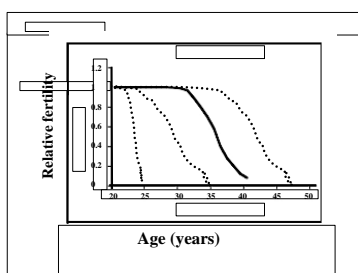


Genetic determinants of ovarian reserve

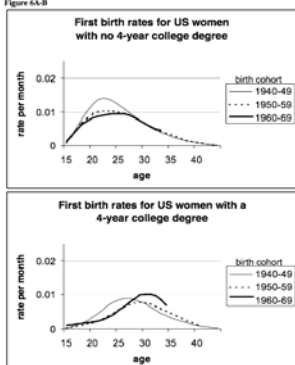
Daniela Toniolo
DIBIT- San Raffaele Scientific Foundation
Milano, Italy

Women fertility starts to decrease around 30 years



From Noord-Zaadstra et al. 2001

Figure 6A-B



Source: June 1985-1995 Current Population Surveys. See text for details.

Steven P. Martin
Department of Sociology
and Maryland Population Research Center
University of Maryland, College Park

October 2002

Premature Ovarian Failure

Disorder of ovulation characterized by:

- Elevated levels of gonadotropins before the age of 40
- Primary or secondary amenorrhea or early menopause
- Ovaries reduced or absent
- Ovarian follicles reduced or absent

Corresponds to about 10% of female sterility
Its relevance is increasing with the increase in the age of first child bearing

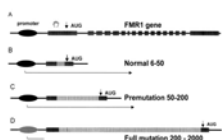
Premature Ovarian Failure has a genetic basis

30-40% are familial cases

Mutations in genes responsible for rare genetic forms inherited as autosomal recessive (FSHR), X-linked dominant (BMP15, NOBOX) and syndromic forms (FOXL2, E1F2b)

very low epidemiologic relevance

FRAXA premutation is a common risk-factor

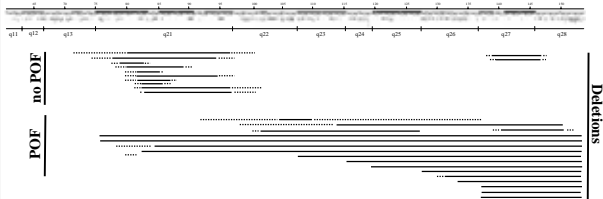


20-30% of women with premutation have POF
(OR=21; 95%CI:15-27)

3-5% of POF affected women carry a FRAXA premutated allele
(about 13% of familial cases-about 2% of sporadic cases)

POF seems to behave also as a complex disorder
caused by several risk genetic factors

Large deletions of the X chromosome are associated with POF



the rarity of small monosomies is in favor of a cumulative effect of the large deletions on several genes of the X chromosome long arm and of POF as a complex disorder

1. Analysis of chromosomal rearrangements and elucidation of a novel mechanism responsible for the X-linked POF critical region

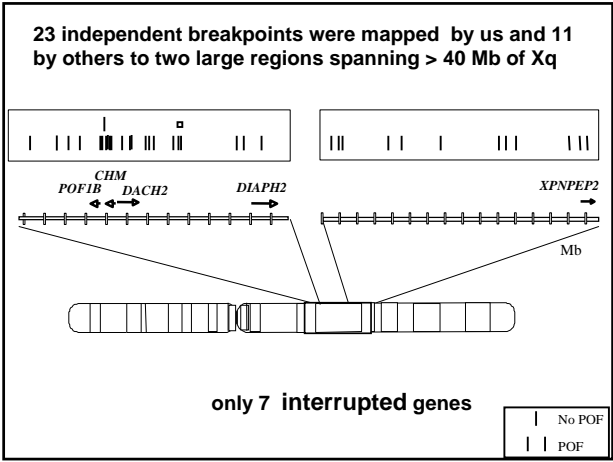
2. POF as a complex disorders: new evidence for risk factors

