EUROPEAN SCIENCE FOUNDATION
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Exploratory Workshop Scheme

Standing Committee for the European Medical Research Councils (EMRC)

ESF Exploratory Workshop on

Molecular Signalling in Cardiovascular and Oncological Diseases: Similar and Shared Pathways

Pisa (Italy), 13-15 July 2008

Convened by:

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Executive Summary

The Exploratory Workshop “Molecular signaling in cardiovascular and oncological diseases: similar and shared pathways” derived from the intention to put together researchers in cardiovascular diseases and oncology, molecular biology and information technology to evaluate similar and shared pathways and molecular signals during disease development. The definition of similarities and differences at the molecular level appears important for multiple reasons: 1) to investigate the multifactorial role in cell to cell communication, 2) to understand the pathophysiological mechanisms of diseases, 3) to prospect possible therapeutical interventions.

This extensive collaboration strategy in multidisciplinary fields can offer a new background platform for the interdisciplinary research, by inducing also a constructive brainstorm among basic research investigators and clinicians and reducing the excessive fragmentation in medicine.

The idea of the workshop originated in the environment of the Clinical Physiology Institute of the Italian Research Council (IFC-CNR), where the peculiarity of interdisciplinarity was mandatory from its foundation in 1968. At the beginning, the fields of activities have been mainly cardiopulmonary and metabolic diseases. The main features are: a multidisciplinary scientific/technological approach, based on the coexistence of medical, engineering, physics, informatics, mathematics, chemistry and biology research units; a comprehensive approach to clinical research, made possible by the presence, in the same environment, of hospital units, laboratories of animal research and epidemiological research groups. For these reasons IFC-CNR research has an integrated and multidisciplinary approach open to new fields of interaction. Three particular conditions solicited the idea to put together and explore cardiovascular and oncological research. The Positron Electron Tomography Laboratory started years ago studies in the field of cardiac microcirculation and ischemic myocardial metabolism and later on it became a powerful tool in oncological diagnostic procedures. Secondly, many patients who received chemotherapies developed heart failure symptoms and the IFC-CNR cardiologists had to afford the complexity of therapeutic decisions and to interact with the oncology specialists. Finally, in the biological laboratory the cellular and sub-cellular research favoured the study of shared phenomena and the analysis of similarities and differences in cellular signaling.

In order to follow the purposes of the workshop the presentations were made without any specific sequences between cardiovascular and oncological fields, as well as with random commixture of basic and clinical research investigators. A large possibility of discussion was given to the participants after each presentation and at the end of the sessions. By overcoming the differences due to the specific branch of expertise, all the participants gave a fruitful contribute to the principal points of discussion by underlining potentialities, difficulties and weaknesses of a joint research for the future in a friendly and constructing way**.** Different substances have been analysed: endothelins and endothelin receptors, natriuretic peptides, HIF1α, VEGF, TNF-α, P and E-selectines, VCAM, ICAM, NFkB, HRG, PIGF, aquaporins, profiling 1, osteopontin, mitochondria ATPbinding cassette protein-1, hexokinases, protein disulfide isomerase A3, EMMPRIN. Vessel heterogeneity together with endothelial and smooth muscle cells heterogeneity and the role of micro environment on cell motility have been underlined. Important concepts and mechanisms, as intercellular interactions, matrix content and cellular activations, cellular proliferation, immune evasion, and targeting therapy have been described. As far as the “omics” and the data archives, their potential and strong help in research developing in the fields of pathophysiology and pharmacology has been emphasized, as well as the existence of many conflicting data It will be necessary for the next future to acquire other elements and to aggregate different information by multiple independent studies.

Scientific Content

The workshop, starting from an overview of the endothelin axis in cancer and cardiovascular field, takes into consideration different cellular species, different substances involved in cell to cell interaction, the role of extracellular environment in normal tissues, in cancer and in atherosclerosis, and important concepts as survival skip and immune evasion, targeting therapies and chemoresistance, data warehouse on genes and proteins, genotyping, polymorphisms and environmental multiparameter effects.

