differentiated non-medullary carcinomas ($p = 0.04$). Winn et al. [48] suggest the combined use of MLH-1 and CDX2 together with calretinin. In that study, MLH1 and CDX2 were positive in 21% and 19% of medullary carcinomas as opposed to 60% and 55% of the poorly differentiated non-medullary carcinomas ($p = 0.02$ and $p = 0.03$ respectively). The differential staining pattern for calretinin was the most striking with 73% of the medullary carcinomas as opposed to 12% of the poorly differentiated non-medullary carcinomas staining positive ($p < 0.0001$). A CDX2 negative, MLH1 negative, and calretinin positive immunohistochemical phenotype had an 82% positive predictive value for correctly distinguishing medullary carcinoma from poorly differentiated non-medullary carcinoma (Table 2).

Other sites

Very rare cases of carcinomas with lymphoid stroma of the oesophagus, usually referred to as lymphoepithelioma-like carcinoma [52-55], and the small bowel, usually referred to as medullary carcinoma [56,57], have been described in the literature. In the former, the association with EBV is controversial. To our knowledge, no lymphoepithelial carcinoma of the anus has been reported.

Table 2. Immunophenotype of medullary colorectal carcinoma compared to poorly differentiated non-medullary colorectal carcinoma [from Winn, Kanstrup]

	Medullary carcinoma	**Poorly differentiated non-medullary carcinoma**
CK-20	11-44%	45%
CK-7	0-13%	21-23%
CDX-2	19-78%	55-68%
MUC-2	11-60%	36-59%
MLH-1 (and PMS-2)	11-21%	55-60%
Calretinin	44-73%	12-27%

Conclusion

Carcinomas with lymphoid stroma represent a distinct morphological subtype of gastrointestinal cancer. On the morphological level, they may be separated into medullary and lymphoepithelioma-like cancers. The former are characterized by predominantly syncytial growth and dense lymphocytic infiltration that prevails at the tumour periphery. Lymphoepithelioma-like cancers are made up of small clusters and aggregates of tumour that are broken up by large numbers of intratumoural lymphocytes. Diagnosis of gastrointestinal carcinoma with lymphoid stroma should always prompt further investigations, aiming at the detection of EBV infection and MSI-H. The majority of carcinomas with lymphoid stroma occurring in the stomach are lymphoepithelioma-like cancers. On the molecular level, these cancers are positive for EBV. In the colon medullary cancers prevail. These tumours are almost invariably MSI-H due epigenetic silencing of MLH-1.

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The Roles of Lipoproteins, Diet, and Peripheral Macrophages in Atherosclerotic Disease

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Abstract

We consider lipid accumulation in atherosclerosis, with emphasis on mechanisms mediating atheroma growth at peripheral sites. Macrophages normally recycle all dead cell components, including membranes. Membrane lipids are exported, as cholesterol or cholesterol esters, by lipoproteins for disposal by the liver or, as triglycerides or phospholipids, for lipid storage or re-use. Membranes of somatic cells, such as red blood cells, incorporate fatty acids that reflect dietary intake. When excessive saturated and trans-unsaturated fats are incorporated in cells, and the cells die, macrophages cannot fully recycle the membrane lipids, setting up a vicious cycle of lipid overload, cell death, recruitment of macrophages, and cell proliferation. Semi-liquid masses of partially oxidized fatty acids and cholesterol, foamy macrophages, and proliferating stromal cells accumulate in arterial walls. The dramatic increase of atherosclerotic disease since 1920 reflects superabundant nutrition and altered dietary composition, along with reduced exercise and smoking. The dietary changes included increased saturated and trans-unsaturated fats. The biochemical basis of epidemic atherosclerosis includes a partial metabolic block in β-oxidation caused by unsaturated fatty acid intermediates. Responses of cells to un-degraded saturated and trans-fatty acids include production of inflammatory cytokines, alteration of macrophage signaling pathways, and altered lipid-handling enzymes. Prevention of disease is an ideal approach, but pharmacological inhibitors including statins, PCSK9 inhibitors, or limiting macrophage catabolism of lipids by reducing carnitine availability, may limit progression of atherosclerosis.

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Lipoproteins and receptors

The discovery of the low density lipoprotien (LDL) receptor [1] was a pivotal event in study of atherosclerotic disease. Subsequent work created a complex web of clinical and experimental work focusing largely on plasma lipid components including high density lipoproteins (HDL), LDL, and triglycerides, with an expanding armamentarium of lipid-modifying drugs [2]. The use of plasma lipid profiles to monitor risk of atherosclerois risk is well reviewed elsewhere, and here we focus instead largely on the peripheral sites where atherosclerosis develops. Plasma lipid carriers are used by many different organs. This includes LDL and very low density lipoprotein (VLDL) receptors in skeletal and heart muscle, as transporters for lipids used in metabolism [3]. There are also specialized roles for lipoproteins; e.g., cortisol is made from cholesterol delivered to the adrenal via the HDL receptor Scarb1 -- absence of which causes greatly increased ACTH, but normal cortisol [4]. A surprising variety of lipoprotein defects have consequences on skeletal mass or joint disease [4,5]. Effects on the central metabolic pathways that regulate lipids have unexpected consequences such as deficiency of apolipoprotein E protecting mice from obesity and nonalcoholic fatty liver disease [6]. There are too many examples to enumerate. That said, although heart attacks and strokes account, together, for nearly 40% of deaths in “first world countries”, people do not have hereditary lipoprotein, or lipoprotein receptor, defects. Development of atherosclerotic disease reflects in major part environmental factors, diet, exercise, and smoking, and the pathology occurs entirely at peripheral sites and centers on macrophage metabolism. We consider here diet and macrophage function in atherosclerosis. We review the biochemical mechanisms affected by dietary fat that determine how and where atherosclerotic lesions accumulate. A central concept is that lipids carried on lipoproteins are not transferred into atherosclerotic lesions. Lipids are carried by lipoproteins; they are fuel for cell growth and metabolism, and in some cases, such as oxidized phospholipids at peripheral sites, are toxic to cells, typically macrophages, that receive these modified lipids. This reflects that lipoproteins are the acceptors of lipids; in atherosclerotic lesions they are processed by macrophages.

Historical perspective

Prior to World War II, atherosclerosis as a cause of death was uncommon. It was known in antiquity [7]. But, for most of history, it was a problem largely of rich individuals with rich diets, and a few unfortunates with genetic predilections, then unknown. As an illustration, deaths, in the over age 40 population group, attributed to ischemic heart disease from 1870-1980 are shown (Figure 1), from a century of autopsies at St Barts, London [8]. Many consistent reports noted the same disturbing trend around 1950. The median age of patients with myocardial infarction, depending on diagnostic criteria and population, is in the mid to late 60s, with two-thirds 45-74 years [9,10]. Since the turn of the 21st century, there are encouraging signs that atherosclerotic disease is declining with smoking decreased by half to two thirds since 1965, improvements in diet, and metabolic inhibitors to normalize blood pressure and control LDL cholesterol.

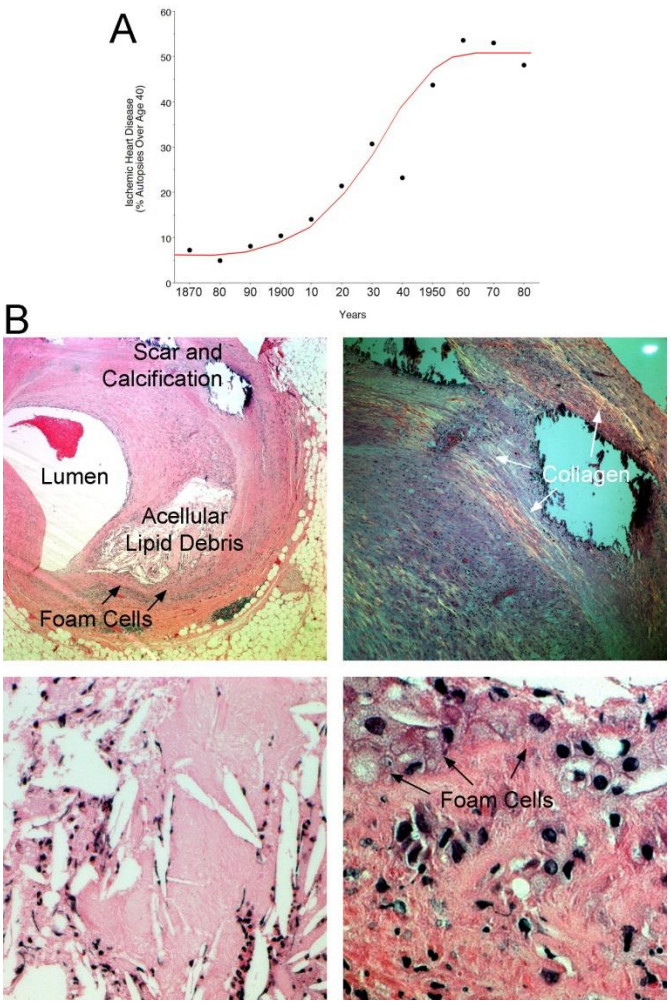

Figure 1. Atherosclerosis incidence and histological features.