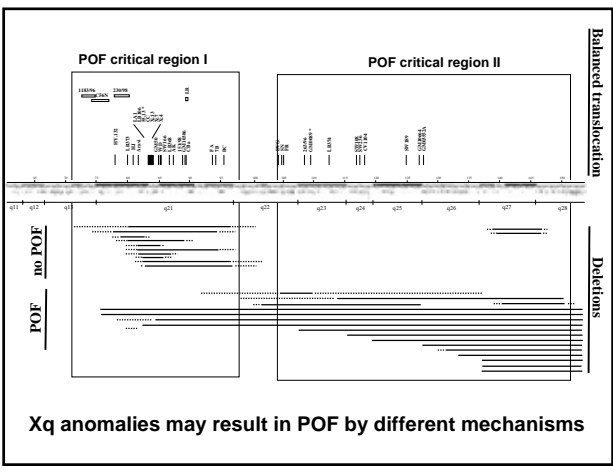
3. A new approach to the study of POF

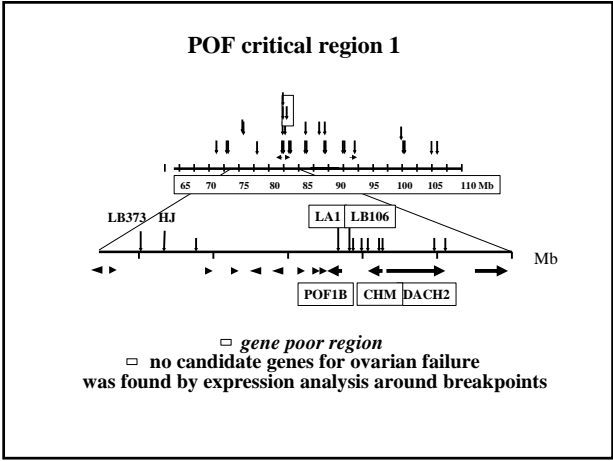
1. Analysis of chromosomal rearrangements and elucidation of a novel mechanism responsible for the X-linked POF critical region

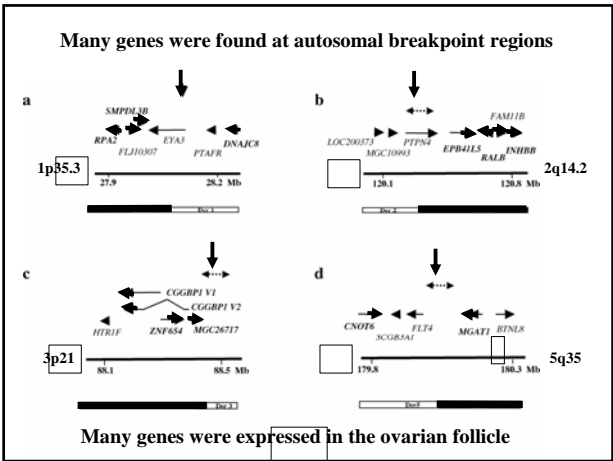
2. POF as a complex disorders: new evidence for risk factors

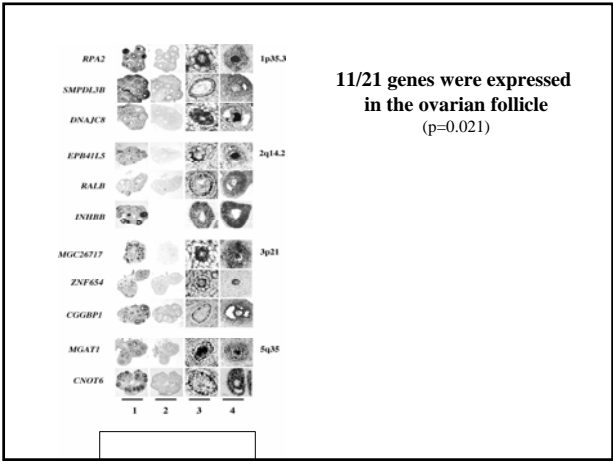
3. A new approach to the study of POF











The critical region 1

1. low recombination
2. gene poor
3. rich in LINE sequences
4. gene expression down regulated in oocytes
5. heterochromatic like organization

The epigenetic organization of the critical region 1 during oogenesis spreads to the autosomal genes translocated to it and cause silencing or mis-regulation of genes required for oocytes maturation

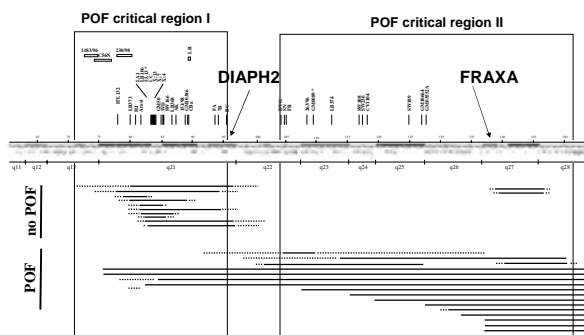
Genes involved in balanced translocations represent novel candidates for POF

