

## GENOMIC ASSESSMENT IN TESTIS CANCER PATIENTS (GATE)

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#### INTRODUCTION

Testicular cancer represents 1% of adult neoplasms and 5% of urological tumours, with three to ten new cases per 100,000 males/per year in Western societies. Its incidence has increased during recent decades, particularly in industrialised countries, and keeps to rise. At diagnosis, 1-2% of cases are bilateral and the predominant histology is germ cell tumours (GCT) (90-95% of cases) [1].

Epidemiological risk factors for the development of TC are components of the testicular dysgenesis syndrome (which encompasses cryptorchidism), hypospadias, decreased spermatogenesis and impaired fertility [1-3] or disorders/differences of sex development [1]. Additional risk factors include family history of TC among first-degree relatives and the presence of a contralateral testicular tumour or germ cell neoplasia "in situ" [1, 4-6]. Recent genome-wide association studies revealed detectable susceptibility loci leading to an increased relative risk to develop TC [1, 7-11].

Infertility is a disease of nearly endemic proportions, affecting up to 15% of couples of reproductive age. Overall, a pure male factor infertility underlies the problem in at least 30% of cases.

As a whole, up to 50% of the primary infertility cases are either unexplained or classified as idiopathic, and most likely this involves that mostly could be attributed to a genetic aetiology [12]. Our preliminary data from a cohort of idiopathic infertile patients as well as genetic data from other cohorts and also previous reports showed that a large proportion of them, ranging from 40% to 20%, respectively, is associated with mutations of single genes, which are not included in the routine diagnostic work-up [12].

Growing clinical data shows that infertile men are at increased risk of developing unfavorable age-related comorbidities in almost 10% of cases [12-14]. Indeed, infertility *per se* is now considered not only an epiphenomenon of diseases but a disease, with relevant clinical implications and health-related outcome [12]. Indeed, it has become evident that infertile men are less healthy than age-comparable fertile men [12,15]. Associations have been described between infertility and non-malignant chronic diseases [15]. Moreover, data supports a link between male infertility and the risk of developing malignant diseases [16].

#### AIMS

- 1) Define genetic determinants of testis cancer (unilateral vs. bilateral)
- 2) Define genetic determinants of metachronous bilateral testis cancer vs. unilateral
- 3) Define genetic determinants of infertility vs. fertility, and the subsequent risk of developing testis cancer

#### METHODS

We foresee to enroll 100 testis cancer patients (Group 1), along with 100 infertile (Group 2) and 100 fertile men (Group 3) (UO1 = URI/UO Urologia IRCCS Ospedale San Raffaele, Milan, Italy).

UO1 will collect clinical variables for each participant: date of birth; ethnicity; complete medical history, including a compilation of the Charlson Comorbidity Index (CCI); history of undescended testis/testes; measured Body Mass Index (BMI); waist circumference; presence of varicocele; testicular volume; hormonal parameters; semen parameters (if available); partner's age; and, partner's health status (for Group 2 and Group 3).

Notably, UO1 will collect clinical data and biological material (e.g., samples of semen, stool, urine, blood) of a cohort of 200 white-European, non-Finnish infertile and fertile men. Likewise, liquid tissues to determine the genetic profile will be collected from 100 same-ethnicity testis cancer patients (either unilaterale and bilaterale).

UO2 = Genomic lab, Prof. Giorgio Casari, UniSR, Milan, Italy) will perform genomic analysis by whole-exome sequencing (WES) of the whole cohort of participants.

### ***Genetic variants identification and selection***

GATE will perform genomic analysis by whole-exome sequencing (WES) of: Group 1: testis cancer patients (n. 100 in prospective and retrospective blood samples available); Group 2: idiopathic primary infertile men [12] (n. 100 in prospective and retrospective blood samples available); and, Group 3: fertile men, as for WHO reference criteria [12] (n. 100 in prospective and retrospective blood samples available).