A. Atherosclerosis as common pathology expanded rapidly around the time of world war II. Deaths from ischemic heart disease, of autopsies at St Bartholomew's Hospital, London, 1870-1980. Data from Table 4 of Finlayson, 1985 [8], are used with permission. Numbers of autopsies attributed to ischemic heart disease divided by the number of autopsies, in patients over age 40; data average the decade beginning with the date on the abscissa. In 1870 46% of deaths were over age 40, this increased to 70% at 1935, and then abruptly to 80-90% after world war two. A drop in the percent of ischemic deaths during world war two years (1940s) is ignored in the curve fit, but it might represent wartime rationing and related effects.

B. Features of an atherosclerotic lesion.

Top left. A low power image, 3.5 mm across of a coronary artery ~75% narrowed by atherosclerosis. The intima is expanded; in the lower portion of the lesion is caseous debris.

Bottom left. A high power image of the cell debris (300 µm across) showing clear areas where lipid was removed in processing and cell debris without nuclei or preserved cell membranes.

Top right. Medium power image (0.6 mm across) in polarized light to show the dense collagen (bright) around a mineral nodule in the scarred, inactive region of this lesion.

Bottom right. High power (image 150 µm across) shows the vacuolated, or physaliferous, macrophages "foam cells" at the border of the acellular debris.

Dietary fatty acids: Saturated and unsaturated, natural and artificial

Fatty acids have a carboxyl group at one end and a tail of carbons, with a variable number of double bonds, in cell membranes mostly 16 to 20 carbons overall; C18:2 indicates an 18 carbon fatty acid with two double bonds; C18:1 Δ9 indicates that a double bond occurs between carbons 9 and 10. Double bonds in cis configuration (Figure 1A) are Z isomers in formal IUPAC nomenclature; trans-bonds are E isomers. Fatty acid unsaturation in natural membrane

phospholipids fatty acids is always cis, double bonds are created at 3-carbon increments (that is, not conjugated). Exceptions include trans bonds between carbons 2 and 3 from the parent carboxyl chain in specialized derivatives including sphingosine, an important amino-alcohol synthesized from serine and palmitoyl CoA, and 2,3 trans bonds used during catabolism of double bonds at odd-numbered carbons, which are out of phase with 2,3 reductases (Figure 1). Linoleic acid, C18:2 Δ9,12 cis, cis, is an essential nutrient. Linoleic acid is required for synthesis of arachidonic acid; arachidonic acid makes up 10 to 20% of red cell membrane fatty acids, and arachidonic acid is the substrate for synthesis of prostaglandins. This 20 carbon fatty acid formally is 5Z,8Z,11Z,14Z-5,8,11,14-eicosatetraenoic acid. An alternate ω, or omega, nomenclature counts double bonds from the distal end of the fatty acid. It is used to designate fatty acids with double bonds near the tail, 3 or 6 carbons from the end, that cannot be synthesized by mammals but which occur in vegetable and fish oils; linoleic acid is thus, ω6, ω9-octadecadienoic acid. Simplified names, giving acids as stearate "-ate" rather than stearic acid "-ic acid", are used subsequently.

Sources of fatty acids: Differences in length and saturation

Animal fats contain ~ 50% saturated fatty acids (Figure 2A, top), of which ~ 25% is C18:0, stearate, and roughly twice as much C16:0, palmitate, as stearate. Fatty acids from vegetables, e.g., soy oil, contain mostly unsaturated fatty acids with a large proportion of polyunsaturates, including variable amounts of ω-3 and ω-6 unsaturates. Trans bonds occur in some natural fats, but in central regions of long chain fatty acids they are not natural. Usually they result from partial hydrogenation. Unsaturated fatty acids with trans-bonds are stiff and have high melting points; the singly-unsaturated cis-fatty acid oleate (Figure 2A, middle) is a liquid; its tran-isomer, elaidate, is a solid (Figure 2A, bottom). Elaidate ((E)-octadec-Δ9-enoic acid) is the key 18-carbon monounsaturated trans fatty acid, with the trans bond at the C9-10 position, Δ9. Artificial trans-fatty acids accounted for ~2.6% of individual energy intake, and over 7% of fatty acid calories, in the US at the turn of the 21st century [11]. Unsaturated fatty acids can be converted to saturated fatty acids by bubbling hydrogen through them at high temperatures in a closed container. Industrial hydrogenation was developed by the noted French chemist Paul Sabatier in the late 1800s; he received the Nobel Prize for direct hydrogenation in 1912 [12].

In vegetable oils, typically 90% of fatty acids are C18, with most unsaturated and a high proportion doubly and triply unsaturated, all of the unsaturation cis. Hydrogenation reduces double bonds; partial hydrogenation reduces some double bonds and converts others from natural cis-unsaturated bonds to trans-unsaturated bonds: Trans isomers have lower free energy, and thus are strongly favored. Elaidate is one of many trans isomers that occur with partial hydrogenation. Double bonds in the carbon chain may be moved to make various isomers with double bonds at various positions between C4 and C14; when eaten they are used in cell membrane phospholipids in a similar distribution to the unsaturated fatty acids in food [13]. But the quantitatively predominant trans fatty acid in partially hydrogenated oils is elaidate, with the double bond smack in the middle [14], the most stable isoform (Figure 2A, bottom).

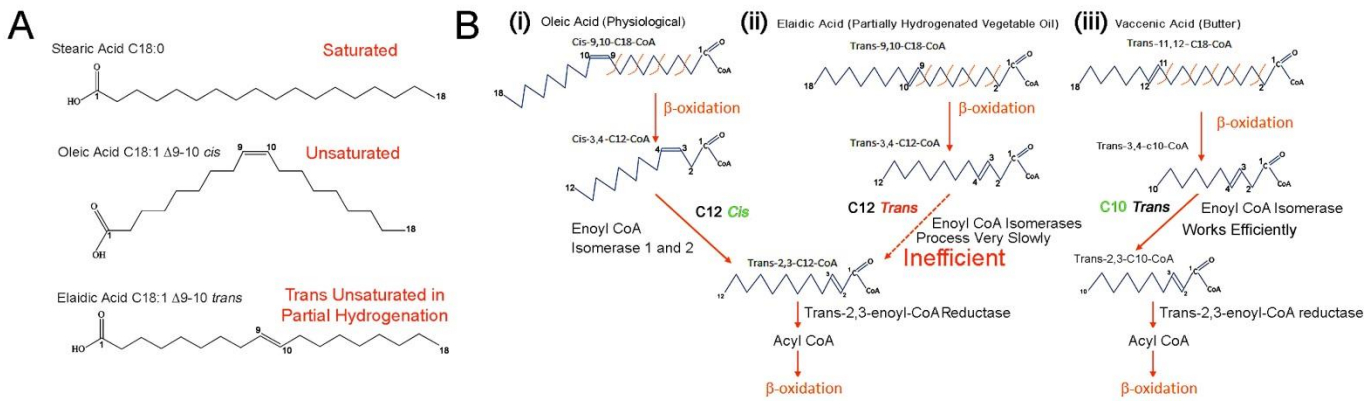

Figure 2. Unsaturated, cis and trans, and saturated fatty acids, and idiosyncratic substrate specificiites of enzymes that process double bonds during catabolism.

A. The fatty acids. Animal fats contain typically 50% saturated fatty acids, of which ~ 25% is C18:0 (stearate, illustrated) and ~ twice as much C16:0 (palmitate). Unsaturated cis fatty acids have lower melting points than their saturated counterparts, and increase membrane flexibility; oleate (middle) is a liquid. Unsaturated fatty acids with trans bonds are stiff and have higher melting points; elaidate (bottom), the major trans-fatty acid in partially hydrogenated vegetable oils, is solid at 37 °C.

