Study of a supplement and a genetic test for lymphedema management

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Abstract. Malformations in the lymphatic vasculature, injury, surgery, trauma or toxic damage may lead to swelling of the limbs caused by inefficient lymphatic uptake and flow (lymphedema). Lymphedema can be congenital or acquired. Primary lymphedema is rare and caused by mutations in single genes, secondary lymphedema is more common and caused by a trauma in association with a genetic predisposition. We decided to develop a genetic test that would determine the genetic predisposition to the onset of lymphedema and to predict the course of the disease by analyzing polymorphisms involved in leukotriene B4 (LTB4) synthetic pathway, and variants involved in the onset of secondary lymphedema. There are not many compounds available for the treatment of the negative effects of lymph accumulation, we therefore designed a food supplement based on the hydroxytyrosol, that has anti-oxidant, anti-bacterial and anti-inflammatory activities. (www.actabiomedica.it)

Key words: lymphedema, hydroxytyrosol, leukotriene B4, food supplement

Introduction

Malformations in the lymphatic vasculature, injury, surgery, trauma or toxic damage may lead to lymphedema, a swelling of the limbs caused by inefficient lymphatic uptake and flow (1). Lymphedema is classified as primary when congenital and secondary when acquired (2). Usually, primary lymphedema is determined by a mutation in a single gene, whereas secondary lymphedema is associated with a trauma, but genetic predisposition may be involved.

Since secondary lymphedema is quite common in the population (lymphedema affects 200 million people worldwide and around 3 million people in the United States), we decided to develop a genetic test that would determine the genetic predisposition to the onset of lymphedema and to predict the course of the disease by analyzing polymorphisms involved in leukotriene B4 (LTB4) synthetic pathway, LTB4 is the major mediator of inflammation (3) (Table 1). It promotes lymphatic endothelial cells growth at low concentrations, but causes lymphatic endothelial cell injury at high concentrations (4). We also included variants involved in the onset of secondary lymphedema, in order to predict the predisposition to lymphedema after trauma, surgery or infection.

Since there are not so many compounds available for the treatment of the negative effects of lymph accumulation, we also designed a food supplement based on the hydroxytyrosol (HT), extracted from olive trees. HT is a compound with anti-oxidant, anti-bacterial and anti-inflammatory properties. We previously reviewed in a previous work the promising properties of HT in the treatment of the effect of lymph accumulation by blocking leukotriene B4 generation (5).
Table 1. Polymorphisms that can predispose to secondary lymphedema and/or modulated the clinical course of lymphedema