1. Analysis of chromosomal rearrangements and proposal of a new mechanism for the POF critical region

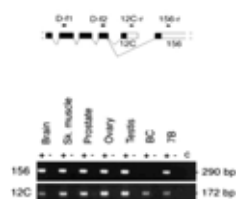
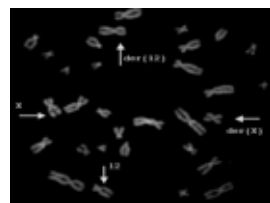
2. POF as a complex disorders: new evidence for *risk factors*

3. A new approach to the study of POF

Which are the genes involved in X monosomies ?

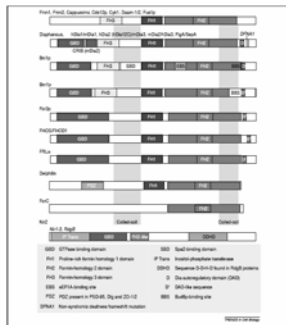


The DIAPH2 gene was found interrupted in a X:12 familial balanced translocation that interrupted the 3' end of the gene



Bione et al. 1998

The DIAPH2 gene and the formin homology family

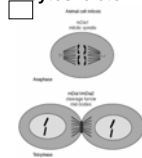


From: Wallar & Alberts
TCB 2003

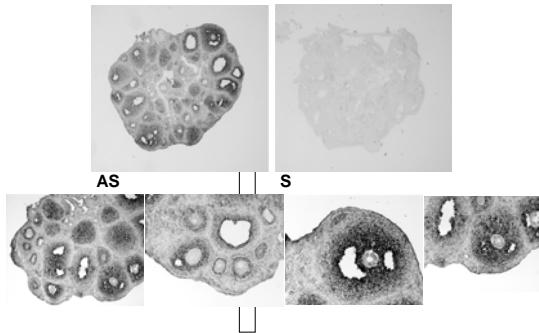
The Diaph2 protein is part of the evolutionarily conserved Formin Homology (FH) protein family

Formins share a common architecture of conserved domains

Formins direct dynamic remodeling of the cytoskeleton

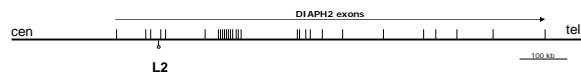


DIAPH2 is expressed in mouse ovaries

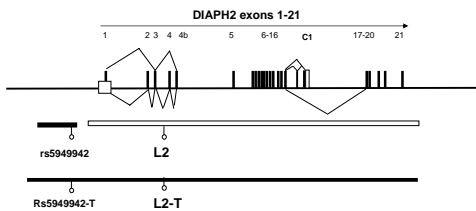


CD1 mice: P20 ovaries

One DIAPH2 intronic variant showed distortion in genotypes distribution between POF patients and controls



The L2 variant is not the risk factor: study of DIAPH2 haplotypes containing L2



identified the TT haplotype, which is more strongly associated to POF

TT haplotype association in North Italian POF cohort

	TT frequency in POF	TT frequency in Controls	One-tail X- square p value
Cohort 1 (POF=161, Con=477)	0,180	0,115	0,0014
Cohort 2 (POF=161, Con=471)	0,164	0,110	0,0058
Combined (POF=322, Con=948)	0,171	0,111	6x10-5
POF only familial cases (POF=135, Con=948)	0,181	0,111	0,0004

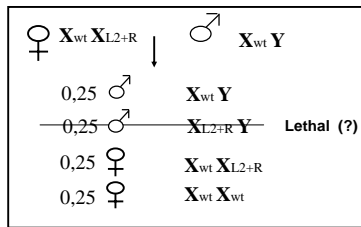
The TT haplotype is 6% more frequent in POF than in controls: OR 1.69