B. Degradation of unsaturated fatty acids of odd, but not even, numbered double bonds requires enoyl Co-A isomerase (ECI), and ECI processes trans-bonds inefficiently. The degradation of unsaturated fatty acids at odd carbons from the terminal acid group adds to the complexity of β-oxidation. When a double bond occurs in an odd numbered position, this out of phase with trans-2,3 CoA reductase. This is adjusted by enoyl CoA reductases (two isoforms) that move the double bond from 3,4 to 2,3-trans. Fatty acids with cis double bonds at even positions are degraded similarly but by cis-2,3 CoA reductase (not shown). The enoyl CoA reductases are NADPH requiring enzymes in mitochondria.

Both cis- and trans-double bonds of odd numbered carbons are out of phase with the reductase. These fatty acids, when present in large quantities, reduce the efficiency of β-oxidation, but the effect varies idiosyncratically with the type of double bond, cis or trans, and with its position in the fatty acid. Inefficient processing of Δ9 trans C18 fatty acids by ECIs lead to accumulation of trans-C12 and trans C-14 intermediates, which are poor substrates for fatty acid β-oxidation and may inhibit β-oxidation by competing for rate limiting enzymes. Oleate at 30 μM measurably suppresses β-oxidation, but only by ~ 10% the quantitative effect of elaidate at the same concentration [33]; vaccenic acid, with a C10 rather than C12 intermediate, is much less toxic to β-oxidation than elaidate [60].

The purpose of hydrogenated oils in food is to replace saturated fat "shortening", such as in baked goods. Saturated fatty acids and trans fatty acids improve shelf-life of foods and preserve taste and textures. Hydrogenated oils have similar food characteristics to saturated fatty acids [15], and were for a long time seen as a preferable substitute for saturated fat. With metabolic study and the advent of lipid profile screening, this conclusion was reversed. In

addition to the abnormal stiffness of membranes that include artificial trans-fatty acids, the enzymes that reduce the double bonds during catabolism have idiosyncratic substrate specificities, causing a back-up of degradation intermediates, discussed below.

The prevalent saturated fatty acids from animal fats are palmitate (C16:0) and stearate (C18:0), 90% of saturated fatty acids consumed, typically two-thirds palmitate [16]. A large majority, ~80%, of tissue membrane fatty acids is palmitate [17]. There is increasing evidence asserting that both trans fatty acids and saturated fatty acids in excess (long chain in particular) have damaging effects on the health of humans, including obesity, diabetes, and heart disease [18]. Diets including of high amounts of long-chain saturated fatty acids or small amounts of trans fatty acids promote atherosclerotic lesions [16].

Not all trans-fatty acids are manufactured, and not all fats with trans double bonds are toxic. Naturally occurring trans fatty acids include an unusual conjugated fatty acid, conjugated linoleate (C18:2, Δ9 cis, 11 trans) and vaccenic acid (C18:1 Δ11 trans (or ω-7 trans, counting from the non-carboxy end of the fatty acid)). Conjugated linoleic acid and vaccenic acid are produced in ruminant cattle, occuring in meat and dairy products. These natural trans fatty acids are handled by the same catabolic machinery as any fatty acids, but they cause fewer problems than elaidic acid (Figure 2B (iii)), reflecting that the enzymes idiosyncratic activity on different chain-length substrates, and also that quantitative loads differ. These differences are experimentally confirmed *in vivo*; in pigs, comparing diets with butterfat and corn oil (2-4% natural trans fat) to partially hydrogenated soy oil (30% trans fat, mainly elaidate) showed that the butterfat was harmless while the hydrogenated soy oil caused fatty streaks in arteries and increased unsaturation of membrane fatty acids [19]. Studies of oxidation of these and other fatty acids *in vitro* are considered below.

Progressive atherosclerosis: A vicious cycle of necrosis, reactive macrophages and intimal expansion

The vascular intima amasses caseous lipid-rich cellular debris known as plaque, with the plaque surrounded by reactive macrophages, many with lipid inclusions, foam cells, surrounded by proliferating stromal cells (Figure 1B). Healthy vascular intima has no cellular debris, no reactive macrophages, and stable stromal composition. In studies of the composition of plaque, researchers found that the caseous lipid debris is a mixture of cholesterol and oxidized phospholipids, with fat-engorged macrophages, and proliferating smooth muscle cells; there are complex biochemical interactions of macrophage and smooth muscle components [20,21,22]. The caseous debris includes dead cells, macrophages and stromal cells. The normal role of the macrophage is phagocytosis and processing of cellular debris, including lipids; the completeness and speed of this process determines whether the vessel wall is restored to normal or plaque will form. Ignoring partial oxidation effects, the profile of lipids in an atherosclerotic vascular sections and in red blood cells from the same subject is essentially the same, in fatty acid chain length, saturation, and percent cholesterol (30-40%). This reflects that membranes of dead cells are the source of the un-degraded lipids. In long-standing plaque, typically some regions are converted to scars with dense collagen and mineral nodules, while regions around caseous debris remain to show active, progressive disease (Figure 1B).

Effect on atherosclerosis of trans or excessive saturated fat, and exacerbation by immune mechanisms

Once cell membrane lipids accumulate in a vascular wall, how atheroma progression can be avoided is a key question that has no satisfactory answer. Parts of the problem include diet, excess dietary saturated fat, and modified dietary lipids, particularly artificial trans fat. A complicating factor is antibody-receptor mediated inflammation, particularly vis Fcγ, which will briefly be discussed. Other major modifiers of disease are the role of exercise in maintaining healthy circulation, exacerbation of atherosclerosis by smoking, and inborn errors of lipid metabolism affecting a small part of the population. These are important, but outside of our scope.

Although lipoproteins are not unidirectional conduits, in large part low density lipoprotein (LDL) moves cholesterol from the diet or produced in the liver to peripheral tissues; high-density lipoprotein (HDL) is used by peripheral sites to transport cholesterol for central disposal or to deliver lipids and cholesterol to other organs including the adrenal gland [23] and for bone synthesis [24].

Epidemiological work shows that artificial trans fat consumption increases the risk of atherosclerosis [25]. There are many studies supporting this view; trans fatty acid consumption increases total serum cholesterol and low density lipoprotein cholesterol, and decreases high density lipoprotein-cholesterol [26]. There are also many studies showing that consuming a large portion of dietary calories as saturated fat is unhealthy. These studies point to mechanisms that differ significantly from trans-fat consumption. In a landmark study comparing directly trans fat and saturated fat in young healthy volunteers [26], the saturated fat largely increased low density lipoprotein-cholesterol, while trans fat also reduced high-density lipoprotein cholesterol. This work also showed that dietary fatty acids appear rapidly in red blood cell membranes in essentially the same proportion as in the diet consumed. Many other studies have produced consistent results [27].

Macrophages have receptors for HDL and LDL, and take up cholesterol, oxidized or native, from lipoproteins. This process involves several binding and transport proteins [28]. When macrophages are presented with more cholesterol or lipid than can be processed and exported, lipid laden "foam cells" result (Figure 1B). This transformation is fostered not only by too much lipid, but by response via antibody-mediated processes via the Fcγ receptor, particularly isoform III [29]. While the mechanisms are complex, the Fcγ isoform signaling definitely contributes to cytokine and cellular pathways that drive macrophage overload and contribute to the vicious cycle of atheroma growth. In particular, people who have immune-related diseases and develop antibodies to oxidized lipids that form complexes with LDL are associated with acceleration of atherosclerotic disease [30]. The mechanism includes induction of inflammatory cytokines and growth factors.

