The Power of Genomics:
Improving genetic detection of antibiotic resistance in *Mycobacterium tuberculosis*

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APHL TB Meeting
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Isoniazid & Pyrazinamide
Isoniazid (INH) Mechanism of Action

INH (prodrug) → KatG → INH-NAD adduct (active compound) → Mycolic acids (essential for cell wall integrity and growth) → InhA
Isoniazid (INH)

1) Effective for treating both latent and active TB disease
2) Well tolerated with low toxicity
3) Administered orally
4) Depending the country, 7-45% of TB is \( \text{INH}^R \)
5) \( \text{INH}^R \) is highly associated with subsequent treatment failures, and overwhelmingly precedes the acquisition of resistance to other drugs
Primary Loci Associated with INH Resistance

- **Rv1910c** (katG regulatory region)
- **furA**
- **katG** (activates INH)
- **fabG1** (inhA regulatory region)
- **inhA** (target of active INH)

(probably not directly involved in INH resistance)

- **oxyR** (ahpC regulatory region)
- **ahpC** (compensates for physiological KatG inactivity)
- **ahpE** (helps keep AhpC reduced and active)
*katG* and *inhA* mutations confer ~90% of INH resistance

<table>
<thead>
<tr>
<th>Drug</th>
<th>Locus</th>
<th>No. of isolates</th>
<th>Accuracy values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
<td>Susceptible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>With mutation</td>
<td>Without mutation</td>
</tr>
<tr>
<td>INH</td>
<td><em>katG</em></td>
<td>181</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td><em>inhA</em></td>
<td>35</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td><em>katG</em> and/or <em>inhA</em></td>
<td>192</td>
<td>20</td>
</tr>
</tbody>
</table>
We want to “close the gap” between current 90% accuracy and desired 100% accuracy for INH resistance detection...

Need to find new or uncharacterized mutations that might cause INH resistance...

To do this, sequence genomes from INH-resistant strains lacking common INH$^R$ mutations
Strain Selection

Drug Survey (MLB)/PETTS Collections/ MDDR
- 314 clinical isolates from U.S. and abroad (MLB)
- 1278 MDR-TB isolates (PETTS)
- MDDR isolates (12 months)

Phenotypically INH resistant
- Agar proportion

Genetically susceptible (no *katG* S315T or *inhA* promoter mutations)
- Hain LPA test
- Sanger
- Pyro

Final included strain count = 53
MICs performed to quantify and confirm INH resistance

Alamar blue assay (work done by Alexandra Mercante, Ph.D.)

• Colorimetric...growth of bacilli turns the well pink
• Results in 7-9 days
• Plate must be prepared by the technician

(Trek) Sensititre

• Growth is determined visually
• Results in 10-21 days
• Plate is pre-loaded with 1st and 2nd line TB drugs
INH MIC DATA
Whole genome sequencing workflow

1. Grow in broth
2. Extract DNA (Zymo ZR Fungal/Bacterial DNA Miniprep Kit)
3. Quantify DNA (Qubit broad range assay)
4. Fragment DNA on Covaris sonicator and prepare library (NuGen Ovation Ultralow v2)
5. Sequence genome (Illumina MiSeq)
6. Assemble genome from sequencing reads and compare against H37Rv pansusceptible strain for mutations (SeqMan NGen/SeqMan Pro/Unified Variant Pipeline [Ezewudo et al])

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**Diagram Notes**
- Grow in broth
- Extract DNA (Zymo ZR Fungal/Bacterial DNA Miniprep Kit)
- Quantify DNA (Qubit broad range assay)
- Fragment DNA on Covaris sonicator and prepare library (NuGen Ovation Ultralow v2)
- Sequence genome (Illumina MiSeq)
- Assemble genome from sequencing reads and compare against H37Rv pansusceptible strain for mutations (SeqMan NGen/SeqMan Pro/Unified Variant Pipeline [Ezewudo et al])
Mutations Identified

- **katG regulatory region**
  - Rv1910c
  - furA
  - katG

- **inhA regulatory region**
  - fabG1
  - inhA

- **ahpC regulatory region**
  - oxyR’
  - ahpC
  - ahpD

- **fabG3**

Mutations Identified:


- g-142a, g-88a, -84tc ins, c-81t, g-74a, c-57t, c-54t, c-52t, g-51a, g-48a, -47t ins

- S25, P11P, M78L, D97D, E149G

Regulatory regions:

- **katG** (activates INH)
- **inhA** (target of active INH)
- **ahpC** (compensates for physiological KatG inactivity)
- **fabG1** (unknown role in INH resistance)
### Which mutations were most common in our sampling?