An excess of female children among carriers of the TT haplotype

	Total female	No info	without child	Females with child	Total child	F	M	F/M	p-value
North Italian cohort									
POF	322	69	154	99	170	93	77	1,21	0,102
Familial POF	135	8	67	60	108	64	44	1,45	0,054
Not familial POF	187	61	87	39	62	29	33	0,88	0,895
HI carriers									
POF:HI+	144	29	62	53	91	54	37	1,46	0,075
Familial POF:HI+	66	3	29	34	60	42	18	2,33	0,002
Not familial POF:HI+	78	26	33	19	31	12	19	0,63	0,209
TT carriers									
POF:TT+	104	20	45	39	63	38	25	1,52	0,101
Familial POF	45	2	20	23	36	27	9	3,00	0,003
Not familial POF	59	18	25	16	27	11	16	0,69	0,336
Non TT carriers									
POF	183	40	95	48	82	41	41	1,00	1
Familial POF	72	5	40	27	50	24	26	0,92	0,777
Not familial POF	111	35	55	21	32	17	15	1,13	0,724
TT not determined									
POF	25	9	14	12	25	14	11	1,27	0,549
Familial POF	18	1	7	10	22	13	9	1,44	0,394
Not familial POF	17	8	7	2	3	1	2	0,50	0,564

How to explain the excess of female children ?

Is the DIAPH2 L2 risk-allele lethal in males?



Expected: sex ratio distortion in the offspring of POF familial cases carrying the L2 risk-allele

2F : 1M instead of the expected 1F : 1M

Conclusions 2

- ☐ The DIAPH2 gene is a new **susceptibility gene** for POF
- ☐ Two copies of the **DIAPH2** gene must be functional to ensure succesfull oogenesis and early embryogenesis
 - *DIAPH2* transcript is highly expressed in the ovary and in particular in follicular granulosa cells
 - *Diaph2* may be involved in cytoskeleton reorganization during Oogenesis
 - As demonstrated for the *dia* protein of *Drosophila*, defective cytokinesis would result in impaired germ and follicular cells due to polyploidy
- ☐ The DIAPH2 result is in favor of the idea that POF is a complex disorder caused by several risk factors

1. Analysis of chromosomal rearrangements and proposal of a new mechanism for the POF critical region

2. POF as a complex disorders? new evidence for risk factors

3. A new approach to the study of POF

Search for association

in the Italian POF cohort of >550 women/1100 controls
and in a second cohort of early menopause women

Candidate gene analysis

1.X linked genes, selected based on their localization in Xp or Xq and their expression in ovary from database information: about 50 genes

2.Autosomal genes identified in our study: 25 genes

3.Other genes involved in POF:

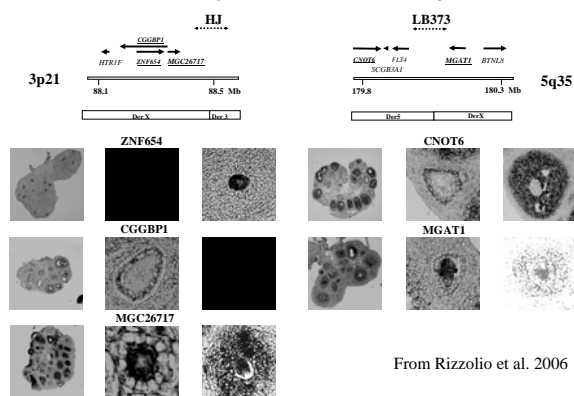
BMP15 and other TGF β family genes,

Genes involved in hormonal pathways (inhibin, FGFR et al):

20 genes

4. Genes identified in animal models (mouse and sheep)

Autosomal genes flanking POF associated breakpoints were expressed in mouse ovary



An allele in MGAT1 is associated with late onset Premature Ovarian Failure (POF)

Name	Allele	Frequency in POF	Frequency in Controls	p value	Corrected	OR
All (POF=667, Con=559)						
501	G	0,706	0,649	0,0035	0,028	1,2
601	A	0,887	0,873	0,3044		
666	A	0,154	0,149	0,7428		
>35 (POF=497, Con=559)						
501	G	0,713	0,649	0,0022	0,0176	1,37
601	A	0,888	0,874	0,3461		
666	A	0,159	0,149	0,5327		

The G allele of the SNP 501 is 6.4% more frequent in late onset POF

MGAT-1 encodes N-acetylglucosaminyltransferase1 (GlcNAc-T1), an enzyme that initiates complex and hybrid N-glycan synthesis

MOLECULAR AND CELLULAR BIOLOGY, Nov. 2004, p. 9920-9929
0730-7580/04/\$08.00+0 DOI: 10.1128/MCB.24.22.9920-9929.2004
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Vol. 24, No. 22

Inactivation of the *Mgat1* Gene in Oocytes Impairs Oogenesis, but Embryos Lacking Complex and Hybrid N-Glycans Develop and Implant

Shaolin Shi,¹ Suzannah A. Williams,¹ Antri Seppo,^{1,†} Henry Kurniawan,¹ Wei Chen,^{1,‡} Zhengyi Ye,² Janey D. Marth,² and Pamela Stanley^{1*}

Conclusions

▪By searching for risk factor for POF, among candidate genes, we have identified 2 genes, the X-linked gene *DIAPH2* and the *MGAT1* gene, on chromosome 5.