Mechanism-specific effects of different fatty acids on macrophage lipid processing

Artificial trans fats, particularly elaidate, are metabolic inhibitors even when elaidate comprises only a few percent of dietary fat (Figures 2, 3B). It was demonstrated 10 years ago, in rat models, that elaidate stalls β-oxidation of fatty acids [31]. There are many differences between rodents and humans, but it a similar incomplete block in elaidic acid β-oxidation occurs in humans. The intermediate that

accumulates in humans predominately is the 12 carbon trans fatty acid [32] (Figure 3B) rather than the 14 carbon intermediate in rat liver [31]. When human macrophages are exposed to elaidate, together with accumulation of intermediates from incomplete β-oxidation (Figure 3B) [32], there are profound changes in metabolism that include increases in the ATP-binding cassette sub-family G-1, ABCG1, steroyl CoA desaturase-1 (SCD), and hydroxymethylglutaryl CoA desaturase-1 (HMGCS1) [33]. ABCG1 is a key mediator of cholesterol and phospholipid transport, and regulator of cellular lipid homeostasis. ABCG1 is a strong candidate for regulation of inflammation and atherosclerosis [34]. The protein encoded by the SCD gene desaturates stearate, 18:0 [35]. It it is linked to downstream increases in doubly unsaturated, 18:2, relative to 18:1 fatty acids, 18:1/18:2 being a marker of atherosclerotic disease progression (Figure 3A) [35,36]. The HMG reductase, one step distal to this synthase, is also highly expressed in macrophages. It is the statin target, the rate-limiting cholesterol synthesis enzyme [37]. Both SCD and HMGCS1 are implicated in regulation of inflammation and in lipid-related metabolism and pathology [34,35]. How these lipid pathway proteins are induced by trans-fat exposure [33] is not established. In addition, exposure of macrophages to elaidate uniquely down-regulates zinc binding proteins and promotes a zinc importer, resulting in a two-fold increase in intracellular zinc activity [33]. This would be expected directly to potentiate pathways, including activation of NF-κB, that depend on zinc as cofactors.

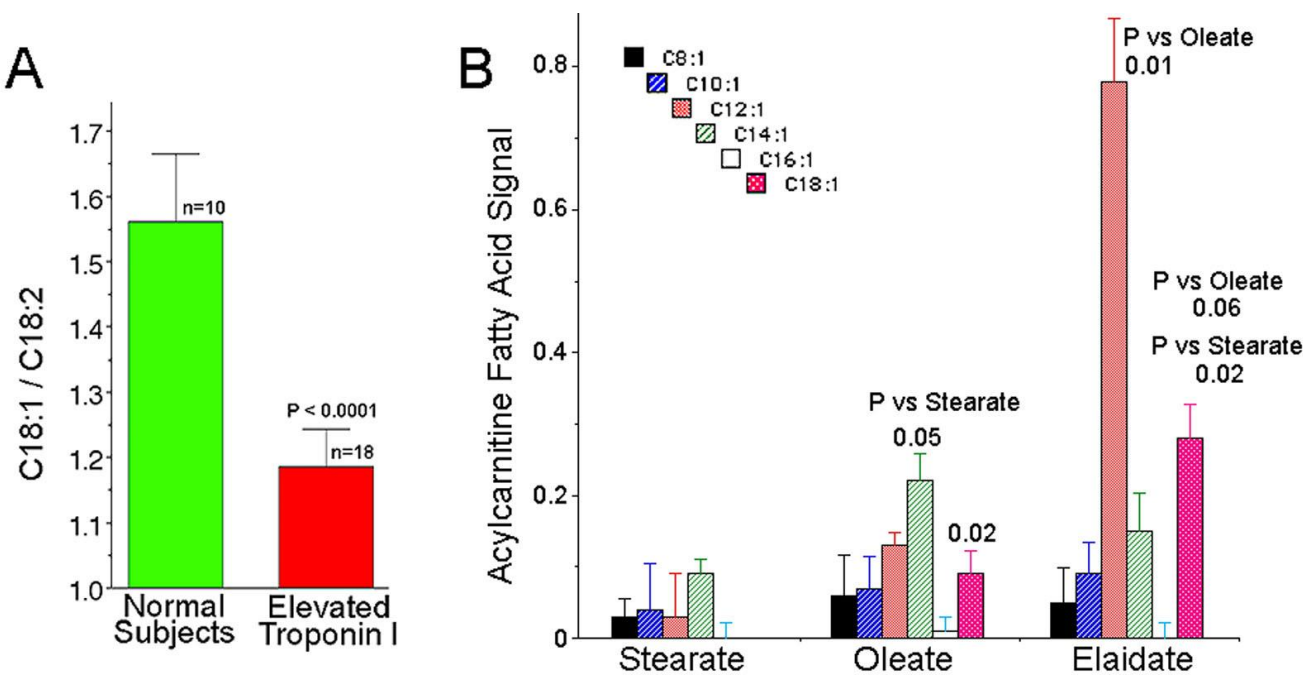

Figure 3. Key fatty acid cellular pathology: Membrane fatty acids change with atherosclerosis, and the effect of 18:1 fatty acids in macrophages on accumulation of carnitine intermediates. The graphs use data published in Sepulveda et al. [36] and Zacherl et al. [32], with permission.

A. The ratio of 18:1 to 18:2 red cell membrane fatty acids in normal and troponin-positive subjects. Analysis of human red cell membranes by GC/MS after hydrolysis and methyl esterification [36]. There is a large and significant increase in 18:2 in the troponin-positive group (right bar). This

correlates with increased stearoyl CoA desaturase in vitro in macrophages exposed to elaidate [32]. Data are mean ± SEM for n=10 (normal subjects) and n=18 (positive troponin subjects).

B. Acylcarnitine intermediates accumulating in supernatants of macrophages at five days in elaidate, oleate, or stearate, at 30 µM. Carnitine is the small-molecule carrier used in the cytoplasm and serum for free fatty acids; analysis was by direct MS/MS as described [32]. The fatty acids were added on albumin carriers, at 30 µM to cultures of human macrophages for five days, and the supernatants harvested. Note that significant increases occur with oleate (middle panel) relative to the unsaturated (stearate) control, although elaidate causes a dramatically and significantly larger quantitative effect on accumulation of C12:1 and C18:1 intermediates relative to oleate or stearate. The C18 carnitine peaks may reflect reversal of the pathway to re-synthesize C18:1. N=4, mean ± SEM.

In contrast, exposure of macrophages to large amounts of saturated fatty acids results in stimulation of cells by mechanisms including toll-like receptor 4 (TLR4), with downstream effects including production of TNF [33] and macrophage chemotactic protein-1 (MCP-1) [38], both of which contribute to the vicious cycle of proliferation and cell recruitment in atherosclerosis. There is a substantial related literature including a role of cytokines in excess lipid accumulation and apoptosis, independent of the NF-κB pathway [39,40]. The effect of saturated fatty acids is partly counteracted by ω-3 fatty acids. These effects are linked to peroxisome proliferator-activated receptor-γ (PPARγ) expression induced by saturated fatty acids [41]. It should be noted that these pathways are exacerbated by mechanisms such as Fcγ receptor mediated activation [29,30].

While the inter-relationships of the pathways are complex, the antibody-mediated pro-atherogenic effect is definitely, at least in large part, attributable to antibodies to oxidized lipids on LDL [42,43]. This association that supports the drive to reduce LDL availability (by suppressing LDL cholesterol synthesis), thus to reduce atherosclerotic progression. In saying this, it should be noted that LDL and HDL are cholesterol and lipid transporters, and both are essential, in some quantities, to normal lipid trafficking, despite the problems that occur due to increased fats in the diet, decreased exercise, as well as smoking and other factors (Figure 1A).

Thus, the available data support mechanisms promoting macrophage lipid accumulation and apoptosis due to artificial trans fats or excess saturated fatty acids which are, at least in part, independent (Figure 4). This model includes associations with unknown mechanisms, but the outcomes are consistent with clinical and experimental studies. Some studies of mechanisms including the association of stearoyl CoA desaturase with saturated fat diet have shown negative correlations [44], in contrast to the effect of trans-fatty acids [33].

Additional pathways altered by fatty acid exposure in toxic concentrations

An effect of saturated or trans fatty acids in macrophages includes acute inflammation, and although simple pathways provide important insights (Figure 4), it is important to keep in mind that the physiology is complex, that additional downstream pathways are involved, and that cells other than macrophages are affected. For example, palmitate (C16:0) or oleate (C18:1 cis) at 30 µM elicits stress responses in many different cell types including cardiac myocytes, where increased intracellular calcium at pre-

apoptotic levels occurs [45]. Cardiac myocytes regulate metabolism including of fatty acids during pathological changes including cardiac hypertrophy, with enzymes induced including HMGCoA reductase [46], highlighting the physiologically integrated nature of fatty acid response in cells other than macrophages [32,33].

