Prognostic and predictive factors from FNAC material using molecular tools

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§ Alphagenics Biosystem
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Breast cytology activity (FNA) in Trieste - Italy

FNA 2004-2018

12.591 FNA in 15 YEARS
Breast Cytological diagnostic diagnostic Categories in Trieste 2004-2018

Total : 12,591 FNAC in the last 15 years
Total number of breast abnormalities investigated entirely in Trieste in the period 2004-2018:

13,805 lesions

- FNA sufficient for definitive diagnosis: 90.5%
- Core Biopsy: Necessary for diagnosis (TRU-CUT or VAB/MAMMOTONE): 6.5%
- Sent DIRECTLY TO SURGERY: 3.0%
## Quality cytological indicators in the entire period 2004 – 2018 in Trieste Breast Unit

<table>
<thead>
<tr>
<th>Indicators</th>
<th>Period 2004-2018</th>
<th>Standard values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute Sensitivity</td>
<td>84.4%</td>
<td>&gt; 60%</td>
</tr>
<tr>
<td>Complete Sensitivity</td>
<td>96.9%</td>
<td>&gt; 80%</td>
</tr>
<tr>
<td>Specificity</td>
<td>67.2%</td>
<td>&gt;60%</td>
</tr>
<tr>
<td>VPP C5</td>
<td>99.9%</td>
<td>&gt;95%</td>
</tr>
<tr>
<td>VPP C4</td>
<td>74.6%</td>
<td>&gt;70-80%</td>
</tr>
<tr>
<td>VPP C3</td>
<td>7.4%</td>
<td>&lt;20%</td>
</tr>
<tr>
<td>False-negative rate</td>
<td>0.4%</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>False-positive rate</td>
<td>0.1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Inadequate rate</td>
<td>7.51%</td>
<td>&lt;25%</td>
</tr>
<tr>
<td>Inadequate rate from cancers</td>
<td>2.7%</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>Suspicious rate</td>
<td>18.9%</td>
<td>&lt;20%</td>
</tr>
</tbody>
</table>

*ALL QUALITY STANDARDS WERE SATISFIED*
TREND POSITIVE PREDICTIVE VALUES of C5

High diagnostic reliability
TREND of 2019
Period January-May: 59% of C5 lesions with subsequent B5 Core biopsy
Molecular profiling of breast cancer has gained popularity due to the possibility of studying the biological spectrum of the disease and to evaluate prognostic and predictive characteristics that can lead to more accurate therapeutic decisions.
Indications on micro histological sampling (needle biopsy) for the determination of hormone receptors and more useful prognostic markers:

- women aged <= 40 years
- patients with cancer > = 2 cm
- patients with comorbidity that are not eligible for surgery
Neoadjuvant treatment post biopsy

<table>
<thead>
<tr>
<th>YEAR OF DIAGNOSIS</th>
<th>N° OF NEOADJUVANT TREATMENTS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010-2011</td>
<td>10</td>
</tr>
<tr>
<td>2012-2013</td>
<td>10</td>
</tr>
<tr>
<td>2014-2015</td>
<td>20</td>
</tr>
<tr>
<td>2016-2017</td>
<td>28</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
</tr>
</tbody>
</table>

For the biennium 2018-2019 trends is increasing: only in 2018, 16 women were treated with neoadjuvant therapy before surgery

* EXCLUDED 1°LINE THERAPY IN METASTATIC NOT OPERABLE PATIENTS
CLINICAL PROTOCOL: I.O.R.T. AS EXCLUSIVE TREATMENT IN EARLY STAGE BREAST CANCER

PDTA second update December 2017
(last revision December 2018)

• **PRE-OPERATIVE MALIGNANT DIAGNOSIS**

  *(Needle biopsy or Cytological cell block) OF INVASIVE BREAST CARCINOMA WITH NON-LOBULAR ISTOTYPE AND WITH FAVORABLE BIOLOGICAL PROFILE (LUMINAL)*

NEEDLE BIOPSY or….
In an era of personalized medicine, with an increasing need for molecular testing, cytologic specimens comprise a crucial component in providing prognostic and predictive informations for clinical management of patients.

The status of ER, PR, and Her2 in breast carcinoma
Breast cancer subtype

• PRE-OPERATIVE HISTOLOGICAL DIAGNOSIS

(with needle biopsy or cell block) OF INVASIVE BREAST CARCINOMA WITH NON-LOBULAR ISTOTYPE AND WITH FAVORABLE BIOLOGICAL PROFILE (LUMINAL)

The molecular "signature" of mammary lobular carcinomas is the loss of E-cadherin protein expression as evidenced by immunohistochemistry, whereas ductal carcinomas are typically E-cadherin positive.

