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New look inside human breast ducts with Raman imaging. Raman candidates as diagnostic markers for breast cancer prognosis: mammaglobin, palmitic acid and sphingomyelin.

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Abstract
Looking inside the human body fascinated mankind for thousands of years. Current diagnostic and therapy methods are often limited by inadequate sensitivity, specificity and spatial resolution. Raman imaging may bring revolution in monitoring of disease and treatment. The main advantage of Raman imaging is that it gives spatial information about various chemical constituents in defined cellular organelles in contrast to conventional methods (liquid chromatography/mass spectrometry, NMR, HPLC) that rely on bulk or fractionated analyses of extracted components. We demonstrated how Raman imaging can drive the progress on breast cancer just unimaginable a few years ago. We looked inside human breast ducts answering fundamental questions about location and distribution of various biochemical components inside the lumen, epithelial cells of the duct and the stroma around the
duct during cancer development. We have identified Raman candidates as diagnostic markers for breast cancer prognosis: carotenoids, mammaglobin, palmitic acid and sphingomyelin as key molecular targets in ductal breast cancer in situ, and propose the molecular mechanisms linking oncogenes with lipid programming.

**Keywords**: breast cancer markers, Raman imaging, metabolism of lipids.

**Introduction**

The role of cancer treatment is a central target in the medical research. The slowing down in the treatment of cancer observed since the late 1990s and the harmful whole-body secondary effects of chemo/radio therapies emphasize the urgent need to develop different approaches to cancer diagnostics, treatment and monitoring responses to therapy. Current diagnostic and imaging methods are often limited by inadequate sensitivity, specificity and spatial resolution [1]. Cancer diagnosis requires better screening of early stages of pathology and monitoring patient responses to treatment. Better diagnostics requires understanding mechanisms of metabolic alterations for the synthesis of proteins, nucleic acids and lipids related to the development of cancer. Raman imaging may bring revolution in understanding of cancer biology [2-7]. The approach presented in the paper is ideally suited to explore cancer phenotype by monitoring the biochemistry and morphology of cells necessary for survival, proliferation, differentiation, cell death, and expression of many specific functions. There is increasing evidence on important role of altered lipid biosynthesis in cancer metabolism and tumor development [8-16]. This paper focuses on the emerging understanding of the role of lipids and lipogenic pathway regulation in breast cancer. We will provide unique insight into vibrational
features of cells/tissues and intracellular processes occurring in cancerous human breast tissue. We will demonstrate that Raman imaging opens a new era as the label-free, minimally-invasive molecular detection tool allowing us to monitor cancer development, because it gives spatial information on molecular compositions of biomolecules in defined cellular compartments of cells without destroying the tissue in contrast to current diagnostic and imaging methods [17].

**Experimental methods**

All procedures were conducted under a protocol approved by the institutional Bioethical Committee at the Medical University of Lodz, Poland (RNN/45/14/KE/11/03/2014). We have studied ductal in situ carcinoma (G1, G2) and normal human breast tissue. All tissue samples were snap frozen and stored at -80 °C. One part of each type was cryosectioned with a microtome (Microm HM 550, Sermed) into 6 µm-thick sections for Raman analysis. The thin cryosectioned tissue samples (without staining and paraffin embedding) have been examined by Raman imaging. After spectroscopic analysis these sections were stained and histologically examined. The adjacent part of the tissue was paraffin embedded and also cut into 6 µm-thick sections for conventional histological analysis. Raman spectra and images were obtained with an alpha 300 RA (WITec, Ulm, Germany) model equipped with an Olympus microscope coupled via the fiber of a 50 µm core diameter with an UHTS (Ultra High Throughput Spectrometer) spectrometer and a CCD Camera Andor Newton DU970N-UVB-353 operating in standard mode with 1600x200 pixels at -60 °C with full vertical binning. The incident laser beam (SHG of the Nd:YAG laser (532 nm)) of alpha 300 RA was focused on the sample through a 40x dry objective (Nikon, objective type CFI Plan Fluor C ELWD DIC-M, numerical aperture (NA) of 0.60, and
a 3.6–2.8 mm working distance) to the spot of 1 µm. The average laser excitation power was 40 mW, with an integration time of 0.2 s. Rayleigh scattered light was removed using an edge filter. The samples were irradiated by a laser at 532 nm. Spectra were collected at one acquisition per pixel and a 600 lines/mm diffraction grating. Prior to the basis analysis, each spectrum was processed to remove cosmic rays, increase the signal-to-noise ratio via spectral smoothing (Savitzky-Golay method [18]), and correct for biological autofluorescence. The large number of spectra collected in this study required the use of automated removal method for all of the spectra, which is critical to remove sources of variability arising from autofluorescence and substrate contamination. After baseline removal, the dominant remaining source of distinction between spectra is the intensity of the Raman features, arising from the variable amount of biological material within the sample. Data acquisition and processing was performed using WITec Project 2.10. The 2D array images of tens of thousands of individual Raman spectra were evaluated by the basis analysis method. In this method, each measured spectrum of the 2D spectral array is compared to basis spectra using a least squares fit. Such basis spectra are created as the average spectra from different areas in the sample. The weight factor at each point is represented as a 2D image of the corresponding color and mixed coloring component. The color code of Raman maps were based on the integrated Raman intensities in specific regions (sum option in the filter manager in the Witec project Plus 2.10). Using a lookup table, bright colors indicate the highest intensities, whereas dark colors indicate the lowest intensities of the chosen region [19].