Endothelins are potent vasoconstricting peptides, playing an important role in tissue differentiation, cell proliferation, mitogenesis in tumour and stromal cells. They contribute to signaling cascades in angiogenesis, inhibition of apoptosis, matrix remodeling and metastases. Endothelin-1 (ET-1) is an angiogenetic factor, acting in neovascularization in concert with VEGF, it has an indirect angiogenetic effect via Hypoxia-inducible factor-I alpha (HIF-Iα) as well as a direct modulation of the lymphatic endothelial cells. By inhibition of HIF-Iα (silencing by RNA interference) ET-1 is completely inhibited, while the VEGF production is blocked.

The lymphoangiogenic root can be important in metastasis spreading; furthermore, also the communication between cells involving the gap-junction proteins connexins can be responsible for tumour invasiveness. ET promotes ovarian carcinoma cell migration and invasion (chemotaxis/chemoinvasion). As far as treatment, the skip of the tumour to another survival pathway becomes relevant. ET in cardiovascular side can induce structural and functional alterations both in macro and micro-vasculature, affecting vascular smooth cells and myocytes; ET-1 stimulates proliferation, cell adhesion and thrombosis. Related clinical disorders are pulmonary hypertension, systemic hypertension, acute coronary syndrome, stroke, heart failure. In this last condition, elevated levels of ET-1 correlate significantly with the recurrence of events in the follow up. ET is overexpressed in the failing heart, in a non homogeneous manner, as well as natriuretic peptides ANP and BNP, which show an opposite effects. During myocardial ischemia there is a paradoxical coronary vasoconstriction. Both ET and NO appear to be involved in such phenomenon. Relatively to the cross talk among cells it is important to underline that macrophages release ET-1.

Following ET, the endothelial cells are examined: they are essential players in leukocyte recruitment and angiogenesis; their behaviour is dynamically controlled by the microenvironment. Heterogeneity of endothelial cells is a characteristic of different vasculatures. Cultured cells show a loss of control after few hours. Vessel microdissection is likely the way of solving this problem: it is possible to obtain the map of vascular responses by different stimuli. For therapeutic purposes, immunoliposomes could guarantee targeting the organ, instead of giving a drug which is diluted in the whole body, with high doses and multiple adverse effects.

Targeting VEGF (vascular endothelial growth factor) receptor signaling modulates vessel functionality. A novel possibility is building blood vessels: the stem cell models are important in vascular biology. VEGF has different isoforms which induce sprouting angiogenesis. In response to acute VEGF stimulus, vessels demonstrate leakiness.

Human histidine-rich glycoprotein (HRG) promotes vessel functionality: it is synthesized exclusively into the liver; however, it becomes associated with platelets and megakaryocytes and be released after thrombin stimulation, showing a regulatory role in haemostasis and innate immunity. By HRG

treatment the perycite coverage of tumour vessels increases; furthermore, tumour hypoxia decreases in HRG overexpressing fibrosarcoma. PIGF, the placental growth factor, is significantly down-regulated in macrophages purified from HRG expressing tumours. HRG inhibits tumour growth with maintained vessel functionality: in these conditions cytotoxic substances increase and this mechanism might be the cause of tumour growth inhibition.

By considering smooth muscle cells (SMCs), these cells too demonstrate heterogeneity, with two types of phenotypes, spindle and rhomboid cells (S-SMCs, R-SMCs).
R-SMCs are responsible for intimal thickening. In vitro resting R-SMCs strongly express calvasculin (S100A4)**,** a member of S100-related calcium-binding proteins, involved in their migration and proliferation, also implicated in metastatization. The S100A4 modulation by siRNA in R-SMCs reduces proliferation. An important finding is the high expression of S100A4 in pig coronary restenosis, as well as in human atherosclerotic lesions and restenosis.

Is it possible to postulate that normalized vasculature is the way for a better delivery of drugs? In this contest a significant element is the microvascular heterogeneity. Endothelial cells heterogeneity in different tumour vessels rather than in different organs could be a crucial point to be considered; in the same tumour, differences could be present at a different stages implying differentiated responses and responsiveness to chemotherapeutic agents. Among the various markers of angiogenesis, fibronectine, an extracellular adhesion molecule, and endoneglin, a type I membrane glycoprotein located on cell surface and part of the TGF beta receptor complex, seem to be the most promising.

Aquaporins (AQPs) are a family of small (~30 kDa/monomer) hydrophobic, integral membrane proteins, widely expressed in the animal and plant kingdoms. AQPs transport water and, in some cases, also small solutes such as glycerol.