<table>
<thead>
<tr>
<th>Number of strains</th>
<th>Number of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>katG_R463L</td>
</tr>
<tr>
<td>12</td>
<td>ahpCprom_G-142A</td>
</tr>
<tr>
<td>10</td>
<td>ahpD_M78L</td>
</tr>
<tr>
<td><strong>10</strong></td>
<td>(inhA_prom G-154A) fabG1_L203L</td>
</tr>
<tr>
<td>5</td>
<td>inhA_I194T</td>
</tr>
<tr>
<td>4</td>
<td>katG_V1A</td>
</tr>
<tr>
<td>4</td>
<td>(inhA_prom C-40T) fabG1_G241G</td>
</tr>
<tr>
<td>4</td>
<td>fabG3_I6l</td>
</tr>
<tr>
<td>3</td>
<td>ahpC_prom_C-52T</td>
</tr>
<tr>
<td>3</td>
<td>ahpC_prom_G-48A</td>
</tr>
<tr>
<td>3</td>
<td>ahpC_prom -47insT</td>
</tr>
<tr>
<td>2</td>
<td>katG_W328R</td>
</tr>
<tr>
<td>2</td>
<td>(katG_prom G-757T) Rv1910c_G144G</td>
</tr>
<tr>
<td>2</td>
<td>inhA_G3G (silent)</td>
</tr>
<tr>
<td>1</td>
<td>katG_P29P (silent)</td>
</tr>
<tr>
<td>1</td>
<td>katG_W107STOP</td>
</tr>
<tr>
<td>1</td>
<td>katG_G123fs</td>
</tr>
<tr>
<td>1</td>
<td>katG_N138S</td>
</tr>
<tr>
<td>1</td>
<td>katG_S140N</td>
</tr>
<tr>
<td>1</td>
<td>katG_W161R</td>
</tr>
<tr>
<td>1</td>
<td>katG_W161Q</td>
</tr>
<tr>
<td>1</td>
<td>katG_G169S</td>
</tr>
<tr>
<td>1</td>
<td>katG_T203T (silent)</td>
</tr>
<tr>
<td>1</td>
<td>katG_P232A</td>
</tr>
<tr>
<td>1</td>
<td>katG_G234E</td>
</tr>
<tr>
<td>1</td>
<td>katG_G285R</td>
</tr>
<tr>
<td>1</td>
<td>katG_W300R</td>
</tr>
<tr>
<td>1</td>
<td>katG_S315G</td>
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<tr>
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<td>katG_S315N</td>
</tr>
<tr>
<td>1</td>
<td>katG_S383L</td>
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<tr>
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<td>katG_E402STOP</td>
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<td>katG_F408L</td>
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<tr>
<td>1</td>
<td>katG_L415P</td>
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<tr>
<td>1</td>
<td>katG_V423fs</td>
</tr>
<tr>
<td>1</td>
<td>katG_A478del</td>
</tr>
<tr>
<td>1</td>
<td>katG_G490D</td>
</tr>
<tr>
<td>1</td>
<td>katG_D573G</td>
</tr>
<tr>
<td>1</td>
<td>katG_K600ins_ggg</td>
</tr>
<tr>
<td>1</td>
<td>katG_A606P</td>
</tr>
<tr>
<td>1</td>
<td>katG_G630fs</td>
</tr>
<tr>
<td>1</td>
<td>katG_T677fs</td>
</tr>
<tr>
<td>1</td>
<td>katG_N701D</td>
</tr>
<tr>
<td>1</td>
<td>(katG_prom G-433C) furA_R20G</td>
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<tr>
<td>1</td>
<td>inhA_I21T</td>
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<td>1</td>
<td>ahpC_P44R</td>
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<td>1</td>
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<td>1</td>
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<tr>
<td>1</td>
<td>ahpC_prom_G-51A</td>
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<tr>
<td>1</td>
<td>ahpD_S25 (silent)</td>
</tr>
<tr>
<td>1</td>
<td>ahpD_P11P(silent)</td>
</tr>
<tr>
<td>1</td>
<td>ahpD_D97D(silent)</td>
</tr>
<tr>
<td>1</td>
<td>ahpD_E149G</td>
</tr>
<tr>
<td>1</td>
<td>fabG1_prom_T-130C</td>
</tr>
</tbody>
</table>