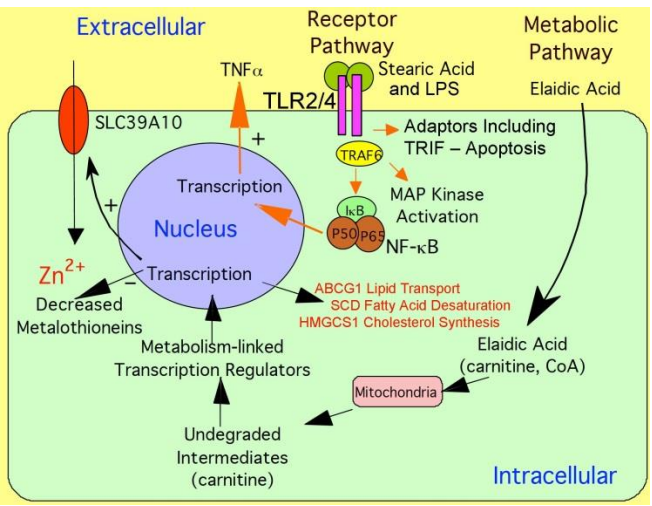

Figure 4. Trans-fatty acid and unsaturated fatty acid macrophage activation pathways.

Incomplete metabolism of the artificial trans-fatty acid elaidate [28] changes expression of many metabolic regulatory enzymes [29]. Many changes secondary to elaidate (C18:1 Δ9 trans) and its metabolites occur by undefined intermediate steps. These include increased expression of HMGCoA synthase, the rate-limiting enzyme for cholesterol synthesis, stearoyl-CoA desaturase, which unsaturates membrane fatty acids, and ABCG1, which transports phospholipids and cholesterol. Elaidate decreases synthesis of metallothioneins, reducing Zn^{+2} binding capacity, and increases expression of SLC39A10, increasing Zn^{+2} importation. Zinc homeostasis is crucial to regulation of the inflammatory response, allowing limited response while preventing cellular injury. Receptor mediated effects of fatty acids, including stearate and elaidate, or substances including lipopolysaccharide, activate TLR2/4 and related receptors. This is normally self-limited in a few hours. However, some fatty acids, particularly stearate, escape down-regulation and chronically stimulate receptor-mediated pathways with production of TNFα continuing at least to 44 hours. This correlates with maintenance of TLR4, which is downregulated in other fatty acid treatments, and maintenance of downstream signals. This mechanism may be reinforced by antibodies to oxidized LDL complexes, and thus sensitive to availability of LDL. Alternate pathways may be activated including apoptosis [53,54]. Trans-fatty acid metabolic changes and uncontrolled inflammatory signals by long-chain saturated fatty acids and immune complexes may all contribute to the accumulation of undegraded cell membrane debris in atherosclerosis, with the combined mechanisms creating more rapid and severe damage than any pathway alone.

Saturated fatty acids cause the release pro-inflammatory cytokines in serum in addition to TNF and MCP-1[33,38], including C-reactive protein, IL-6 [47] and IL-1, which implies the involvement of the liver, since the liver makes C-reactive protein and other acute phase reactants [48]. Additional serum factors, including chemokines, are reviewed elsewhere [49].

Thus, macrophages are primary, but not the sole, cellular agents responding to membrane fatty acids in catabolic pathways. In macrophages, in addition to the pathways above, response linked to TLR4 includes cyclooxygenase activation, an effect specific for the long-chain saturated fatty acids [50], such as TNF production after 44 hours incubation [33]. An underlying concept is that macrophages responding to prolonged activation

by the innate immunity-related, TLR pathway convert to "M1 macrophages". Other macrophages are "M2 macrophages". M1 macrophages mediate intense reactions to bacterial infection and to tissue damage including arterial lesions. These macrophages, in damaged tissue and with triggering of the innate immune system, activate glycolysis for anaerobic energy production [51]. Prolonged glycolysis byproducts include reactive oxygen species, a process that can be driven by increased glucose transporter activity [52].

Lipotoxic cell death

One of the difficulties in mechanisms of atheroma growth is what proportion of the effect is due to direct cell death. There definitely are high concentrations of oxidized phospholipids in contact with macrophages. The atheroma is a semiliquid mass mainly of cholesterol and partially oxidized phospholipids, around which foam cells and cell ghosts abound. With macrophages *in vitro*, long term survival is possible with 30 μM fatty acids on albumin carriers, while 100 μM fatty acids, particularly stearate, cause cell death [32,33]. *In vivo*, the situation is more complex with oxidized phospholipids interacting with LDL and antibodies [30], so it is impractical to determine toxic concentrations of components. Many modified lipids contribute to lipotoxicity acylglycerols and ceramides [53]. Ceramide is the sphingosine-fatty acid cell membrane component. It accumulates in large quantities in atherosclerotic lesions [54], indicating that processing ceramide may be important in progressive atherosclerosis. Ceramide is also a signaling molecule for apoptosis; it is important in cycles of cell death and atheroma growth [55]. It is widely regarded as important but difficult to separate physiologically from other mechanisms. The ceramide pathway amplifies the toxic effect of fatty acids via TLR4 [56].

The central role of the TLR signaling pathway for cell death by the lipopolysaccharide-saturated fatty acid pathway has been shown using macrophages null for TLR2 and the fatty acid scavenger receptor CD36 [57]. Other work has focused on TLR4, which clearly is important in foam cell production, but may be of secondary importance in cell death. Alternative work using TLR4 knockouts [58], in contrast, found that lipopolysaccharide and fatty acids caused macrophage apoptosis by a TLR4 pathway that was independent of the NF-κB signaling which mediates many other cellular effects, as discussed above. Since all of this work is supported by definitive knockout mouse models, a logical conclusion is that multiple TLR pathways, including TLR2 and TLR4, can mediate macrophage cell death with lipotoxic signals, depending on the cellular context, and that pro-apoptotic adaptor proteins [59] are involved in the macrophage cell death pathways.

Accumulation of intermediates of toxic fatty acids, and correlation with clinical disease

A distinguishing characteristic of trans fat metabolism is that intermediates reach high concentrations. The process by which fatty acids are broken down in the mitochondria, β-oxidation, is slowed during elaidate metabolism, as was demonstrated in study of rat liver mitochondria [32]. Other work showed that for vaccenic acid, C18:1 Δ11 trans (the double bond between carbons 11 and 12), where serial acetyl CoA removal creates a C10:1 intermediate, does not hinder further oxidation markedly. Whereas elaidate, C18:1 Δ9 trans (the double bond between carbons 9 and 10), where serial acetyl-CoA removal creates a C12:1 intermediate (Figure

2B), results in a significant metabolic block [60] (Figure 3B). In this case there are secondary changes that include induction of HMGCoA desaturase, the rate limiting enzyme for cholesterol synthesis. In considering this pathway, it should be noted that there are substantive differences in fatty acid oxidation between rodents and humans, making it essential that human cells are used to confirm results in rodents [61]. Further, there are large differences between central metabolism in the liver and peripheral metabolism, such as in macrophages.

Direct analysis of lipids in human atherosclerotic plaque confirm completely the importance of elaidate in atheromata [62]. In human macrophages, exposure of cells to mixed triglycerides loaded on serum, inclusion of 7% elaidate as partially hydrogenated soy oil resulted in a several fold increase in 12:1 carnitine intermediates in supernatants [32]. Analysis of cell membrane fatty acids after exposure to 30 µM elaidate resulted in increased stearoyl CoA desaturase activity with increased 18:2 membrane fatty acids, a change that mirrors that seen in patients with myocardial infarctions [36]. In human macrophages, radiolabeling at C1 or C9,10 unsaturated fatty acids showed that elaidate (C18:1 Δ9 trans) enters β-oxidation at rates at least as great as that of oleate (C18:1 Δ9 cis), but has greatly slowed rates of oxidation at the trans-bond [32]. However, this did not occur in hepatocytes, a difference attributed to greatly increased expression of enoyl CoA isomerases in the liver cells [32]. Overall, this work implicates elaidate as a metabolic inhibitor with serious consequences for lipid intermediate accumulation by mechanisms that are different from those occurring with large amounts of long chain fatty acids including stearate (C18:0). Similar changes occur in arterial smooth muscle [63].

Clinical studies, pre-dating the macrophage biochemical work, showed correlations of elaidate in red cell membranes and atherosclerotic progression, or implicated the burden of membrane trans-fats as a risk factor for atherosclerotic disease [64,65,66,67]. Interpreting this work is complicated by considerations including total dietary fat and the proportion of saturated and unsaturated fat, including ω3 and ω6 fatty acids. It is clear that elaidate should be avoided, without significant contrary opinions, and that diets including very large quantities of long chain fatty acids are unhealthy, in keeping with the studies of peripheral fat metabolism.