Results and discussion
Raman images allow to look inside the biochemical composition of cancerous cells in the lumen, the duct and the extracellular matrix surrounding the duct. To understand information that is provided from Raman vibrational spectra of the normal and cancerous breast tissues, we need to associate these features with the breast morphology. Briefly, the normal organization of ducts in the human breast demonstrates lumen surrounded by epithelial cells aligned in a polar manner so their apical side faces the lumen. These cells are surrounded by the basement membrane. Fibroblasts align the basement membrane and this entire structure is surrounded by the stroma, which is predominantly, but not exclusively, composed of connective tissue and adipose tissue. Schematic basic structure of epithelial tissue, stromal and adipocyte cells around the normal breast duct is presented in Fig 1 and compared with a microscopy image of the normal human duct that will be analyzed in the paper.

Most cancers, including breast cancer, begin in the epithelium cells. During cancer development in the duct (ductal carcinoma in situ (DCIS)), the normal polar organization of the luminal epithelial cells is lost, as these cells proliferate. The epithelial cells completely fill the lumen. In invasive, or infiltrating, cancer, the epithelial cells migrate and invade through the basement membrane and into the surrounding stroma. We will analyze the normal and cancerous ducts (DCIS) by Raman imaging to learn if this method is capable of identifying morphological and biochemical alteration in the human breast duct in cancer development.

Figure 2 shows the Raman image of the normal breast duct (P123) compared with the H&E-stained histological image, and microscopy image, as well as the characteristic vibrational Raman spectra for different areas of the breast tissue.
Figure 3 shows the Raman image of the cancerous breast duct (DCIS, G1 and G2, P115) compared with the H&E-stained histological image, microscopy image and the characteristic vibrational spectra for different areas of the breast tissue. According to the histopathological assessment, the pathology in Fig. 3 represents the early stage of cancer development in situ ductal carcinoma, where, in contrast to infiltrating carcinoma, the epithelial cells do not migrate yet through the basement membrane into the surrounding stroma.

One can see that in both cases there is an almost perfect match between the morphological features obtained from histological images, microscopy images and Raman images.

The Raman image presented in Fig. 2A reveals all morphological features of the normal duct. One can see (white-blue line around the black duct) that normal epithelia cells are lined along the intact basement membrane and do not proliferate inside the lumen and outside through the basement membrane.

The Raman spectra presented in Fig. 2D show the biochemical composition of the substructures. The epithelial cells (white-blue colour in Figs. 2A and 2D) are dominated by monounsaturated oleic acid derivatives composed of glycercyl trioleate and carotenoids. Indeed, the characteristic Raman vibrations of carotenoids with resonance peaks at 1158 and 1528 cm\(^{-1}\) are clearly visible in Fig 2D (top blue spectrum). The peaks at 2852, 2878, 2888, 2931, 2954, 3009 correspond to the vibrations of monounsaturated oleic acid [12,20,21]

The lumen is empty (black colour) with no Raman spectra indicating that there are no epithelial/mesenchymal cells inside the normal duct. In the Raman image, the basement membrane is not resolvable from the epithelial cells due to its thinness and
indistinctive Raman features. The stroma (pink, yellow, green colours in Fig. 2A) is dominated by lipid/protein profiles typical for fibroblasts and the connective tissue.