Their classical role in facilitating trans-epithelial fluid transport is well understood, as in the urinary concentrating mechanism and gland fluid secretion; AQPs are also involved in swelling of tissues under stress, as in the injured cornea and the brain in stroke, tumor and infection. Recent analysis of AQP-knockout mice has revealed unexpected cellular roles: 1) AQPs facilitate cell migration, as manifested by reduced tumor angiogenesis in AQP1-knockout mice; 2) AQPs that transport both glycerol and water regulate glycerol content in epidermis and fat, and consequently skin hydration/biosynthesis and fat metabolism; 3) AQPs might also be involved in neural signal transduction, cell volume regulation and organellar physiology. The many roles of AQPs could be exploited for clinical benefit, as example as diuretics, in the treatment of brain swelling, glaucoma, epilepsy, obesity, cancer, pulmonary edema and chronic heart failure.

Profilin 1 (Pfn1) is one of Actin binding proteins, playing a role in Actin cytoskeleton remodelling control, in cell-matrix and cell to cell adhesion regulation, in endothelial cell proliferation and migration. By silencing Pfn1 in HUVECs (RNA-interference) a significant reduction in the formation of actin filaments and focal adhesions can be obtained, as well as a reduction in the dynamics of cell to cell adhesion, the inhibition of cell migration, some defects in membrane protrusion in terms of its magnitude and directional persistence, inhibition of cell growth without compromising cell survival, suppression of matrigel-induced early cord morphogenesis of endothelial cells. On the other hand, in mice genetically engineered to overexpress the human profilin 1 gene in vascular smooth muscle cells, SMC size increases , narrowing the lumen through which blood flows. Actin is transformed from a loosely configured protein into a more rigid fibrous state enhancing SMC rigidity. Mice develope high blood pressure at six months of age (approximately equal to middle age in adults).

Osteopontin is a secreted protein, multifunctional molecule, present in bones, kidney and epithelial linings. It has been demonstrated that has clinical relevance in chronic inflammation,

several types of cancer, autoimmune diseases, obesity, cardiovascular disease, restenosis (independent risk factor) and vascular calcification.

A relevant role in cellular homeostasis and in hypoxia conditions is played by Hypoxia inducible factor-1 (HIF-1), a transcription factor that functions as protective element for cardiac cells. There is evidence that HIF-1 can also trigger apoptosis, possibly when cellular responses are inadequate to meet energy demands under hypoxic conditions.

The mitochondria ATPbinding cassette protein-1 (mABC1), when overexpressed in cardiomyocyte mitochondria, reduces cellular ROS and increases oxygen consumption.

Hexokinases I and II (HK) are responsible for glucose phosphorylation (glucose-uptake regulation). HK I and HK II overexpression reduces oxygen species (ROS); HK II overexpression increases also contractility in cardiomyocytes and improves mitochondria function.

Protein disulfide isomerase A3 (PDI) is a highly hydrophobic protein mainly localized inside the rough endoplasmic reticulum (RER). In RER, it cooperates to correct folding of native neo-synthesized proteins. PDI may also locate in the plasma membranes where interacts with extracellular environment including exposed domains of membrane proteins, extracellular matrix proteins and circulating factors. Phosphorylation degree of PDI can increase its hydrophilicity enhancing the probable interaction with extracellular components. It has been demonstrated that PDI may interact with extracellular trombospondin-1 (TSP-1) inducing its refolding by disulfide rearrangements: such structural changes expose a cryptic RDG motif allowing the binding of TSP-1 to integrin receptors. For a specific block of cell migration it will be important to select the most representative targets and develop gene-specific drugs (ribozymes) to approach functional studies by gene knock-down technology.

In the field of angiogenesis, PIGF appears as a novel target. Infact, VEGF promotes the angiogenic switch in tumours, however certain tumours both in mice and humans are resistant to VEGF inhibitors. The 95% of antiangiogenic agents available are anti VEGF. They induce significant side effects: hypertension, arterial thrombosis, impaired wound healing, bleeding due to vessel dysregulation. PIGF is undetectable in healthy tissues, but it is highly upregulated in tumours. Monoclonal antibodies anti PIGF inhibit the growth of VEGF inhibitor resistant tumour and they also show sensitizing effect in chemotherapy. They impede tumour vessel formation and lymphoangiogenesis, without regression of healthy vessels, demonstrating a safety profile.