Fatty acid and metabolic pathology are linked, in part, by specialized membrane structures

Additional data on cell signaling that is important to place fatty acid receptor response in perspective relates to specialized cell membrane regions where saturated acyl chains of sphingolipids and cholesterol align to form "lipid rafts" with sizes on the order of 100-200 µm, which accumulate TLRs [68]. These complexes, at least in part, promote activation of the TLRs as dimers when reactive oxygen species are present [69], which supports for the synergistic effects of reactive oxygen species (ROS) and fatty acid/ lipopolysaccharide complexes. The mechanism involved, largely specific for saturated fatty acids (Figure 4) can include activation of TLR heterodimers including components other than TLR2/4 [70]. There is strong evidence that the lipid raft is as an integrator of response of metabolic and inflammatory diseases with TLR-ligand signaling [71].

Outward transport of lipid degradation products from macrophages

It is important to consider that in health macrophages may remove excess lipid, in major part as cholesterol, thus interrupting the process of atheroma growth. In brief, the key apolipoproteins involved in reverse transport of cholesterol are apolipoprotein E, largely found in intermediate density lipoproteins, which is produced by macrophages and liver cells [72] and apolipoprotein A1, the key component of high density lipoproteins "good cholesterol" carriers, overexpression of which has been shown to disperse lipid from foam cells *in vivo* [73]. Macrophage ATP-binding cassette proteins ABCA1 and ABCG1 are key receptors involved in cholesterol export by macrophages [74]. The role of β-oxidation and cholesterol synthesis, blocking of β-oxidation by elaidate, and secondary cell responses including increased synthesis of unsaturated long chain fatty acids [32,33], discussed above.

Additional metabolic changes in macrophages that may affect lipid handling

As macrophages fight to control atherosclerotic damage and convert to foam cells, inflammatory signals downstream of TLRs are prominent in the serum. An unexpected component that may promote synergistic pathology from saturated and toxic trans-fatty acids is an increase in macrophages of zinc activity. This was discovered when genome-wide screening of mRNAs in macrophages exposed to elaidate relative to oleate for two days showed a dramatic decrease in expression of several zinc-chelating metallothioneine proteins together with increased expression of a specific zinc importer, SLC39A10 [75], which correlate with an increase in elaidate exposed macrophages of zinc activity, "free zinc", by a factor of two [33]. Intracellular zinc physiology is complex, and most work has focused on low serum and intracellular zinc as requirements for normal cell signaling including by the NF-κB mechanism [76]. However, high intracellular zinc can promote apoptosis [77] by a mechanism involving Bax activation [78]; in macrophages, this might promote cell death and atherosclerosis. In contrast, in macrophages high concentrations of zinc produce inflammatory cytokines, possibly by enhancing NF-κB activation [79]. However, TNF production was not increased in macrophages with increased intracellular zinc exposed to 30 μM elaidate for two days [33], and it is possible that changes in intracellular zinc might reflect, also, cellular defense mechanisms against fatty acid toxicity.

Peripheral macrophage-related effects of pharmacological Inhibitors of LDLR, HMGCoA reductase, and mitochondrial fatty-acid uptake

Notwithstanding our hopeful outlook on preventative measures to reduce the incidence of progressive atherosclerosis, diet and exercise are frequently inadequate or not practical to control the problem. A new class of LDL cholesterol-lowering drugs is in phase III testing in humans is based on inhibiting PCSK9, the proprotein convertase subtilisin/kexin type 9. PCSK9 binds an LDLR EGF-like-repeat domain, the effect being to prevent LDLR from recycling to the cell surface, targeting its degradation. In the liver this has the effect of lowering LDL cholesterol production by greatly reducing LDLR-dependent cholesterol transport [80]. In cell models of atherosclerosis, secreted PCSK9 has been shown to reduce LDLR in macrophages, reducing processing of oxidized lipids, and hence limiting inflammation and cell death [81]. Statins, HMGCoA synthase inhibitors,

are usually regarded as a means to reduce cholesterol production for LDL loading. In addition, they play specific roles in peripheral macrophages. Statins reduce proliferation of macrophages [81], and reduce ABCG1-mediated cholesterol efflux [82]. Additionally, statins suppress Fcγ-antibody-mediated phagocytosis [83], which may lessen the effect of antibodies to oxidized phospholipids, discussed above. A promising additional tool to disrupt the vicious cycle of plaque formation is to limit β-oxidation by limiting the supply of carnitine. This new approach might intervene before macrophage overloading can occur. In a mouse model it shows promising effects on atherosclerosis progression [85].

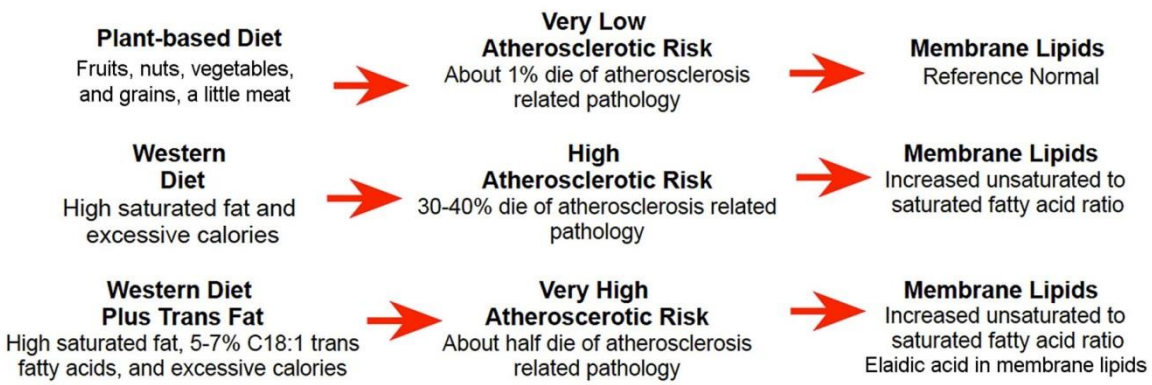

Figure 5. Dietary fats, cell membranes, and atherosclerosis risk.

Traditional diets, typically fruits and vegetables with limited meat, contain high proportions of poly-unsaturated linolenic and α-linoleic acids, low saturated fatty acids, and no artificial trans fatty acids. This diet, with normal calorie intake and physical activity, does not support atherosclerosis. Diets with excessive saturated fat increase LDL cholesterol and mediate lipid toxicity by complex mechanisms that include inflammatory growth factors and antibodies to oxidized LDL. Diets with the artificial trans fatty acid elaidate accelerate atherosclerosis by mechanisms that are at least in part different, but complementary. Both saturated fat and trans fat mechanisms are associated with higher risk of atherosclerotic disease. The estimates shown are the authors', and include components from other risk factors than diet alone.

Conclusion

Large amounts of saturated fat, or relatively modest amounts of the trans-fatty acid elaidate contribute to the development and progression of atheromata. Independently, inborn errors of metabolism contribute to atherosclerosis. The effects of excessive overall dietary fat, smoking, and infrequent exercise, are the common associations. The adverse macrophage cellular metabolic effects of C16 and C18 fatty acids largely are mediated by TLR pathways, while effects of C18:1 Δ9 trans, elaidate, appear at least in large part to reflect inability of the macrophage to degrade this fatty acid at a normal rate. Development of atherosclerosis overall reflects the inability of macrophage degradation of fatty acids, cholesterol synthesis, and back-transport to keep up with accumulation of cell membrane debris. This pathology is complicated by inflammatory cytokine production by atheroma-associated macrophages, by response to oxidized phospholipids by mechanisms including Fcγ activation, and by trans-fat metabolite associated changes in macrophage protein expression. A

conservative approach to the problem (Figure 5) includes encouraging a diet with modest amounts of animal fat and other saturated fat, elimination of modified fats containing elaidate, and use of metabolic inhibitors to control atherosclerosis progression when conservative approaches are inadequate.

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Prognostic impact of progesterone receptor expression in HER2-negative Luminal B breast cancer

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Abstract

Aim: The new classification of breast cancer is based on microarray studies. Within the estrogen receptor (ER) positive breast carcinoma subtype further subgroups could be identified. In the present study, we analyzed the Her2 negative, highly proliferative subgroup (Luminal B1-like, LUMB1) with emphasis on their clinicopathological characteristics and progesterone receptor (PR) expression.