| Gene      | Gene function (GeneCards)                                                                 | rs ID, alleles                          | Association                                                                 | Ref. |
|-----------|------------------------------------------------------------------------------------------|-----------------------------------------|-----------------------------------------------------------------------------|------|
| **LTB4R2** | Chemotaxis mediation of granulocytes and macrophages                                      | rs1950504, A/G                          | Enhanced ROS generation/AKT phosphorylation under LTB4 low-dose conditions. | 6    |
|           |                                                                                         | rs4987105, C/T                          | Enhanced cell motility under low-dose ligand stimulation                    |      |
| **ALOX5** | Catalyzes the first step in leukotriene biosynthesis and has a role in inflammatory processes | rs59439148, del(GGGG/GG)2/3/2/del(G)/C/dup(G)/C/| Determination of the expression levels of ALOX5. Two copies of a minor variant of the ALOX5 | 8    |
|           |                                                                                         | dup(GGGG/GG)2/3                          | GG genotype is associated with modest increase in body mass index.          |      |
|           |                                                                                         | rs4769874, G/A                          | A-allele potentiates the expression of ALOX5 and/or the function of FLAP    | 9    |
| **LTA4H** | Epoxide hydrolase that catalyzes the final step in the biosynthesis of leukotriene B4      | rs17525495, C/T                         | T allele associated with lower levels of LTA4H. The presence of the T allele | 10   |
|           |                                                                                         | rs1978331, C/T                          | significantly increased the proportion of Crohn’s patients requiring         |      |
|           |                                                                                         |                                          | glucocorticoids                                                            |      |
| **MMP2**  | Metalloproteinase involved in remodeling of the vasculature, angiogenesis, tissue repair, inflammation | rs1030868, G/A                          | A allele, higher risk of secondary lymphedema                               | 11   |
|           |                                                                                         | rs2241145, G/C                          | C, higher risk of secondary lymphedema                                      | 11   |
| **CEACAM1**| Cell-cell adhesion molecule with roles in angiogenesis, modulation of immune response. Inflammasome activity reduction. Blood vessel remodeling through endothelial cell differentiation and migration. Vascular permeability regulation | rs8110904, G/A                          | A, higher risk of secondary lymphedema                                      | 11   |
|           |                                                                                         | rs8111171, G/T                          | T, higher risk of secondary lymphedema                                      | 11   |
| **FOXC2** | Transcriptional activator. Involved in the mesenchymal tissue formation                   | rs199772307, G/A                        | AA genotype more frequent in lymphatic filariasis patients, influence on the severity of lymphedema | 12   |
|           |                                                                                         | rs34221221, A/G                          | G allele, increased expression                                              | 13   |
| Gene      | Gene function (GeneCards)                                                                 | rs ID, alleles          | Association                                                                                                                                                                                                 | Ref. |
|-----------|------------------------------------------------------------------------------------------|-------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| **TNF**   | Cellular responses to cytokines and stress. It regulates the immunological response to infections | rs1800629, G/A          | High percentage of TNFa homozygotes GG in patients with dermato-lymphangio-adenitis in obstructive lymphedema of lower limbs                                                                                |      |
| **TLR2**  | Key role in the innate immune system. It is expressed in macrophages, B lymphocytes, mast cells | rs121917864, C/T        | Low percentage of CT heterozygotes and TT homozygotes in patients with dermato-lymphangio-adenitis in obstructive lymphedema of lower limbs                                                                       | 14   |
| **TLR4**  | Key role in the innate immune system. It is expressed in macrophages, B lymphocytes, mast cells | rs4986791, C/T          | High percentage of CT heterozygotes and TT homozygotes in patients with dermato-lymphangio-adenitis in obstructive lymphedema of lower limbs                                                                       |      |
| **VEGFA** | Growth factor active in angiogenesis, vasculogenesis and endothelial cell growth. Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels | rs699947, C/A           | -2578C>A Lower or higher expression                                                                                                                  | 15   |
|           |                                                                                         | -1154G>A                | A allele, lower expression                                                                                                                          | 15   |
|           |                                                                                         | -460C>T                 | T allele, increased promoter activity                                                                                                                  | 15   |
|           |                                                                                         | +405G>C                 | C allele, lower or higher expression                                                                                                                  | 15   |
|           |                                                                                         | +936C>T                 | T allele, lower expression                                                                                                                          | 15   |
| **HGF**   | Role in angiogenesis, tumorigenesis, tissue regeneration                                  | rs5745652, C/T          | CC genotype is associated with lower serum HGF levels                                                                                              | 16   |
|           |                                                                                         | rs2074725, C/A          | CA and AA genotypes are associated with lower serum HGF levels                                                                                     | 16   |
| **CYP26B1** | Involved in the metabolism of retinoic acid                                             | rs2241057, A/G          | G allele associated with higher levels of retinoic acid catabolism and reduced retinoid availability                                                  | 17   |
| **PROX1** | Critical role in neurogenesis, development of the heart, eye lens, liver, pancreas and lymphatic system | rs340874, T/C          | CC genotype is associated with higher nonesterified fatty acids levels, lower glucose oxidation, higher accumulation of visceral fat | 18   |
| **RORC**  | Essential for lymphoid organogenesis                                                     | rs11801866, A/T         | T allele, higher risk of secondary lymphedema, might affect transcription factor binding sites                                                        | 19   |
|           |                                                                                         | rs12128071, G/A         | It might affect transcription factor binding sites                                                                                                 | 19   |
|           |                                                                                         | rs12045886, A/G         | G allele, secondary lymphedema predisposition after breast cancer surgery                                                                        | 19   |

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Table 1 (continued). Polymorphisms that can predispose to secondary lymphedema and/or modulated the clinical course of lymphedema