In contrast to the normal duct in Fig. 2, the cancerous duct presented in Fig. 3 shows that the normal organization of the epithelial cells is lost and the lumen is filled with the cancerous cells. It would be extremely interesting to learn what chemical substances are inside the lumen during cancer development, because monitoring biochemical alterations would drive the progress on mechanisms of cancer to limits just unimaginable a few years ago. For our best knowledge this a first report about the chemical structure of concerous and normal human breast ducts.

To identify distribution of various biochemical components in the defined cellular compartments of the breast tissue we have created Raman images of pure components. To obtain Raman images of the distribution of pure components we used the basis analysis method, where each recorded spectrum at a given pixel is fitted as a linear combination of basis spectra (chosen as pure components) by using partial least square method. Details are described in the section Experimental methods.

The Raman images that have been created allowed to look inside the biochemical composition of cancerous cells in the lumen, the duct and the extracellular matrix surrounding the duct.

Fig. 4 shows distribution of pure components inside and around the normal human duct (P 123) obtained from the basis analysis with eight components as a reference set: arachidonic acid, palmitic acid, oleic acid, sphingomyelin, collagen, mammaglobin-A, β-carotene and stearic acid.

One can see from Fig 4 that the normal breast duct is empty inside (dark colour of the glass) while the epithelial cells and the basement membrane of the duct are dominated
by oleic acid with a significant amount of β-carotene, mammaglobin-A, and palmitic acid.

Fig. 5 shows distribution of pure components inside and around the cancerous human duct (P 115) obtained from the basis analysis with eight components as a reference set: arachidonic acid, palmitic acid, oleic acid, sphingomyelin, collagen, mammaglobin-A, β-carotene, and stearic acid.

A detailed inspection into Figures 4 and 5 demonstrates that the protein/lipid profiles inside the duct, the luminal epithelial cells, the basement membrane, and the extracellular matrix are markedly different for the normal duct and the cancerous duct. First, one can see from Fig. 5 and 3A that sphingomyelin, saturated acids (palmitic acid) and mammaglobin dominate the biochemical composition inside the cancerous duct in contrast to the empty lumen of the normal duct (Fig. 4 and 2A). Second, the comparison between Fig. 5 and 4 clearly shows that the composition of the epithelial cells of the duct also changes with the cancer development. In contrast to the normal duct dominated by oleic acid and large amount of β-carotene, the cancerous duct is dominated by collagen with no presence of carotenoids. This result was extremely surprising for us, because our results published so far identified β-carotene in the adipose cells of the stroma around the normal duct [10]. Now, for the first time carotenoids have been identified in the epithelial cells of the duct. Thus, carotenoids, which play diverse functions including antioxidant properties to defend against ROS (reactive oxygen species), may be very useful for the biodiagnostics of cancer.

The extracellular matrix around the cancerous duct is dominated by a network consisting of complementary regions of sphingomyelin and collagen.
To summarize, the picture that emerges from our results is as follow: the most intriguing are the cells inside the cancerous duct, the lumen of the cancerous duct is filled with the cells overexpressing palmitic acid, sphingomyelin, and mammaglobin in comparison with the normal duct, which is empty. Therefore, Raman spectra and images provide evidence that the disordered epithelial cells that lost the polar organization and proliferate into the lumen of the duct are composed almost exclusively of saturated fatty acids/triglycerides with a dominant component of sphingolipids and proteins such as mammaglobin. It is worth emphasising a large amount of mammaglobin inside the cancerous lumen monitored by the Raman imaging in this paper. Mammaglobin has already been suggested to be important protein biomarker that is overexpressed in reverse transcription-PCR assays and in human peripheral blood using surface enhanced Raman scattering nanoparticles [22-29].

Moreover, our results clearly demonstrate that the composition of the epithelial cells surrounding the lumen of the cancerous duct changes dramatically in comparison with the normal duct. First, in contrast to the normal duct that are abundant with carotenoids (characteristic Raman resonance peaks at 1158 and 1528 cm\(^{-1}\)), there is a complete depletion of these compounds in the cancerous duct. Second, the cancerous duct contains significantly smaller amount of monounsaturated fatty acids and triglycerides than the epithelial cells of the normal duct dominated by oleic acid, but significantly increased level of saturated lipids (palmitic acid) and sphingomyelin. The Raman profile of the extracellular matrix around the cancerous duct is dominated by the contribution from the CH\(_3\) vibrations (2949 cm\(^{-1}\)) suggesting enhanced protein production. This finding is supported by the low frequency region – where the Raman shift from 1444 cm\(^{-1}\) (fatty acids) to 1454 cm\(^{-1}\) (proteins), 1654 cm\(^{-1}\) (fatty acids ) to 1665-1670 cm\(^{-1}\) (proteins) is clearly visible from the spectra in Fig. 3D. The
extracellular matrix around the cancerous duct contains also significantly higher level of sphingomyelin than the matrix around the normal duct (green colours in Fig. 4 and 5).