Toll-like receptor (TLR4) is a transmembrane lipopolysaccharide receptor. Its activation causes the release of antimicrobial peptides, inflammatory cytokines and chemokines, and costimulatory molecules that initiate the innate immune response to common gram-negative bacteria. TLR4 also interacts with respiratory syncytial virus, heat-shock proteins, fibronectin, fibrinogen, and hyaluronic acid. A TLR4 defective mice does not remodel carotid artery in ligation model: there is in fact, a big increase in collagen, resulting in increased stiffness of the vessel. Furthermore, in the model of myocardial infarction, without TLR4 the matrix turnover increases and reperfusion injury decreases. A new vascularization takes place in the unstable plaque.

An inflammatory condition initiates and promotes atherosclerosis. Adherent platelets activate endothelium and interact with monocytes: EMMPRIN (Extracellular Matrix Metalloproteinase Inducer) on monocytes in acute myocardial infarction can be a potential regulator, inducing inflammatory reaction. Platelet α granules contain EMMPRIN; their stimulation transfers EMMPRIN on the cell surface. EMMPRIN is expressed on atherosclerotic lesions and it is reported to induce cancer cell dissemination and tumour progression. By blocking EMMPRIN the tumour size reduces. By EMMPRIN, pancreas carcinoma cell lines induce MMP (metalloproteinases) expression in stromal cells. Pancreas cancer is extremely fibrotic. Tumour

cells attract pancreas stellate cells which contain a lot of vitamin A: their activation produces many substances such as collagen I and III, fibronectin. Around carcinoma there is a high number of activated stellate cells, which are inhibited by anti PDGF (platelet-derived growth factor).

Also cervical cancer is related to inflammation. The presence of inflammatory cells has a negative implication for patients. The cervical cancer cells circumvent elimination by the immune system (immune evasion). A poor survival is related to TGF-β1 (Transforming growth factor beta 1) activation, MMP-2 activity, MMP-7 expressed in endothelial cells. Cervical cancer cells are resistant to TGF- β1 mediated growth inhibition: immunotherapy is not a good solution for the eradication of these cells.

The problems to be afforded are: can the environment be modulated? Is it possible to alter the balance?

Other open question: is it better to inhibit EMMPRIN instead of 2MMP? In the past, anti MMP1 and anti MMP2 are already demonstrated toxic for junctions. The difficulty is to get antibodies inside the tissues.

In cancer there are alternative vascularization strategies: 1) neoangiogenesis, 2) angioblastic angiogenesis (neonatal), 3) vessel cooption, 4) glomeruloid vessel formation (it is used to evaluate tumour malignancies, 5) tumoral vessels – tumoral sinuses – vasculogenic mimicry – mosaic vessel. Examples:A) Tumour induced neoangiogenesis: ductal breast cancer; single endothelial cells inside. B) Vessel cooption: non small cell lung cancer; no angiogenesis. C) Vessel cooption (peritumoral neoangiogenesis) in melanoma (no intratumoral angiogenesis), perycites provide a way of communication. D) Glomeruloid vessels in brain micrometastasis.

Relatively to the postneonatal vasculogenesis, important point to be considered are: bone marrow stem circulation and possible markers; presence of substances in healthy subjects; WT1 (Wilms' tumor suppressor gene) expression in microvessels; c-kit loss in circulation and in endothelial cells. There are kinase targets in various angiogenesis forms. Tumour can switch from one type to another.

Preclinical investigators and pharmacologists cooperate to strategies to target the cancer vasculature. Not only endothelial cells, but also other cells allow the stabilization of the vasculature of metastasis. It is important to consider endothelial precursor cells and combination of therapies with vascular disrupting agents (VDA). With rare exceptions efficacy of a therapeutic treatment appears of short duration: the patients become refractory by a mechanism of escape treatment. VDA –tubuling binding induce a damage of the existing vasculature of tumours: most of these drugs are colchicines derivatives. Colchinol ZD6126 causes microtubule depolarization (effect on endothelial cell shape, change the morphology, disruption of capillary like structures, with reversible effects washing the agent, on HUVEC cultures). In vivo, in neovessel there is the shut down of the vasculature (tumour necrosis). The effect is more evident in large tumours.