Strong expression of E-cadherin immunostaining in cell block
Accurate assessment of biomarkers is fundamental to identifying patients who will benefit from treatment targeting/inhibiting ER and HER2. Incorrect test results can lead to a potential risk of under or overtreatment. There is thus considerable interest in improving and optimizing test results. Assessing ER, PR, and HER2 status by centralized measurement of mRNA levels can aid this by providing a standardized and reproducible technique.
I-SMART
Innovative Molecular Essay
Associated with Therapeutic Response

The I-SMART project is applied in the field of in vitro diagnostics, and includes the development of an innovative kit for a simplified, rapid and non-invasive molecular diagnostic test for personalized cancer diagnosis.

The I-SMART project is aimed at the development of a non-invasive molecular diagnostic kit based on Real-Time PCR technology, capable to perform quickly and easily, the mutational and transcriptional analysis of predicting genes of response to tumor samples.

Through the methodology developed by the I-SMART project, we want to reduce the overall analysis time and eliminate the possibility of losing precious material in the course of nucleic acid purification due to the reduced amount of initial clinical sample.
In the case of breast cancer, which is the most frequent neoplasm for incidence in the female population, correctly diagnosing breast cancer is of fundamental importance for the therapy. The choice of conducting neoadjuvant therapy is based on the diagnosis that is generally performed on micro-biopsy (Tru-Cut), after the patient has been subjected to cytological sample collection by fine needle aspiration (FNAC).

Among the aims of the I-SMART project there is also the development of the assay for samples taken with FNAC generally used only in preliminary cytological analysis. This will allow to perform a molecular analysis on samples taken with non-invasive method (FNAC), instead of the bioptic sampling using a larger gauge trimming needle (Tru-Cut micro-biopsy), avoiding to women the discomfort of this invasive methodology, and also allowing a drastic reduction of the time for the preparatory diagnosis and the costs for its execution.
Results
(update: September 2019)

200 cytological smears (C5) of women with different molecular subtypes breast cancer, defined on surgical specimen or CB (according to St. Gallen 2013)
✓ 84 Luminal A-like
✓ 57 Luminal B Her-2 negative
✓ 7  Luminal B Her-2 positive
✓ 12 HER2 positive non luminal
✓ 40 TNBC

For the analysis we selected out of our C5 breast FNA, smears with different cellularity (most of which had a poor or medium cellularity)

All slides were digitalized with D-Sight Menarini Vysia scanner device, to create a digital morphological copy.
Different types of digitalized smears used for molecular analysis

C/2016.002543
C/2016.002617
Different type of smears used for molecular analysis

C/2015.020592

C/2016.001310
Different type of smears used for molecular analysis

C/2016.005489

C/2017.0013661
Different type of smears used for molecular analysis

C/2016.016096

C/2016.002979
All selected FNA slides counted much more than 50 malignant cells.
Method

• After removing the coverslip (Xilene for about 24 hours) and scraping the cytological material from the slide with a blade, the cytological material was rinsed and resuspended in a specific buffer and sent to molecular biomarker profiling......
Effect of different sample preparation method on selected markers detection – Luminal A and Luminal B

Qiagen extraction kit

Lysis SOLUTION R

Lysis SOLUTION D

samples in Solution R and D were incubated for 30’ at 50-60°C

The 2 new techniques (Solution R and Solution D) are concordant with traditional method (Trizol/Qiagen)
Effect of different sample preparation method on ESR1 detection in LumA sample

- Qiagen extraction kit
- Lysis Solution R
- Lysis Solution D

Amplification Plot

- GAPDH
- ESR1

Qiagen extraction kit
SOLUTION R
SOLUTION D
The advent of quantitative real-time RT-PCR (Q-RTPCR) techniques for the measurement of gene expression has allowed the accurate determination of the expression levels of target genes in cells and tissues. To control for experimental variations in the amount of RNA used in each Q-RTPCR and batch-to-batch variations in PCR reagents, coincident measurement of so-called “housekeeping” genes has been used for the normalization of target gene expression data.

GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues

Robert D. Barber, Dan W. Harmer, Robert A. Coleman, and Brian J. Clark
Pharmagene Laboratories Limited, Royston, Hertfordshire, United Kingdom
Submitted 15 January 2005; accepted in final form 8 March 2005
Effect of different sample preparation method on ERBB2, ESR1 and PGR detection in KRT19 negative sample (5694)
ESR1 expression level in different sample types

LumA

LumB

Basal like

Her2 (3+)

ER positive
PGR expression level in different sample types

- LumA
- LumB
- Basal like
- Her2 (3+)
LUMINAL - A samples according to IHC

20-\(\text{Ct}_{\text{GOI}} - \text{Ct}_{\text{REF}}\)
ESR1 RT-qPCR Performance with respect to IHC

Optimal cut-off method: Youden
Optimal cut-off point: 13.40176

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
<th>Lower Limit</th>
<th>Upper Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>0.879</td>
<td>0.816</td>
<td>0.927</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.969</td>
<td>0.892</td>
<td>0.996</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>0.985</td>
<td>0.946</td>
<td>0.991</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>0.775</td>
<td>0.677</td>
<td>0.967</td>
</tr>
<tr>
<td>Positive Likelihood Ratio</td>
<td>28.134</td>
<td>7.182</td>
<td>110.207</td>
</tr>
<tr>
<td>Negative Likelihood Ratio</td>
<td>0.125</td>
<td>0.081</td>
<td>0.193</td>
</tr>
</tbody>
</table>

Distribution of ESR1
LUMB samples according to IHC

LUMB HER2-

LUMB HER2+

\(20\times (\text{Ct}_{\text{GOI}} - \text{Ct}_{\text{REF}})\)
**PGR RT-qPCR Performance with respect to IHC**

**Optimal cut-off method:** Youden
**Optimal cut-off point:** 13.32542

<table>
<thead>
<tr>
<th>PGR</th>
<th>Value</th>
<th>Lower_Limit</th>
<th>Upper_Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>0.865</td>
<td>0.793</td>
<td>0.919</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.905</td>
<td>0.815</td>
<td>0.961</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>0.94</td>
<td>0.877</td>
<td>0.965</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>0.798</td>
<td>0.702</td>
<td>0.911</td>
</tr>
<tr>
<td>Positive Likelihood Ratio</td>
<td>9.145</td>
<td>4.504</td>
<td>18.569</td>
</tr>
<tr>
<td>Negative Likelihood Ratio</td>
<td>0.149</td>
<td>0.095</td>
<td>0.233</td>
</tr>
</tbody>
</table>
Concordance between HER-2 status determined by qPCR in Fine Needle Aspiration Cytology (FNAC) samples compared with IHC and FISH in Core Needle Biopsy (CNB) or surgical specimens in breast cancer patients

Claudia Rodriguez, Voichita Suciu, Audrey Poterie, Ludovic Lacroix, Isabelle Mirand, Amélie Boichard, Suzette Delaloge, Jacqueline Deneuvel, Sandy Azoulay, Marie-Christine Mathieu, Alexander Valent, Stefan Michiels, Monica Arnedos, Philippe Vielh

From a total of 154 samples from patients who had nodular breast lesions and attended the 1-day-stop clinic at the Gustave Roussy from March 2013 to October 2014, qPCR was able to determine the HER2 status in a mean of 3.7 days (SD 3.1). The overall concordance with standard HER2-testing was very high: 97% (95% CI 0.94 to 0.99); sensitivity was 96% (0.87e1), specificity 98% (0.95e1) and positive and negative predictive values 88% (0.75e1) and 99% (0.98e1), respectively.

In conclusion,......HER-2/neu status determined in FNAC samples using qPCR is fast, reliable, highly concordant with standard IHC/FISH testing and presents high specificity and negative predictive values.
HER2+ (non luminal) samples according with IHC

20-(Ct_GOI - Ct_REF)

- ERBB2
- ESR1
- PGR
Effect of different sample preparation method on selected markers detection – Her2

The circumferential membranous staining pattern which is characteristically seen on histologic sections was replaced by this dark, opaque staining pattern on the cell-transferred direct smears.

This is due to the three-dimensional nature of the tumor cell clusters on the direct smears compared with the more two-dimensional nature of the histologic sections.

Possible cut-off for HER 2 POSITIVITY (3+)

> 22
ERBB2 RT-qPCR performance with respect to IHC

Optimal cut-off method: Youden
Optimal cut-off point: 21.48205

<table>
<thead>
<tr>
<th>ERBB2</th>
<th>Value</th>
<th>Lower_Limit</th>
<th>Upper_Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>0.958</td>
<td>0.789</td>
<td>0.999</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.852</td>
<td>0.791</td>
<td>0.901</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>0.469</td>
<td>0.367</td>
<td>0.973</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>0.993</td>
<td>0.961</td>
<td>0.996</td>
</tr>
<tr>
<td>Positive Likelihood Ratio</td>
<td>6.487</td>
<td>4.506</td>
<td>9.34</td>
</tr>
<tr>
<td>Negative Likelihood Ratio</td>
<td>0.049</td>
<td>0.007</td>
<td>0.333</td>
</tr>
</tbody>
</table>