Fig. 6 shows the comparison between the biochemical Raman spectrum of the cancerous tissue of the duct with the Raman spectra of sphingomyelin, mammaglobin, and collagen. Almost perfect agreement between the Raman spectra in Fig. 6 of the breast tissue in the specific regions with the pure components demonstrates that the stroma is dominated by these components.

One can see that in contrast to the normal duct collagen is the most abundant component of the cancerous breast tissue in the region of stroma, where large, eosinophilic collagen fibres (orange colour in Fig. 5) are formed around the duct. Perfect agreement between the Raman spectra in Fig. 6B of the breast tissue around the cancerous duct with the pure collagen demonstrates that the stroma is dominated by this component. However, one can see from Figures 5 and 6C that in the network of collagen represented by the orange colours the areas represented by yellow and green structures are incorporated in the stroma.

Fig. 6 shows also the comparison between the Raman profile of yellow-green structures in the region of the extracellular matrix of stroma in Figures 5 and 6C with the Raman spectra of sphingomyelin and mammaglobin. Almost perfect agreement indicates that the second important component in the network around the duct is sphingomyelin. Incorporation of particular lipids, such as sphingomyelin into lipid membranes is known to stiffen a membrane. Such membranes can be described as "a glass state, i.e., rigid but without crystalline order" [30].
In the view of the results presented so far one can propose discrete sequence of biochemical events that lead to malignant transformation of the epithelial cells in the normal breast duct. First, the upregulation of glycolysis [31-36] leads to enhanced synthesis of fatty acids de novo [13-15,37-40]. De novo fatty acids synthesis changes biochemical composition of the epithelium membrane. Our results show evidently that the ratio of saturated to monounsaturated fatty acids increases in the cancerous ductal epithelium cells. Taking into account inhomogeneous distribution of saturated and unsaturated acids we have calculated the ratios for the characteristic regions of stroma, duct, and lumen. For the noncancerous tissue the ratios are the following: a) stroma the average value is equal to 0.13 and standard deviation is equal to 0.01, b) duct the average value is equal to 0.08 and standard deviation is equal to 0.02, c) lumen is empty. For the cancerous tissue the ratios are the following: a) stroma the average value is equal to 0.27 and standard deviation is equal to 0.03, b) duct the average value is equal to 0.85 and standard deviation is equal to 0.3, c) lumen the average value is equal to 0.86 and standard deviation is equal to: 0.3 (more information in supplementary materials).

The proper ratio of saturated to monounsaturated fatty acids contributes to a proper membrane fluidity in the normal duct. Additionally, incorporation of particular lipids, such as sphingomyelin into synthetic lipid membranes is known to stiffen a membrane [30,41]. Our results clearly demonstrate that the sphingomyelin level in the normal epithelial cells of the duct are higher than that in the cancerous cells of the duct, in contrast to the region of the lumen and the extracellular matrix, where sphingomyelin dominates inside and around the cancerous duct. The abnormal proportion between saturated and unsaturated fatty acids in the membranes of the cancerous duct effects fluidity of the membranes leading to distortions and deformations [30,41,42].
The mechanisms how the epithelial cells lose their tight polar organization as well as cell-cell adhesion and gain migratory and invasive properties is still unknown, but they must be related to their lipid composition. The lipid composition of the normal cellular membranes is regulated to maintain a proper membrane fluidity. We have found that cancer cells lipids are enriched with saturated fatty acids, which are almost exclusively identify inside the duct. It indicates that the cells containing higher ratio of saturated fatty acid have been expelled from the duct. It is interesting to notice that the saturated fatty acids are present not only inside the duct but also outside in the stroma surrounding the duct (Fig. 3A and Fig. 5). It may suggest that the process of cancerous cells proliferation into the extracellular matrix is proceeded by releasing a large amount of saturated fatty acids in pre-invasive stages of cancer progression. Saturated fatty acids are less flexible, but they are not prone to oxidation [25]. Activation of an antioxidant signature is necessary to compensate depletion of anti-ROS carotenoids in the cancerous duct that are present in abundant amount in the normal duct (see Raman spectra in Fig. 4 and Fig. 5) [2,5-8]. The balance between the positive (detoxification) and negative (membrane deformation) effects of fatty acid saturation of the epithelial cells prevents cancer development. However, the abnormal proportions between saturated and unsaturated fatty acids affect fluidity of the membrane leading to distortions and deformations. The altered epithelium membrane fluidity can affect lipid packing, viscosity and mechanical tension. Viscosity of the membrane can affect the rotation and diffusion of proteins and other bio-molecules within the membrane, thereby affecting the functions of these molecules, including cadherin-associated proteins involved in regulation and coordination of cell-cell adhesion. For example, epithelial cells express high levels of E-cadherin responsible for tight junctions, gap junctions, and adherents junctions between epithelial cells that are closely connected to
each other. Alteration the functions of these molecules results in loss of cell adhesion, constriction and extrusion of altered apical epithelium cells into the lumen [43,44].