Patients and methods: Our retrospective study concerned the period between 2000 and 2010. 158 patients were selected with ER positive, Her2 negative, Ki67>15% breast cancer. The pathological and clinical data were collected and analyzed. Age, tumor grade and stage, ER, PR, Her2 and Ki67 expression were recorded. The clinicopathological variables were correlated to PR expression.

Results: The mean age of the patients was 57.5 (28-75) years. The ratio of patients younger than 40, was 8.86%. Shorter metastasis-free survival was observed in this young age group (P=0.044). The majority of our cases belonged to the pT1-pT2 stages (41.28% and 44.95%, respectively) whereas pT3 and T4 stage was detected in 5.50% and 8.25% of the cases, respectively. Almost half of the cases had no axillary lymph node metastasis (pN0: 48.91%), 1-3 lymph node metastases were detected in 38.04% (pN1), 4-10 metastatic lymph nodes were identified in 9.78% (pN2) and pN3 stage was found in 3.26% of the cases. Most commonly the tumors were either grade 2 or 3 (44.16% and 45%, respectively). The median value of Ki67 labeling index was 30%. Disease progression was detected in 36.19% of the patients. According to PR expression, a tendency to better prognosis (i.e. longer disease free- and overall survival) was detected in

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cases showing >10% PR positivity. However, no difference was found regarding tumor size, axillary stage, grade and age when comparing lower and higher PR expressing tumors.

Conclusions: LUMB1 breast carcinomas are typically grade 2 and grade 3, the Ki67 labeling index is often 30% or higher. Distant metastases occur in more than one third of the cases. Within this subgroup, those cases with low PR expression represent a poor prognostic cohort. These findings require further investigations in larger number of LUMB1 breast cancer cases.

Introduction

One of the reasons behind the heterogeneity of breast cancer is the phenotypical reflection of their different gene expression profiles. Cancers with different expression profiles show different clinical and prognostic features. Since the advent of microarray based classifications, various molecular classes of breast cancer have been described. The first and most robust molecular classification was published in 2000, when Perou and his group published their work on cDNA microarray studies of human breast cancers and described five major subtypes [24]. Hormone receptor positive breast cancers are treated with endocrine therapy. Although, in the majority of patients the likelihood of recurrence and disease related death decreased considerably due to administration of endocrine therapy, a number of patients are not responding or develop resistence to the treatment with time and therefore have a poorer prognosis. [1, 2]. It was, thus, a logical conclusion that there are subclasses within the group of ER positive breast cancers. Sorlie and co-workers [3] classified the ER-positive breast cancers into Luminal A, B and C subgroups. Expression of ERα was highest in Luminal A cancers. Expression of ER regulated genes were lower in Luminal B és C subgroups. Most recently, the St. Gallen International Consensus Conference [4-8] suggested the following definitions for subclassifying breast cancers based on estrogen (ER), progesterone receptor (PR), Her2 and Ki67 protein expression. Luminal A-like (LUMA): ER-positive and PR-positive, low Ki67 index, Her2-negative, "recurrence risk" low, Her2-negative Luminal B-like (LUMB1): ER-positive and Her2-negative, and at least one of the followings: high Ki67, PR-negative/low, "recurrence risk" high; Her2-positive Luminal B-like (LUMB2): ER-positive and Her2-positive, any PR, any Ki67, Her2-positive (non Luminal): ER-negative, PR-negative, Her2-positive, Triple-negative: ER-negative, PR-negative, Her2-negative.

The prognostic and possible predictive role of low PR expression has been studied more recently. Within the luminal group of breast cancers, low expression of PR characterizes a more agressive, less endocrine therapy sensitive subgroup: lower ER levels, higher proliferation rate, larger tumor size, more positive axillary lymph nodes, aneuploid DNA content and increased expression of epidermal growth factor receptor were found [9]. Cancello and co-workers [10] investigated the role of PR in relation with recurrence in Lumial B breast cancers, considered a less favourable prognostic group. They divided the Luminal B breast cancers into 4 subgroups based on Her2 and PR expression: ER+/PR+/Her2-; ER+/PR-/Her2-; ER+/PR-/Her2+ és ER+/PR+/Her2+. They concluded that in both the Her2 positive and Her2 negative groups, low expression or lack of PR is related to shorter metastasis-free- and overall survival. Considering the difficulties in treating Luminal B breast cancers, many ongoing research aim at identifying newer targets for therapy. In our present study, we investigated LUMB1 breast carcinoma cases from the point of view of PR expression, among others. We

compared the level of PR expression with clinico-pathological and follow-up data.

Patients and methods

ER-positive, Her2-negative breast carcinoma cases with Ki67 index ≥15% diagnosed between the period 2000-2010 were selected and the clinico-pathological data were collected. Follow-up data of the patients were retrieved from the University's database (MedSolution), the pathological data were collected from the files of the 2nd Department of Pathology following approval by the Institutional Review Board (SE-IKEB 77/2007). Overall survival data were provided by the Central Office for Administrative and Electronic Public Services. In our study we considered age of the patients, grade, TNM stage, ER, PR, Her2 and Ki67 expression of the tumors. Age groups were created according to patient's age at the time of the primary diagnosis of breast cancer. In order to elucidate the suspected prognostic significance of age at the primary diagnosis, we analyzed distant metastasis-free survival data at 35, 40 and 45 age thresholds. Tumor grade was defined according to the Nottingham grading system [11], TNM stage was recorded according to the 7th Edition of the American Joint Committee on Cancer and the International Union for Cancer Control (AJCC-UICC) manual [12]. We calculated the distant metastasis-free survival (DMFS) as the period in months elapsed between the diagnosis of the primary tumor and the occurance of the first distant metastasis. Overall survival (OS) was also calculated in months: time elapsed between the diagnosis of the primary tumor to the time of disease related death. In some cases, in the cohort not all of the clinical or pathology data were available, but we didn't exclude these cases from the statistical analyses upon this fact had no interference with the result of the calculation. Details of the immunohistochemical reactions for ER, PgR, Her2 and Ki67 are summarized in Table 1.

Table 1. Antibodies, dilutions and providers used in the study

Antigen	*Provider*	*Clone*	*Dilution*
ER	Novocastra	6F11	1:200
PgR	Novocastra	312	1:200
HER2	Novocastra	CB11	1:150
Ki67	DAKO	MIB1	1:100

Hormone receptor positivity was recorded if >1% of the tumor cells showed positive nuclear reaction, in line with the ASCO/CAP guideline [25]. Concerning PR status we divided our cases into „PR low" and „PR high" subgroups. Since only scattered recent literature data are available regarding the prognostic meaning of low PR expression in breast cancer, we tested our cases at 5, 10 and 20% limits of PR expression. Ki67 labeling index was estimated by eye-balling on a representative tumor slide. Any intensity of positive reaction was considered and the percentage of positive tumor cells was recorded. Her2 status was defined according to the ASCO/CAP recommendations valid at the time of the period under investigation [13, 26]. The study group comprised of 158 patients.

For survival analysis Kaplan-Meier method was used; log-rank statistics was used to characterize the differences between prognostic groups. Categorical data were analysed using Chi-square test and Fischer exact test. Results were regarded statistically significant at $p<0.05$. Statistica 11.0 software was used for each analysis (StatSoft, Tulsa, OK, USA).

Results

Clinical and pathological characteristics of the cases are shown in Table 2.

Table 2. Clinical and pathological characteristics of LUMB1 cancers included in this study

Parameters	N	%
T1	45	41,28%
T2	49	44,95%
T3	6	5,50%
T4	9	8,25%
No data	29	
N0	45	48,91%
N1	35	38,04%
N2	9	9,78%
N3	3	3,26%
No data	46	
Grade 1	13	10,83%
Grade 2	53	44,16%
Grade 3	54	45%
No data	18	41,28%
Treatment:		
Endocrine-and/or Chemotherapy	105	84%
Neoadjuvant therapy	20	16%
No data	33	
Age		
<45	26	16,45%
>45	132	83,54%
Total	158	

Mean age of the patient at the time of the primary diagnosis was 57.51 (range: 25-75). Twenty six of 158 patients (16.45%) were <45, 14/158 (8.86%) were <40 and 5/158 (3.16%) were <35 years. Age, as an adverse prognostic factor was significant regarding DMFS in patients <40 when compared to DMFS of patients >40 (p=0.044) (Figure 1).