Among the possible Next Generation Sequencing (NGS) approaches to discover genetic determinants of human diseases, we selected WES to identify predisposing variants in our cohorts.

WES is a cost-effective approach compared to whole genome sequencing and allows to directly relate the DNA variant to the functional impairment of the gene product. Furthermore, WES gives us better chance to identify relevant variants than a dedicated, and therefore restricted, panel of genes, since the poor genetic information nowadays available on testicular cancer.

### ***DNA extraction***

We will extract the genomic DNA from blood samples using the Maxwell<sup>®</sup> 48 Instrument (Promega, Madison, WI, USA) and the Maxwell<sup>®</sup> RSC Blood DNA Kit (AS1400). The DNA concentration, the purity ratio (260/280; 260/230) and the fragmentation are assessed using the Qubit<sup>®</sup> 3.0 Fluorometer, the Nanophotometer<sup>®</sup> P-Class 300 and the 2100 Bioanalyzer Instrument respectively.

### ***Library preparation***

The WES of all participants will be performed with the Illumina technology, a type of NGS approach. It provides different steps listed below:

1. Enzymatic DNA fragmentation using the SureSelect Enzymatic Fragmentation kit (Agilent);
2. Library preparation, selection and amplification performed using the SureSelect XT HS/Low Input Kit with All Exome V7 RNA Oligos (Agilent);
3. Library dilution, Free Adapter Blocking reagent (FAB), spike-in with the PhiX and denaturation;
4. Loading of S2 flow cells (Illumina) on Illumina<sup>®</sup> NovaSeq<sup>TM</sup> 6000 (Illumina). A pair-end sequencing is performed;
5. The variant calling (vcf file generation) is done with the germline pipeline of Dynamic Read Analysis for GENomics (Dragen, Illumina).

Steps 1 and 2 are performed on the Hamilton MicroLab STAR M automated technology.

### ***Variant interpretation***

Vcf files are annotated with ANNOVAR (DOI: 10.1093/nar/gkq603). To identify testicular cancer predisposing variants, several filtering steps are applied:

1. PASS variants (quality filter);
2. Variants with a frequency less than 1% in both Global and Non-Finnish European (Nfe) GnomAD population;
3. Variants that arise in exonic and splicing regions (until +/- 8 bp from start or end of exon);

After these filters, we have retained only the variants belonging to genes included in a manually curated genes library containing genes related to cancer and spermatogenesis.

At the end, variants are classified according to ACMG guidelines (DOI: 10.1038/gim.2015.30; 10.1038/gim.2017.210)

Starting from an integrated bioinformatics analysis of available data, GATE will help to confirm and prioritize candidate genes causing testis cancer (uni vs. bilateral), idiopathic infertility and idiopathic-infertility cases associated with relevant clinical implications and oncologic health-related outcomes in men.

## IMPACT

Given the existing lack of research on the genetic as well as unhealthy ageing status over men's fertility potential, GATE will further investigate the potential genomic causes of idiopathic male infertility and the pathophysiological mechanisms behind the higher burden of clinically relevant oncological comorbidities observed in infertile men, thus mostly including testis cancer.

Thereof, GATE will address a clinically relevant question and will deliver information to define infertile men at actual risk of developing non-malignant and malignant comorbidities. According to the almost endemic dramatic prevalence of infertility, this is an unparalleled opportunity to identify the aetiologies of chronic disease in these men and develop tailored health prevention strategies with a significant rebound toward the national health systems.

Likewise, GATE will further help to explain the pathobiology behind the development of bilateral synchronous vs metachronous testis cancer in a selected cohort of same-ethnicity white-European, non-Finnish men.

This knowledge will be adopted to develop robust biomarkers/signatures of comorbidity prediction and develop effective tailored prevention strategies and measures of comorbidities development and progression in men with i) idiopathic infertility; and, ii) unilateral testis cancer.

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