| Gene       | Gene function (GeneCards)                                                                 | rs ID, alleles       | Association                                                                 | Ref. |
|------------|------------------------------------------------------------------------------------------|---------------------|-----------------------------------------------------------------------------|------|
| LCP2       | T-cell antigen receptor mediated signaling                                                | rs572192, C/T       | T allele, secondary lymphedema predisposition after breast cancer surgery    | 20   |
|            |                                                                                         | rs6866733, C/G,T    | T allele, secondary lymphedema predisposition after breast cancer surgery    | 20   |
|            |                                                                                         | rs315721, A/G       | AG and GG genotype are associated with a 50% decrease in the odds of        | 20   |
|            |                                                                                         |                     | developing secondary lymphedema                                             |      |
| NRP2       | It binds interacts with vascular endothelial growth factor (VEGF)                         | rs849530, G/T       | TT and TG genotype are associated with 62% decrease in the odds of          | 20   |
|            |                                                                                         | rs849563, T/A,G     | G allele, secondary lymphedema predisposition after breast cancer surgery   | 20   |
|            |                                                                                         | rs16837641, G/A,C,T | A allele, secondary lymphedema predisposition after breast cancer surgery   | 20   |
| SYK        | Regulation of innate and adaptive immunity, vascular development. Plays a crucial role    | rs158689, T/A       | AA and AT genotypes are associated with 3.43-fold increase in the odds of    | 20   |
|            | in the innate immune response to fungal, bacterial and viral pathogens. Activates the   |                     | developing secondary lymphedema                                             |      |
|            | inflammasome and NF-kappa-B-mediated transcription of chemokines and cytokines in        |                     |                                                                             |      |
|            | presence of pathogens. It is involved in vascular development where it may regulate      |                     |                                                                             |      |
|            | blood and lymphatic vascular separation                                                  |                     |                                                                             |      |
| VCAM1      | Pathophysiologic role in immune responses and leukocyte emigration to sites of           | rs3176861, C/T      | CT and TT genotypes are associated with a 45.0% decrease in the odds of      | 20   |
|            | inflammation                                                                             |                     | developing secondary lymphedema                                             |      |
| miR499     | miR-499 gene targets are involved in remodeling and inflammation-related signaling       | rs3746444, A/C,G    | Associated with inflammatory arthritis susceptibility. The A allele         | 21   |
|            | pathways; including fibrogenic and immune-modulator pathways                              |                     | creates an altered target gene set. Disruption of 667 genes of the miR- |      |
|            |                                                                                         |                     | 499a targets and creation of new 763 genes                                  |      |
| CDKN2B-AS1 | Interacts with polycomb repressive complex-1 and -2, leading to epigenetic silencing     | rs1333048, A/C,G    | AA genotype is associated with elevated C-reactive protein plasma levels    | 22   |
| CALCRL     | Receptor for calcitonin-gene-related peptide together with RAMP1 and receptor for       | rs185008808, C/T    | Common colds susceptibility                                                  | 23   |
|            | adrenomedullin together with RAMP3 and RAMP2                                             |                     | Waist-hip ratio                                                             | 23   |
|            |                                                                                         | rs61739909, A/G     |                                                                            | 23   |
|            |                                                                                         | rs10177093, G/C,T   |                                                                            | 23   |
| VEGFC      | Growth factor active in angiogenesis of veins and lymphatics, endothelial cell growth,   | rs2333496, C/T      | T allele, waist-hip ratio increase                                           | 24   |
|            | stimulating their proliferation, migration, permeability of blood vessels               |                     |                                                                            |      |
|            |                                                                                         | rs7664413, C/T      | T allele, secondary lymphedema predisposition after breast cancer surgery    | 20   |

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Table 1 (continued). Polymorphisms that can predispose to secondary lymphedema and/or modulated the clinical course of lymphedema

| Gene   | Gene function (GeneCards)                                                                 | rs ID, alleles     | Association                                                                 | Ref. |
|--------|------------------------------------------------------------------------------------------|-------------------|------------------------------------------------------------------------------|------|
| EPHB4  | Regulation of cell adhesion and migration, angiogenesis, blood vessel remodeling, permeability | rs314313, T/A/C/G  | G allele, Crohn’s disease/ulcerative colitis/inflammatory bowel disease susceptibility | 25   |
|        |                                                                                         | rs314311, T/G      | T allele, low density lipoprotein cholesterol levels decrease                 |      |
| PLA2G4A| Hydrolyzes arachidonyl phospholipids for releasing arachidonic acid. Implicated in the initiation of the inflammatory response. | rs1079069, G/T    | G allele, Crohn’s disease/Inflammatory bowel disease                          | 26   |
| IL1R1  | Mediator involved in cytokine-induced immune and inflammatory responses.                 | rs949963, C/T      | A allele, secondary lymphedema predisposition after breast cancer surgery       | 27   |
| IL4    | B-cell activation, DNA synthesis stimulation, expression induction of MHC-II on resting B-cells, secretion enhancement and cell surface expression of IgE, IgG, expression regulation CD23 IgE receptor on lymphocytes and monocytes, expression induction of IL31RA in macrophages, autophagy stimulation in dendritic cells | rs2227284, T/C,G  | A allele, secondary lymphedema predisposition after breast cancer surgery       | 27   |
| IL6    | Inducer of the acute phase response, final differentiation of B cells into Ig-secreting cells, lymphocyte and monocyte differentiation, generation of Th17 cells, myokine, increase the breakdown of fats, improve insulin resistance | rs2066992, G/A,C,T | T allele, secondary lymphedema predisposition after breast cancer surgery       | 27   |
| IL10   | Cytokine produced by monocytes, lymphocytes, pleiotropic effects in immunoregulation, inflammation, down-regulation of Th1 cytokines expression, MHC-II, stimulator of macrophages, B cell survival enhancement, proliferation, antibody production | rs1518111, T/C    | T allele, secondary lymphedema predisposition after breast cancer surgery       | 27   |
|        |                                                                                         | rs1518110, A/C,G,T | A allele, secondary lymphedema predisposition after breast cancer surgery       | 27   |
| NFkB2  | Pleiotropic transcription factor ubiquitously expressed involved in inflammation, immunity, differentiation, cell growth, tumorigenesis, apoptosis | rs1056890, G/A,C   | A allele, secondary lymphedema predisposition after breast cancer surgery       | 27   |
| ANGPT2 | Endothelial cell migration and proliferation                                              | rs6990020, C/A,T   | C allele, secondary lymphedema predisposition after breast cancer surgery       | 20   |
| SOX17  | Embryonic vascular development, postnatal angiogenesis                                    | rs12541742, C/G,T  | T allele, secondary lymphedema predisposition after breast cancer surgery       | 20   |