A key enzyme involved in the process of desaturation is the membrane-bound stearoyl-CoA desaturase (SCD) which is the rate-limiting enzyme in the cellular synthesis of monounsaturated fatty acids from saturated fatty acids [14,40]. The starting point involved in fatty acids (FA) and cholesterol biosynthesis is cytosolic acetyl-CoA with repeated coupling of acetyl groups that produces a basic 16-carbon saturated FA: palmitic acid. Further elongation and desaturation generates the diverse spectrum of saturated, monounsaturated (e.g. oleic acid) and polyunsaturated fatty acids synthesized by mammalian cells. The monounsaturated FAs are generated by SCD, which induces a double bond at the Δ9 position of the palmitic and stearic acid.

Our results indicate that cancer development must be related to decreased level of stearoyl-CoA-1 expression that is responsible for diminished level of monounsaturated FAs in the cancerous duct in comparison with the normal duct. The decreased level of stearoyl-CoA-desaturase reduces the synthesis of de novo unsaturated fatty acids and inhibits β-catenin signalling in breast cancer cells reducing proliferation but activating an antioxidant signature, because saturated fatty acids are less prone to oxidation than unsaturated fatty acids. It has been suggested that controlling of lipid synthesis and uptake occurs by upregulating SREBP, a master transcriptional factor, via the oncogenic signalling PI3K/Akt/mTOR pathway [14,35].

The mechanism of de novo unsaturated fatty acids synthesis is inhibited by depletion of sterol regulatory element-binding transcription factor 1 (SREBP1) and 2 (SREBP2), resulting in mitochondrial dysfunction, the accumulation of reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress in immortalised human epithelial cells.[44]
In this critical situation for cancer cells survival, sphingolipid synthesis regulates lipid homeostasis. Sphingolipid synthesis is required for the activation of SREBP [45,46]. Sphingolipid de novo synthesis increases in rapidly dividing cells like cancer cells. The effects of different long chain sphingoid bases are derived from sphingomyelin or glucosylceramides.

Our results provide clear evidence that sphingolipids play an important role in cancer development. The biochemical analysis of Raman spectra presented in this paper allowed to identifying sphingolipids as important components of the de novo lipid synthesis. We have found enhanced production of sphingomyelin in the lumen of the cancerous duct and around the duct from in comparison to the normal duct (see Fig. 4 and Fig. 5).

Lack of monounsaturated fatty acids in the epithelial cells of the duct transformed into cancerous, in the lumen and in the extracellular matrix suggests that the palmitic acid does not transform into the oleic acid but choose the other pathway presented in Fig. 7. The initial step in de novo sphingolipid synthesis is the condensation of L-serine and palmitoyl-CoA on the endoplasmic reticulum and is catalyzed by serine-palmitoyl transferase. Ceramide is the central product of sphingolipid synthesis. Most of the ceramides are further metabolized to lipids including sphingosine, ceramide-1-phosphate, glucosylceramides and sphingomyelin. Contrary to previous assumptions that ceramides and other sphingolipids found in cell membrane were purely structural elements, they can participate in a variety of cellular signalling: examples include regulating differentiation, proliferation, and programmed cell death (PCD) of cells [35,39].