Sensitivity & Specificity Curves

Distribution of ERBB2 (from IHC)
Effect of different sample preparation method on selected markers detection – Basal Like

Possible cut-off for ESTR/PGR - <13.5
(grey zone between 8 and 12)
Triple Negative Breast Cancer samples according with IHC

$20 \cdot (C_t_{GOI} - C_t_{REF})$

GOI = GAPDH
RT-qPCR expression of the four biomarkers in the molecular breast cancer subtypes

Markers Expression (20-Ct_GOI_Ct_REF)

- ESR1
- PGR
- HER2
- MKI67

Subtypes:
- TNBC
- LUM_A
- LUM_B
- HER2pos
- LUM_B_HER2pos
Bioinformatics Multi Parameters Class Prediction Analysis

- Several Methods: we have chosen Unbiased Artificial Intelligence Approach.
- We tried several AI algorithms with several models (more than 60).
- We obtained the best performance with Deep Learning Grid AI algorithm.

<table>
<thead>
<tr>
<th>Molecular Subtype</th>
<th>HER2pos</th>
<th>LUM_A</th>
<th>LUM_B</th>
<th>LUM_B_HER2pos</th>
<th>TNBC</th>
<th>Error</th>
<th>Error/rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2pos</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00%</td>
<td>=0/12</td>
</tr>
<tr>
<td>LUM_A</td>
<td>1</td>
<td>76</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>9.52%</td>
<td>=8/84</td>
</tr>
<tr>
<td>LUM_B</td>
<td>2</td>
<td>11</td>
<td>39</td>
<td>0</td>
<td>5</td>
<td>31.58%</td>
<td>=18/57</td>
</tr>
<tr>
<td>LUM_B_HER2pos</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>14.29%</td>
<td>=1/7</td>
</tr>
<tr>
<td>TNBC</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>36</td>
<td>10.00%</td>
<td>=4/40</td>
</tr>
<tr>
<td>Totals</td>
<td>17</td>
<td>88</td>
<td>46</td>
<td>6</td>
<td>43</td>
<td>15.50%</td>
<td>=31/200</td>
</tr>
</tbody>
</table>
The potential of Ki-67 IHC and RT-qPCR to predict pathological complete response (pCR) was evaluated using ROC analysis and non-parametric Mann-Whitney Test. Correlation between Ki-67 qIHC and RT-qPCR is only moderate and RT-qPCR was significantly more specific.
This is the first study to compare tumor MKI67 gene expression by RNA and protein assessment in a prospective retrospective neoadjuvant setting. Due to the relatively small sample size, these data should be considered preliminary and worth validating in larger datasets.
MKI67 RT-qPCR performance with respect to IHC

Optimal cut-off method: Youden
Optimal cut-off point: 16.90831

<table>
<thead>
<tr>
<th>MKI67</th>
<th>Value</th>
<th>Lower_Limit</th>
<th>Upper_Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>0.738</td>
<td>0.642</td>
<td>0.82</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.485</td>
<td>0.382</td>
<td>0.588</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>0.603</td>
<td>0.5</td>
<td>0.71</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>0.635</td>
<td>0.526</td>
<td>0.726</td>
</tr>
<tr>
<td>Positive Likelihood Ratio</td>
<td>1.431</td>
<td>1.143</td>
<td>1.792</td>
</tr>
<tr>
<td>Negative Likelihood Ratio</td>
<td>0.541</td>
<td>0.369</td>
<td>0.794</td>
</tr>
</tbody>
</table>
Immunostain of cellblock (x40 original magnification) depicting nuclear immunoreactivity with Ki-67 in tumor cells.

The expression of Ki67 could be very heterogeneous in different areas of the same tumor.
Importance of assessing CK19 immunostaining in core biopsies in patients subjected to sentinel node study by OSNA

Felip Vilardell · Anna Novell · Javier Martin · Maria Santacana · Ana Velasco · M. J. Diez-Castro · Dolores Cuevas · M. Jose Panadés · Serafin González · Antonio Llombart · Edelmiro Iglesias · Xavier Matias-Guiu

Sentinel lymph node from the fourth luminal A CK19-negative breast carcinoma. a Lymph node section showing a macrometastasis of 9 mm in maxim diameter. b View of the metastatic population of cells by means of CKAE1/AE3 pan-cytokeratin immunostaining of the same section. CK19 immunostaining of the same lymph node section (c and d). Only a small proportion of metastatic cells express CK19. a H&E staining, original magnification × 40. b Original magnification × 200. c Original magnification × 40. d Original magnification × 200
“..... there is no correlation between CK19 protein expression and CK19 RNA level neither within the primary breast cancer nor within the metastatic node; moreover, no correlation as well has been found between protein expression in NCB and mRNA level in metastatic lymph nodes. Thus, our results suggest that there is no evidence-based reason to stain every NCB for CK19 before performing OSNA in patients with breast cancer”.......
FNA tested with CK19 IHC:
Positive