- **Yellow** = anticipated to cause resistance
- **Red** = proven to cause resistance in *Mtb* by func. genetics
Functional genetics
using recombineering to test the *katG* V1A mutation
Only a handful of INH\textsuperscript{R}-associated mutations have been confirmed by functional genetics in \textit{Mtb}.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Mutation (mechanism)—mutation frequency at that site among INH\textsuperscript{R} specimens (%)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| \( katG \)       | \( S315T \) (decreased INH-NAD adduct formation)—\( 64.20\% \)  
                      \( T275P \) (unstable KatG protein)—\( 0.20\% \)  
                      \( W300G \) (decreased INH-NAD adduct formation)—\( 0.07\% \) | • Pym et al., 2002 Infect Immun  
                   • Richardson et al., 2009 Int J Tuberc Lung Dis  
                   • Cade et al., 2010 Prot Sci |
| \( furA-katG \) IR | \( g-7a \) (decreased \( katG \) expression)—\( 0.18\% \)  
                      \( a-10c \) (decreased \( katG \) expression)—\( 0.11\% \)  
                      \( g-12c \) (decreased \( katG \) expression)—\( 0.11\% \) | • Ando et al., 2011 Mol Micro |
| \( furA-katG \) upstream deletion | Deletion of the 134 bp fragment (-134 to -1 of \( furA \)) as part of a larger 7.2 kb deletion upstream of \( katG \) (decreased \( katG \) transcription due to loss of a putative promoter upstream of \( furA \) driving \( katG \) expression)—\( \sim 7\% \) | • Hang Siu et al., 2014 AAC |
| \( inhA \)        | \( S94A \) (decreased INH-NAD adduct binding to InhA)—\( 1.21\% \)  
                      \( c-15t \) (increased \( inhA \) expression)—\( 19.21\% \) | • Vilcheze et al., 2006 Nat Med |
| \( mabA/fabG1 \)  | \( g609a/L203L \) (increased \( inhA \) expression from novel promoter)—\( 0.12\% \) | • Ando et al., 2014 Mol Micro |
|                  | \( \text{Total}=85.41\% \) | \( \sim 15\% \) of INH resistance remains unexplained by functional genetics in \textit{Mtb} |

\( \text{Total}=85.41\% \)
How do we choose which mutations to make?

~800-1000 SNPs in clinical isolates vs. H37Rv pan-susceptible reference strain

Focus on INH-associated loci (e.g. \textit{katG}, \textit{inhA}, \textit{ahpC}...)

Ignore mutations that occur in INH-susceptible phylogenetic neighbors (e.g. \textit{katG} R463L)

Analyze predicted impact on protein based on the sequence change

Ignore mutations that have already been proven by functional genetics

Or find mutations in entirely new genes that cause INH resistance!
Recombineering: Cleanly make point mutations in *Mtb* (no leftover genetic changes except desired mutation!)


Recombineering the *katG* V1A Mutation

1. Electroporate recombinogenic *H37Rv* with oligo encoding desired *katG* V1A mutation and an oligo to fix the broken Hyg resistance gene.

2. Select for resistance to hygromycin with or without INH to enriched for recombinants with desired V1A mutation.

3. Grow up colonies in broth without selection, then sequence to check for mutation...grow mutant in the presence of sucrose to select for clones that have lost pJV128.
Recombineering Results (*katG* V1A and S315T control)

Colonies from Hyg50/INH0.2 plates were grown and Sanger sequenced to confirm mutations.

Donor DNA

- **HygFix+katG S315T**
- **HygFix+katG V1A**
- **no DNA**

= spontaneous INH resistance
Plans for Confirmed Mutants

Plate serial dilutions of confirmed mutants on agar with 10% sucrose to select for bacilli that have lost the plasmid.

Grow sucrose-resistant bacilli in broth.

Perform whole-genome sequencing to confirm that mutation is present, plasmid has been lost, and no additional mutations have occurred.