Our results show that the lipid synthesis de novo in cancer transformed epithelial cells occurs via sphingolipid synthesis.
Decreased SREBP decreases triacylglycerol synthesis needed for adaptations to survive stress associated with tumour growth and to satisfy the anabolic demands of proliferation is compensated by sphingolipid synthesis, where palmitic acid forms complex palmitoyl-CoA and produces sphingolipids (Fig. 7). Decreased SREBP results in decreasing stearoyl-CoA desaturase that in turn inhibits β–catenin signalling in breast cancer cells.

**Conclusions**

We looked inside human breast ducts by Raman imaging answering fundamental questions about location and distribution of various biochemical components inside the lumen, epithelial cells of the duct and the stroma around the duct during cancer development. This kind of spatial information by monitoring various chemical constituents in defined cellular compartments of the breast tissue has been reported for the first time. The analysis based on Raman imaging demonstrates that the vibrational signatures by Raman microspectroscopy can accurately predict which breast tissue has normal biochemistry and morphology and clearly distinguishes cancer pathology.

Our results clearly demonstrate that the composition of the epithelial cells surrounding the lumen of the cancerous duct changes dramatically in comparison with the normal duct.

In contrast to the normal duct, which is abundant in carotenoids, there is a complete depletion of these compounds in the cancerous duct. The cancerous duct contains significantly smaller amount of monounsaturated fatty acids and triglycerides than the normal duct dominated by oleic acid, but significantly increased level of saturated lipids (palmitic acid) and sphingomyelin. The biochemical analysis obtained from the Raman spectra allowed to identifying sphingolipids as the components of the de novo
lipid synthesis essential in cancer development. We have identified Raman candidates as diagnostic markers for breast cancer prognosis: carotenoids, mammaglobin, palmitic acid and sphingomyelin as key molecular targets in ductal breast cancer in situ, and propose the molecular mechanisms linking cancer development with lipid programming. The results presented in the paper suggest that metabolic alterations in tumors extends beyond the Warburg effect [21] and the pathways related to the production of fatty acids activated via multiple lipogenic enzymes as well as antioxidants (carotenoids) forming the defense against ROS are equally important.

Acknowledgements

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References


**Fig. 1.** Schematic basic structure of epithelial tissue, stromal and adipocyte cells around the normal duct (A) and microscopy image of normal human breast duct (P123) (B).

**Fig. 2** Raman image of normal breast duct (P123) (A) compared with the microscopy image (B), H&E-stained histological image (the shape of the lumen has been a bit distorted after H&E-staining) (C), and the characteristic vibrational spectra for different areas of the tissue (D), colours of the lines correspond to the colours in the Raman imaging (A).

**Fig. 3** Raman image of the cancerous breast duct (ductal carcinoma in situ, G1 and G2, P115) (A) compared with the microscopy image (B), H&E-stained histological image (C), and the characteristic vibrational spectra for different areas of the tissue (D), the colours of the lines correspond to the colours of the areas in Raman image (A).

**Fig. 4** Raman images of distribution of pure components inside and around the normal human duct (P123) obtained from the basis analysis with 8 components as a reference set: arachidonic acid, palmitic acid, oleic acid, sphingomyelin, collagen, mammaglobin-A, β-carotene and stearic acid and Raman spectra of the pure components used for the basis analysis.

**Fig. 5** Raman images of distribution of pure components inside and around the cancerous human duct (P 115) obtained from the basis analysis with 8 components as a reference set: arachidonic acid, palmitic acid, oleic acid, sphingomyelin, collagen,
mammaglobin-A, β-carotene and stearic acid and Raman spectra of the pure components used for the basis analysis.

**Fig. 6** Comparison of the Raman spectra in the spectral region 700-3200 cm⁻¹ of the cancerous breast duct (ductal carcinoma in situ, G1, P115 represented by green and yellow colours in the Raman image (C) with sphingomyelin (black), mammaglobin (blue) (A), and orange colours in the Raman image (C) with collagen (black) (B).

**Fig. 7** Metabolism of lipids.
HIGHLIGHTS

- Raman candidates as diagnostic markers for breast cancer prognosis: carotenoids, mammaglobin, palmitic acid and sphingomyelin as key molecular targets in ductal breast cancer in situ have been identified.

- The composition of the epithelial cells surrounding the lumen of the cancerous duct changes dramatically in comparison with the normal duct.

- In contrast to the normal duct there is a complete depletion of carotenoids in the cancerous duct.

- The cancerous duct contains significantly smaller amount of monounsaturated fatty acids and triglycerides than the normal duct dominated by oleic acid, but significantly increased level of saturated lipids (palmitic acid) and sphingomyelin is observed.