▪The definite proof of their role may only come from the availability of additional and different very large cohorts of patients and population matched controls

▪Both risk factors represent more common risks than any other identified to data, with the exception of the *FMR1* premutation

▪Our result also indicate that by using this kind of approach on larger scale, e.g. by whole genome genotyping large patients cohorts, it should be possible to identify enough variants to develop a first generation molecular diagnosis for POF and possibly predict the ovarian reserve at young age.



Collaborations: The "NIDO"

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Tanguy Corre
Manuela Testa

DIBIT-HSR
Milano
and IGM-CNR
Pavia

Orsetta Zuffardi
and collaborators
U. of Pavia
for FISH and
Genomic array

Power calculation

The study will exceed 90% probability

- to identify variants associated with POF with allelic frequency in the range of 0.1-0.3 with an odd ratio between 2.2 and 2.5

and

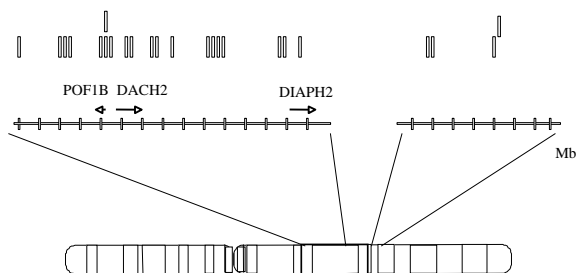
- to establish a first generation diagnostic tool for female fertility

An excess of POF L2 carriers have only females

	1 child (n=13)	2 child (n=15)	3 child (n=2)	4 child (n=1)	5 child (n=1)
Observed	8	7	1	1	0
Expected	6.5	3.75	0.25	0.0625	0.0312

One-cell-X square (d.f.=4)
p-value= 0,001

In the last years we have characterized X;autosome balanced translocations on the long arm of the human X chromosome



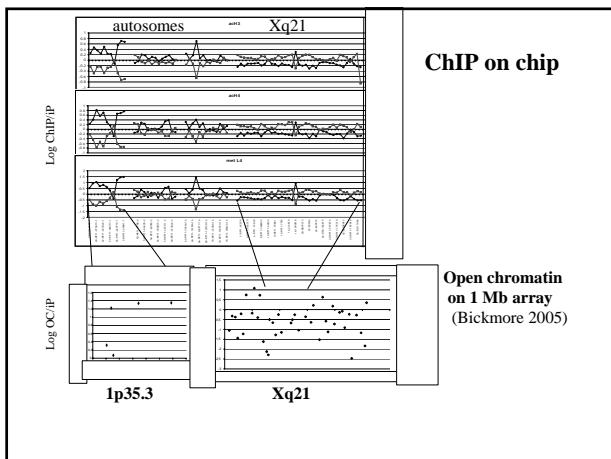
We have identified three genes interrupted by balanced translocations

What is the role of the POF critical region I?

Gene poor region

Low recombination
(similar to centromeres)

Is Xq21 an heterochromatic region?



POF critical region I

Gene poor region

Low recombination
(similar to centromeres)

Closed chromatin

**Low acetylation
and K4 methylation**

Conclusions

The Xq POF critical region can be divided in two portions

Region 1 is an heterochromatic region that seems to exert a position effect on autosomal genes in balanced translocations.

POF associated to region 1 appears to be caused by alteration of the expression of one or several genes translocated to the critical region.

No X linked genes with ovarian specific expression seem to be present on region 1

Region 2, in distal Xq is a relatively more gene rich region

Ovary expressed genes in region 2 may be required in double dose for ovarian function and their deletion or direct modification by mutations should be responsible for POF

May the DIAPH2 gene represent a susceptibility gene for POF?