Test drug resistance caused by mutation in isolation.
Conclusions-I

- Performed MICs and WGS on 46 clinical strains of Mtb that were INH$^R$ by agar proportion but lacked common mutations
  - 8/46 were susceptible to INH (MIC <0.250 ug/mL)
  - 38/46 were resistant to INH (MIC >0.250 ug/mL)
  - 61 different mutations found in INH-associated loci
    - 1 mutation (fabG1 L203L) already proven to confer INH resistance
    - 12 mutations reasonably anticipated to confer INH resistance (present in inhA promoter, inhA ORF, katG ORF)
    - 48 mutations with unknown role in INH resistance (may also include some that are phylogenetic markers)
  - 2/46 strains were highly INH resistant (MIC >4 ug/mL) but had NO mutations in any of the classically associated loci
  - 3/46 strains were INH resistant (range: 0.25-4 ug/mL) and only had 1 mutation in these loci (the katG R463L phylogenetic marker)
Conclusions-II

- Recombineering mutations into H37Rv pansusceptible strain
  - 4/46 strains in our study had the katG V1A mutation
    - 3/4 V1A mutants had an INH MIC >0.5 ug/mL
  - Successfully generated katG V1A, katG S315T, and ahpC c-54t mutations into H37Rv
    - All mutations confirmed by sequencing
    - katG V1A and S315T mutants grew on INH selection (0.2 ug/mL)
  - Developed and successfully tested high-throughput IONTorrent screening method for nonselectable mutations
    - Universal, short amplicon sequencing applicable to any gene
    - INH (0.2 ug/mL) prevented growth of ahpC c-54t transformants
PYRAZINAMIDE
Pyrazinamide (PZA)

- A prodrug that must be converted to its active form, pyrazinoic acid, by the enzyme pyrazinamidase (PZase) which is encoded by the gene pncA.
Pyrazinamide (PZA)

- Active under acidic conditions
- Sterilizing activity against semidormant (persister) bacteria
- Part of standard 4 drug regimen
- Reduces length of treatment from 9–12 months to 6 months for drug susceptible TB
- Use in treatment of MDR-TB increases likelihood of a successful outcome\(^1\)
- Synergistic in new drug regimens\(^2-5\)

PncA, the *Mycobacterium tuberculosis* (Mtb) Pyrazinamidase (PZase)

- Mutations in *pncA* are associated with PZA resistance
- 187 amino acids (561 bp)
- No “hotspot” for mutations
- Multiple substitutions can occur at each position
  - Example: Histidine at position 57 changed to arginine, aspartic acid, leucine, proline, or tyrosine in different isolates
- Some strains contain insertions or deletions in *pncA*
- Some strains contain mutations in the *pncA* promoter region which likely alter the amount of PZase made, therefore affecting PZA susceptibility
Preserving Effective TB Treatment Study (PETTS)

- January 1, 2005–December 31, 2010
- 9 countries
  - Estonia, Latvia, Peru, Philippines, Russia, South Africa, South Korea, Thailand, and Taiwan
- Consecutive, consenting adults with pulmonary MDR TB enrolled at the start of second-line treatment
- Demographic, socioeconomic, clinical, and outcome data was collected
- Baseline and monthly follow-up sputum specimens were collected and cultured for patients with locally confirmed MDR TB
- Isolates were sent to U.S. CDC for additional first and second-line drug susceptibility testing, genotyping, and further analysis
Primary Objectives of this Study

- Determine the frequency and variety of \( pncA \) mutations among baseline PETTS isolates
- Correlate mutations with clinical outcome data
Methods

- 928 baseline isolates were selected
  - 14 excluded (not Mtb, no frozen stock, no amplification)

- Conventional sequencing of *pncA* plus 100 bp upstream
  - Aligned to wild-type *pncA* using DNASTAR Lasergene SeqMan Pro
  - Mutations identified

- Whole genome sequencing (WGS) on a subset of isolates for which conventional sequencing failed
  - Illumina technology
  - Aligned to H37Rv reference using DNASTAR Lasergene SeqMan NGen
  - Mutations identified
### pncA Sequencing Results by Country

<table>
<thead>
<tr>
<th>Country</th>
<th>Number Tested</th>
<th>WT (%)</th>
<th>Mutant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Country A</td>
<td>41</td>
<td>7 (17.1)</td>
<td>34 (82.9)</td>
</tr>
<tr>
<td>Country B</td>
<td>102</td>
<td>24 (23.5)</td>
<td>78 (76.5)</td>
</tr>
<tr>
<td>Country C</td>
<td>111</td>
<td>35 (31.5)</td>
<td>76 (68.5)</td>
</tr>
<tr>
<td>Country D</td>
<td>182</td>
<td>60 (33.0)</td>
<td>122 (67.0)</td>
</tr>
<tr>
<td>Country E</td>
<td>76</td>
<td>16 (21.1)</td>
<td>60 (78.9)</td>
</tr>
<tr>
<td>Country F</td>
<td>283</td>
<td>61 (21.6)</td>
<td>222 (78.4)</td>
</tr>
<tr>
<td>Country G</td>
<td>72</td>
<td>21 (29.2)</td>
<td>51 (70.8)</td>
</tr>
<tr>
<td>Country H</td>
<td>36</td>
<td>20 (55.6)</td>
<td>16 (44.4)</td>
</tr>
<tr>
<td>Country I</td>
<td>11</td>
<td>5 (45.5)</td>
<td>6 (54.5)</td>
</tr>
<tr>
<td>overall</td>
<td>914</td>
<td>249 (27.2)</td>
<td>665 (72.8)</td>
</tr>
</tbody>
</table>
# Frequency of Mutation Types