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Inflammation in lymphedema

The fluid accumulation typical of lymphedema stimulates the activation of the inflammatory response. This inflammation modifies the extracellular matrix that further decreases lymphatic function (29). Patients with lymphedema are characterized by the upregulation of pro-inflammatory genes (e.g. TNF and IL1). In response to these factors, the dendritic cells synthesize digestive enzymes that allow the passage of dendritic cells through the extracellular matrix into the lymphatic vessels (30). However, in presence of lymphatic injury, dendritic cells concentrate in the site where lymph accumulates. Therefore, they produce additional pro-inflammatory factors that make the inflammation chronic (31). Another typical characteristic of lymphedema is fibrosis. This fibrotic evolution is determined by the synthesis of pro-fibrotic cytokines by Th2 cells, such as IL-4, IL-13 and TGF-β1. These cytokines affect the survival, proliferation and migration of lymphatic endothelial cells (32).

Leukotriene B4 synthesis, function, and its inhibition by hydroxytyrosol

Leukotrienes are derived from the oxidation of arachidonic acid catalyzed by an enzyme called 5-li-
poxxygenase (5-LO). This step leads to the formation of the conjugated triene epoxide LTA4. LTA4 is then released by 5-LO and is converted into leukotriene B4 (LTB4) by the enzyme LTA4 hydrolase (LTA4H) (33). LTB4 exerts its biological activity after binding G-protein coupled receptors designated LTB4R and LTB4R2 (34). LTB4 is produced by activated neutrophils and macrophages and has the ability to recruit and activate immune cells. LTB4 at lower concentrations stimulates neutrophil chemotaxis, adherence and migration to venule walls, whereas at higher concentrations stimulates neutrophil lysosomal enzyme release, generation of superoxide radicals, and production of IL-8 and LTB4 (35). Elevated concentrations of LTB4 have been found in secretions in a wide variety of inflammatory conditions including cystic fibrosis, asthma, respiratory distress syndrome, rheumatoid arthritis, inflammatory bowel disease and lymphedema. Excessive neutrophil recruitment and activation by LTB4 may cause tissue damage thereby contributing to the pathological features and progression of lymphedema (36). Interestingly, it was previously shown that the antagonism of leukotriene B4 synthesis or binding to its receptors is able to improve lymphedema in vitro in human lymphatic endothelial cells and in vivo in mouse model (4).

In humans, HT is able to inhibit the 5-lipoxygenase enzyme activity, thereby blocking leukotriene B4 generation (37). Furthermore, 5-LO is a non-heme iron dioxygenase and HT is able to bind the 5-LO iron ions reducing them to a catalytically inactive ferrous form (38).

Conclusions

Lymphedema is a common disorder with a multifactorial origin. In the recent years, it is becoming more and more clear that genetics play an important role in the pathogenesis and progression of this disorder. Therefore, we think that analyzing polymorphisms that predispose to onset of lymphedema or that could modulate the progression of the disease would be of extreme importance to gain insights into the individual genetic background. This could also be exploited to plan a personalized treatment and management of lymphedema. Additionally, the use of food supplement based on the natural phenol, HT, may help in the treatment of the negative effects of lymph accumulation as we previously reviewed (5).

Conflict of interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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