In tumour targeting there is a limited efficacy of IgG (immunoglobulins) if the target is not the sole cause of disease, together with the limited tissue distribution of IgG.

One of the most promising new avenues for the development of more selective and efficacious cancer therapies relies on the antibody-mediated targeted delivery of bioactive agents (e.g., cytokines) to the tumor environment. The identification of quantitative differences in the expression of accessible vascular proteins in metastatic lesions and host organs facilitate the development of antibody-based strategies.

The ligand-based vascular tumour targeting (monoclonal antibodies) ignores the normal tissue. By using a chemical label of the proteins of the pathological vessels, respect to normal tissue, the therapy becames extremely specific and selective.

A powerful help could derive from the characterization of chemical proteomics for the discovery of vascular markers of metastasis (as example, liver metastasis respect normal liver, fibronectin in both, but in neovasculature of liver metastases the alternatively spliced extra-domain A – EDA - of fibronectin is strongly expressed, while being undetectable in most normal organs).

The “omics” as system biology tools represent a very promising area in the interaction with basic research. RNA interference and microRNAs are methods to be considered not only to build new therapeutical agents but also to understand the behaviour of different cells and the mechanisms of homeostasis in various conditions. This way is complex, but it could be a strong tool for knowledge advancement. The developmental pathways during pattern formation and morphogenesis are extremely important to be studied. Proto-oncogenes seem to have implications in many human tumours,.with a tissue specificity. From a methodology point of view, it is important to proceed in steps in a microarray data experiment: hypothesis, design, sample, hybridisation and scan normalization, analysis, data submission. A metanalysis is performed to build up the platform. The data warehouse is organized by experimental factors, disease, organ, with a matrix for each. There is a repository of transcriptomic experiments. The research is made for similarities (gene warehouse), in situ and for microarray overlap. It is evident that there are conflicting data in correlations (genes ↔ proteins). Antibodies are imperfect data. Do different studies give the same results on gene expressions? Most genes are not expressed in most conditions.

The research is conducted in different ways:1) conditions studied per gene, 2) gene expression distribution over conditions, 3) condition-specific genes, disease state. Looking for core mechanisms goes to find dense submatrices. The ongoing work is based on looking for core regulation subsets: given a set of genes, find a subset of conditions for strong co-expression. Latent semantic indexing (LSI) is a technique for information retrieval theory, used in large scale text searching. It is useful to aggregate different information from multiple independent studies. It will be important to get a normal tissue set, considering the large nature variability together with the atlas of signaling pathways (renormalized microarray data atlas).

In the field of DNA bank, single nucleotide polymorphism and candidate gene studies are prominent aspects. The main points to be considered are: 1) genome-wide genotyping without any preconcept about relation with particular disease, 2) Linkage disequilibrium, 3) detection of frequent disease-associated variants, 4) phenotyping is not always the same, 5) rare events are not captured, 6) resequencing procedures, 7) multiparameters from the environment, 8) high time consuming and high cost 9) resequence of fragments: selection of parts of genome (Project READNA in FP7, Revolutionary Approaches and Devices for Nucleic Acid analysis).

By considering common variant loci in type 2 diabetes, there is an overlap between diabetes genes and cancer, but casualty is rarely established. The variant influences some process: modifies risk of diabetes; modifies risk of some cancers. Mechanisms: 1) Shared risk factors, obesity, glucose and lipids. 2) Cell cycle regulation. Allele1, pro-proliferation; Allele2, reduced proliferation. By analyzing type 2 diabetes and coronary disease signals, loci in chromosome 9 close for diabetes, coronary artery disease, aneurysms are found. Among tumours, it is relevant melanoma and other susceptibility as well as pancreatic cancer and diabetic risk. In some conditions diabetes and cancer (prostate) goes in the opposite directions. 3) Signaling pathways. Some suppressor genes could be involved in glucose homeostasis.

In the programme INTERACT of FP6 there are studies of prevalent diabetes and cancer in EPIC cohorts (coincident signals).

Assessment of the results, contribution to the future direction of the field

The intent of the workshop was very ambitious in that scientists from different fields were put together and required to expose their results to other “inexpert” scientists in a mutual brainstorming experience.

The scientific content of the various presentations was certainly elevated and in many cases very new and stimulating.