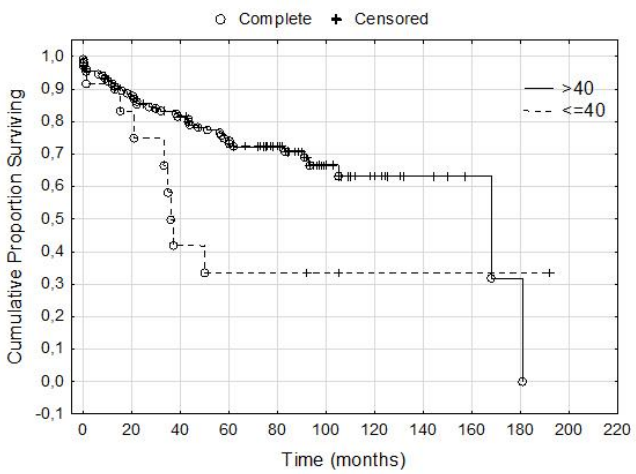

Figure 1. Distant metastasis-free survival according to patients' age (p=0.044).

We had treatment data from 125/158 patients. Neoadjuvant oncological treatment was used in 20/125 cases (16%). In these patients, ER, PR, Ki67, Her2 immunohistochemistry results of the core biopsies were considered, while pT and pN stage was known from the surgical resection specimens. These cases were not considered further in the statistical analyses: we analysed data of 138 patients.

Tumor size was known in 109/138 cases. The majority of the known cases belonged to the pT1 and pT2 stage category (41.28% and 44.95% respectively), 5.50% were pT3. pT4 cases occured in 8.25%. Regarding axillary lymph nodes we could identify 92/138 cases with known regional lymph node status. Negative axillary lymph nodes were present in 48.91% of the cases, 1-3 metastatic lymph nodes were recorded in 38.04%, more than 10 metastatic lymph nodes were present in 9.78% and pN3 was diagnosed in

3.26% of the cases. Tumor grade was known in 120 cases. The majority of the cancers were of grade 2 or grade 3 (53/120, 44.16% and 54/120, 45%, respectively). The median Ki67 labeling index was 30%.

In the adjuvant treated patient group we had follow up data in 105/138 patients. During the period examined 38/105 (36.19%) patients developed distant metastasis, among them 21 patients (55.27%) presented with dissemination to multiple organs. More than half of the solitary metastases (52.94%) localized to the skeletal system.

Relationship between PR expression and prognostic factors

Clinical and pathological variables were analyzed in relation to PR expression. Since there are few data related to the exact role of PR positivity and its extent, we performed analyses with different cutpoints at 5, 10 and 20% PR positivity. Very low PR expression (0-5%) was detected in 53 cases (38.40%), 59 cases showed 0-10% PR positive tumor cells, and 69 cases (50%) had 0-20% PR positivity.

When considering all cases, including the 20 core biopsies, the number of cases in the above three PR expression categories was the following: 63 cases (39.87%) 0-5% PR positive tumor cells, 71 cases (44.93%) 0-10% PR positive tumor cells, 73 cases (46.20%) 0-20% PR positive tumor cells. In our cohort, the cutpoint of PR positivity that divided the patients into a better and a worse prognosis group was at 10%, but only at the level of tendency: cancers that have >10% PR positive cell population show better DMFS (p=0.07) (Figure 2). Regarding age groups, pT categories, tumor grade categories and pN categories, lower and higher (0-10% vs. >10%) PR expressing tumors didn't show any specific distribution. (p=0.661, p=0.061, p=0.541, p=0.403, respectively).

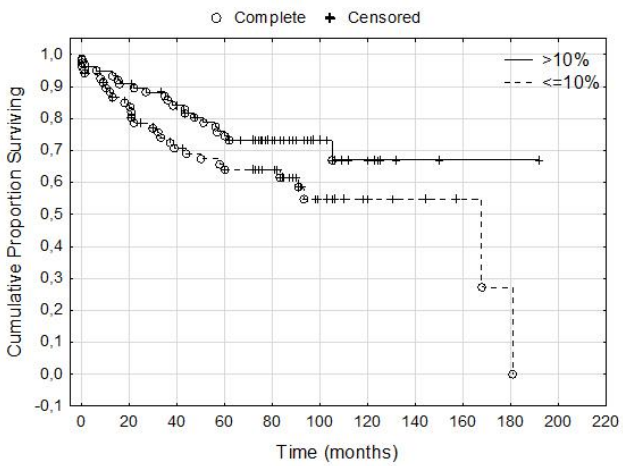

Figure 2. Distant metastasis-free survival according to PR expression at 10% positivity cut-off (p=0.07). A tendency of shorter survival could be detected in low-PR expressing tumors.

Discussion

According to our results, grade 2 and grade 3 cases predominate within the group of Luminal B1 breast cancers. Similar to these results, Park et al. found that among Luminal B subtype breast cancers, higher grade tumors are more common [14]. Many recent studies aim at clarifying the exact prognostic significance of Ki67 labeling index, although the cut-off value has been a subject of discussion: some authors use 10%, 14%, or 20%, yet others suggest the use of the mean or median percentage of Ki67 positive tumor cells as threshold [15]. Despite the reported inter-observer differences in evaluating Ki67 labeling index, it is obvious that higher Ki67 labeling index predicts poorer prognosis. Azambuja and co-workers [16] in a large meta-analysis using 68 studies confirmed the higher risk of relapse and shorter survival in cases with higher Ki67 labeling index. Cheang and co-workers [15] identified 13,25% Ki67 labeling

index as cut-off value for the differentiation of Luminal A from Luminal B breast cancers. The median value of Ki67 labeling index in ER-positive, Her2-negative breast cancer cases was 14% in a multicenter study performed by Cserni and his group [17], but no subtyping of luminal cases was performed. In our present study, we used the 15% cut-off as it was also shown in a study by Cserni and co-workers that approximation of the value of Ki67 labeling index to the closest 0 or 5 is an adequate approach [17].

In our study group of Luminal B1 breast cancer cases the median value of Ki67 labeling index was 30%.

In our patient cohort, more than half (52.94%) of the solitary metastases occured in the skeletal system. Kennecke and co-workers [18] by studying the metastatic pattern of the different molecular subtypes found that in Luminal B breast cancers the most common distant metastatic site was in bones. More recently even more studies are focused on the significance of PR expression that show increased activity between ER and Her2 pathways behind low PR expression. The interaction between ER and Her2 pathways probably downregulates PR [19]. However, there is no consensus in the literature regarding the cut point of relevant PR expression level. Prat and co-workers, based on statistical calculations, defined 20% PR positivity as relevant [20]. In our study, we found that at 10% cut-off of PR expression, a tendency of longer DMFS occured in those patients having >10% PR positivity in the primary tumor. Bardou and co-workers reported longer 5-year survival in ER+/PR+ tumors (82,5%) than in ER+/PR- tumors (73,8%). The same tendency was observed regarding OS. In multivariate analysis, the relative risk of relapse and disease related death was lower in ER+/PR+ and ER+/PR- tumors than in ER-/PgR- tumors. However, they only could show a tendency and not statistically significant difference between ER+/PR+ and ER+/PR- tumors. [21].

Ciriello and co-workers in 2013 [22], by investigating a large cohort of Luminal A breast cancers by means of genetic profiling found at least 4 prognostically different subtypes within the Luminal A subgroup. The prognosis of LUMB1 cancers is regarded poor by many studies' results, therefore any feature that could help to identify a better and a poorer prognostic subgroup could be clinically relevant (9, 15). According to one study, mutation of the TP53 gene is an independent adverse prognostic factor in Luminal B type breast cancer [23]. According to the results of our present study LUMB1 breast cancers show poor prognostic features (the vast majority belongs to grade 2 or 3 category, one third develops distant metastasis, the median Ki67 labeling index is high). These adverse features must encourage further research of this group of breast cancer. LUMB1 breast cancers expressing low levels of PR probably represent a poor prognostic group, that could be proved in much larger cohorts of breast cancer patients. Identification of poorer prognosis in hormone receptor positive breast carcinoma cases may allow more effective oncological approaches.

Conclusions

LUMB1 breast carcinomas are mainly grade 2 and grade 3, the Ki67 labeling index is often 30% or higher. Distant metastases occur in more than one third of the cases. Within this subgroup of breast carcinoma, those cases with low PR expression represent a poorer prognostic cohort presenting with shorter distant metastasis free survival. Further investigations are necessary in larger number of LUMB1 breast cancer cases to elucidate the exact role of PR lacking or low level expression in this subgroup of breast cancer.

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