FNA tested with CK19 IHC:
Negative
All CK19 (IHC) negative samples here.
Analysis of sentinel lymph node (SLN) by means of One-Step Nucleic Acid Amplification (OSNA) is being used increasingly as a very sensitive and quick method for intraoperative axillary staging in patients with breast cancer. This molecular diagnostic assay detects the expression level of cytokeratin 19 (CK19), CK19 immunostaining in core biopsies has been recommended in selecting patients eligible for OSNA analysis because SLNs with metastatic involvement by CK19-negative breast cancers may result in a false negative result by OSNA.
Metastatic breast cancer: mechanisms and opportunities for cytology

D. Martins*, F. Beca*† and F. Schmitt*‡§
*IPATIMUP – Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal, †Department of Medical Oncology, Dana-Farber Cancer Institute/Harvard Medical School, Boston, MA, USA, ‡Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, §Department of Pathology, University Health Network, Toronto, ON, Canada

Accepted for publication 7 April 2014

FNAC is currently the best method for repeated biopsies of a tumor, as it is minimally invasive, safe, cost-effective and can be coupled with modern ancillary techniques, making it ideally placed to monitor biological changes for adaptive therapeutic choices.

The use of biomarkers that until now has represented a limit for the survival of breast FNA as a diagnostic technique of first choice, is about to become the real strength of this technique thanks to new molecular techniques that exploit the quantitative determination of mRNA in place of or in support of protein expression data.
METASTATIC BREAST CANCER AND CYTOLOGY

Utilization of fine-needle aspiration biopsy (FNAB) cytology for the diagnosis of diseases of the breast has been met with both excitement and uncertainty during the last couple of decades. Presently, FNAB for the diagnosis of primary and metastatic breast lesions is on the rise again. This is probably due to:

- its fast turnaround time
- cost efficiency
- minimal invasiveness

characteristics of this sampling modality which are particularly crucial for patients requiring frequent repeat biopsy in the setting of metastatic lesions.

In our experience we use FNAB for diagnosis of loco-regional recurrence in breast cancer:

PERIOD 2004-MAY 2019

-2893 breast cancer surgically treated

87 loco regional recurrence diagnosed through FNAB
**Day 1**
- FNAC
- Recovery from the archive
- Slice
- Deparaffinization

**Day 2**
- RT-qPCR of the biomarkers (Reverse transcription PCR)
- Lysis
- Reverse transcription PCR
- Analysis by proprietary algorithm

**Processing time:**
- 30’ lysis
- 30’ cDNA synthesis
- 90’ RT-qPCR

**Molecular report**
- Molocholar strafification of samples
Cytopathologists have a key role to play in the future of molecular testing, by guiding clinical colleagues toward the appropriate use of cytology specimens for molecular testing and by participating in studies to define the performance of these molecular tests.

- The morphological data with its strength in recognizing the benign lesions from the malignant ones remains the most difficult challenge.

Thank you!
12th EFCS Tutorial

The next EFCS tutorial of cytopathology will be held in Trieste, Italy, from the 8th to the 12th of June 2020.
• **Topics:**
  - Breast
  - Urine
  - Respiratory tract
  - Pancreas and biliary tract
  - Salivary glands
  - Thyroid
  - Serous effusions
  - Gynaecological cytology
  - Soft tissue tumours
• **Tutors:**
  - Christine Bergeron (France)
  - Lukas Bubendorf (Switzerland)
  - Ben Davidson (Norway)
  - Claudio Doglioni (Italy)
  - Ivana Kholova (Finland)
  - Jerzy Klijanienko (France)
  - Giovanni Negri (Italy)
  - Fernando Schmitt (Portugal)
  - Danijela Vrodljak Mozetic (Croatia)
  - Fabrizio Zanconati (Italy)
• The tutorial will be followed by the QUATE examination
Save the date!

12th Annual EFCS Tutorial

Trieste, 8-12 June 2020

University Hospital of Trieste
Strada di Fiume 447, 34149 Trieste, Italy

Local Host: Fabrizio Zanconati (fabrizio.zanconati@asuits.sanita.fvg.it)
EFCS Tutorial Chair: Luigi Di Bonito (luigidibonito@yahoo.it)
See you in Trieste