Several pathophysiological conditions were illustrated and the spectrum of the approaches varied from cellular to molecular biology, from the in vitro to in vivo studies, from possible drugs to actual therapies, from laboratories to clinical arenas.

This wide kaleidoscopic content favoured the discussion and the consequent questions and answers improved the scientific value of the event and accomplished the initial purposes.

The following key points arose from the discussion. 1) the relationships between “sick” cells and the surroundings healthy cells, 2) the cellular heterogeneity as a source of plasticity, 3) the secretion of factors and their role in determining cell fate, 4) the importance of the microenvironment where the pathological events occur, 5) the activation of cells by internal and external stimuli, 6) the revisited role of some molecule families old as endothelins and new as aquaporins in both pathophysiological processes.

The discussion on the various points was very rich, but at the same time very difficult: this came out clear when the participants were invited to propose which points and which possible cooperative approach to be exploited. It was apparent that some people would better cooperate in the immediate future, indicating that a joint research approach could be established.

It was then clear that some fields of investigations appeared less separated than believed and for that coordinate and collaborative approaches are indeed possible. This represents the second important achievement of the workshop as scientists from different fields realized that shared forces could be very useful to study shared pathways.

The third important result is the possible realization of a networking research consortium for an application on system biology area in FP7.

Final programme

July 14, 2008

Morning session

9:00 – 9:15 Welcome to participants by the organisers

9:15-9:30 Presentation of the European Science Foundation by the official ESF representative

9:30 – 10:00 Anna Bagnato: Endothelins in cancer, a translational message

10:00 – 10:30 Antonio L'Abbate: Endothelins in cardiovascular science

10:30 – 11:00 Grietje Molema: Molecular and pharmacological heterogeneity of microvascular endothelial cells

11:00 – 11:30 Coffee break

11:30 - !2:00 Charlotte Rolny: Regulation of VEGFR signaling pathways in vivo and in vitro

12:00 - !2:30 Marie-Luce Bochaton Piallat: Heterogeneity of arterial smooth muscle cells: implications for atherosclerosis and restenosis

12:30 – 13:30 Principal Discussion Points

13:30 – 14:30 Lunch

Afternoon session

14:30 – 15:00 Sonja Loges: Preclinical development of anti-PlGF from genetics to therapeutic perspectives

15:00 -15:30 Maria Giovanna Trivella: Old and new molecules in cardiovascular science

15:30 – 16:00 Dominique de Kleijn: Atherosclerotic plaque and heart: innate immunity and matrix break down

16:00 – 16:30 Coffee break

16:30 – 17:00 Andreas E. May: Regulation of Matrix MetalloProteinases

17:00 – 17:30 Max G. Bachem: On the role of EMMPRIN in tumor progression and the development of plaque instability

17:30 -18:00 Arko Gorter: Immune evasion: role of tumor-derived cytokines in cervical cancer

18:00 – 19:30 General discussion

20:30 Dinner

July 15, 2008

Morning session

8:30 - 9:00 Giuseppe Rainaldi: MicroRNAs and gene expression networks

9:00 –9:30 Misha Kapushesky: Interpreting microarray experiments

9:30 – 10:00 Ivo Gut: Genetic polimorphisms

10:00 – 10:30 Mark McCarthy: From epidemiology to causal pathways: etiological overlap between type 2 diabetes and cancer revealed by genetic studies

10:30 – 11:00 Open questions

11:00 – 11:30: Coffee break

11:30 – 12:00 József Tímár: Molecular markers and molecular targets in human melanoma

12:00 – 12:30 Dario Neri: Tumor targeting

12:30 – 13:00 Raffaella Giavazzi: Pharmacological strategies to develop combination therapies with inhibitors of angiogenesis

13:00 - !4:00 Lunch

14:00 – 16:00 General discussion and perspectives

Statistical information on participants

Countries	Gender	1939-48	1949-58	1959-68	1968-79
1. Italy	M 2				
	F 3				
2. Germany	M 2				
	F 0				
3. Switzerland	M 1				
	F 1				
4. Netherlands	M 2				
	F 1				
5. France	M 1				
	F 0				
6. United Kingdom	M 2				
	F 0				
7. Belgium	M 0				
	F 1				
8. Sweden	M 0				
	F 1				
9. Hungary	M 1				
	F 0				
Total Males	11	2	2	6	1
Total Females	7	0	2	3	2

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