<table>
<thead>
<tr>
<th>Mutation Type</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single SNP only in pncA</td>
<td>448 (67.4)</td>
</tr>
<tr>
<td>SNP only in promoter</td>
<td>32 (4.8)</td>
</tr>
<tr>
<td>Multiple SNPs</td>
<td>18 (2.7)</td>
</tr>
<tr>
<td>Indel only</td>
<td>166 (25.0)</td>
</tr>
<tr>
<td>Indel and SNP</td>
<td>1 (0.2)</td>
</tr>
</tbody>
</table>
SNPs in \textit{pncA}

- 517 total SNPs (33 in promoter, 484 in ORF)
- Mutations found in 104 (out of 187) codons
- 177 unique SNPs identified
- Mutations identified in 4 promoter positions
- A(-11)G was the most frequently observed SNP overall and was found in isolates from 7 countries.
- 10 synonymous mutations (Ser18, Thr47, Ser65, Gly75, Gly150)
- 6 nonsynonymous mutations known not to cause resistance* (Val130Ala, Val163Ala, Val180Ala), however, three of these were in isolates with a second SNP

Indels observed in \textit{pncA}

- 167 total indels
  - 1 nucleotide - 19,952 nucleotides
    - 140 indels <3 nt
    - 27 indels \geq 3nt
- 14 isolates with \textit{pncA} entirely deleted
Outcomes

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Mutant</th>
<th>SNP</th>
<th>Indel</th>
<th>Promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>治愈率</td>
<td>55.1%</td>
<td>42.4%</td>
<td>46.6%</td>
<td>30.7%</td>
<td>42.4%</td>
</tr>
<tr>
<td>完全缓解率</td>
<td>7.3%</td>
<td>21.5%</td>
<td>16.8%</td>
<td>36.8%</td>
<td>12.1%</td>
</tr>
<tr>
<td>失效率</td>
<td>5.3%</td>
<td>10.6%</td>
<td>9.9%</td>
<td>12.3%</td>
<td>12.1%</td>
</tr>
<tr>
<td>死亡率</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>默认</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>转出</td>
<td></td>
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<tr>
<td>未知</td>
<td></td>
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</table>

图示说明了不同基因突变类型（WT、Mutant、SNP、Indel、Promoter）的治愈率、完全缓解率、失效率、死亡率等比例。
Conclusions

- Mutations were identified in 78% of isolates.
- Mutation rate varied from 44%-83% per country.
- SNPs were the most frequent type of mutation.
- SNPs were identified in 55% (104/187) of \textit{pncA} codons.
- Individuals with WT \textit{pncA} were less likely to have a negative outcome.
Future Steps

- Examine how clusters affect these data
- Evaluate the potential association of specific mutations and outcomes
- Compare genetic and phenotypic DST data
- Examine the mechanism of PZA resistance in isolates with WT *pncA*, but phenotypically resistant
- Assess which individuals were treated with PZA
  - Does this affect the outcome?
  - Does this affect the frequency of acquired resistance?
Acknowledgments-I

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  - Melisa Willby, Jeremiah Khayumbi, Tracy Dalton, Peter Cegielski
Acknowledgments-II

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Gail Starks*
Andrey Borisov*
Michael Chen*
Lauren Cowan
Lois Diem
Denise Hartline
Jamila Franklin
Dorothy Kaminski
Heather Alexander
Beverly Metchock
Tom Shinnick
Kathrine Tan*
Lizzy Smith

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Charlotte Kvasnovsky*
Melanie Wolfgang*
Lizzy Smith*

Tropical Disease Foundation, Manila, Philippines
Janice Campos Caoili*
Grace Egos
Maricelle Gler
Ruffy Guilatco
Nellie Mangubat
Imelda Quelapio
Thelma Tupasi

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Kai Kliiman
Piret Viiklepp
Manfred Danilovitz
Tiina Kummik
Klavidia Levina

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Girts Skenders
Ingrida Sture
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