Variants in regulatory regions may interfere with correct gene expression thus representing **risk-factors** for POF

Such variants may be present in the population in a relative high frequency as they would not be negatively selected until women reproductive age increased

**As a consequence:
Case-control association study to evaluate putative distortion
in alleles and/or haplotypes distribution
among POF patients and controls**

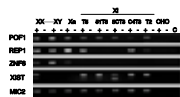
Genes interrupted by the breakpoints

POF1B

POF1B gene was found interrupted in its third intron by the breakpoint of patient LA1 who experienced very early secondary amenorrhea

POF1B is a novel gene with no homology to known genes

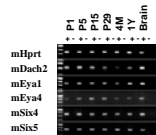
POF1B gene is not subject to X inactivation



DACH2

the DACH2 gene is interrupted in its eighth intron by the breakpoint of patient GM5011A

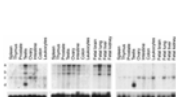
DACH2 is one human homologue of the *Drosophila dachsund* gene mainly involved in developmental processes



DIAPH2

interrupted between its two alternative last exons by the breakpoint of a X;12 translocation

the DIAPH2 gene is one of the two human homologues of the *Drosophila dia* gene that causes sterility in mutants fly



LB106

	MK4/ActH3	MK4/ActH4	ActH4/ActH3	MK4/ActH3	MK4/ActH4	ActH4/ActH3	MK4/ActH3	MK4/ActH4	ActH4/ActH3
MGC10993	6.49	2.30	2.82	7.70	1.87	4.12	0.84	1.23	0.68
PTPN4	3.98	2.33	1.71	5.41	1.98	2.73	0.74	1.17	0.63
EPBF1	5.60	26.84	0.21	3.08	6.01	0.51	1.81	4.46	0.41
EPB4.1L5	9.65	27.84	0.35	2.70	5.13	0.53	3.57	5.43	0.66

LA1

	MK4/ActH3	MK4/ActH4	ActH4/ActH3	MK4/ActH3	MK4/ActH4	ActH4/ActH3	MK4/ActH3	MK4/ActH4	ActH4/ActH3
RPA2	0.88	0.82	1.07	0.95	1.27	0.75	0.93	0.65	1.44
SMPDL3B F1	2.44	1.51	1.62	7.16	5.10	1.41	0.24	0.30	1.15
SMPDL3B	5.80	4.06	1.43	13.49	6.72	2.01	0.43	0.60	0.71
FLJ10307 f2	4.43	2.79	1.59	13.33	5.25	2.54	0.33	0.53	0.63
FLJ10307	4.66	4.53	1.03	8.56	8.23	1.04	0.54	0.55	0.99
FLJ10307 iso	2.27	1.82	1.25	4.68	4.94	0.95	0.49	0.37	1.32
EYA3 F1	1.02	1.87	0.54	1.64	3.50	0.47	0.62	0.54	1.16
EYA3	0.48	1.02	0.47	0.96	1.79	0.54	0.50	0.57	0.88
EYA3 F2	0.90	1.37	0.66	1.02	1.70	0.60	0.88	0.81	1.09
PTAFR	4.40	3.63	1.21	4.66	2.66	1.75	0.94	1.37	0.69
DNAJC8	0.70	1.75	0.40	0.60	1.74	0.35	1.16	1.01	1.15

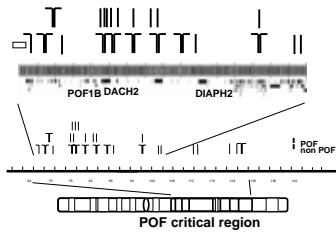
An excess of heterozygotes at the L2 locus is associated with POF

	Ct	CC+tt
POF	107	146
Controls	125	282

Chi-square test (1d.f)
p=0,0024

The Xq21-22 POF critical region

A total of 26 independent breakpoints were mapped in the Xq21-22 interval



Two emerging evidences:

The majority of the breakpoints (23/26) occur in a gene-poor region
Chromatin effect?

Only three genes were found interrupted
Are they responsible genes for POF?

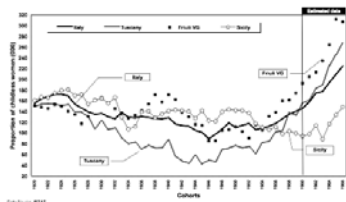
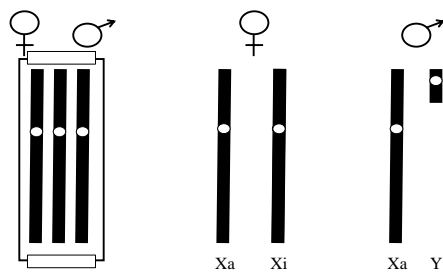


Figure 3. Trends in Completed Fertility by Generation in Italy, Friuli Venezia Giulia, Sicily and Tuscany. Cohorts: 1920-1948 (Source: ISTAT)

Different gene dosage of the X chromosome

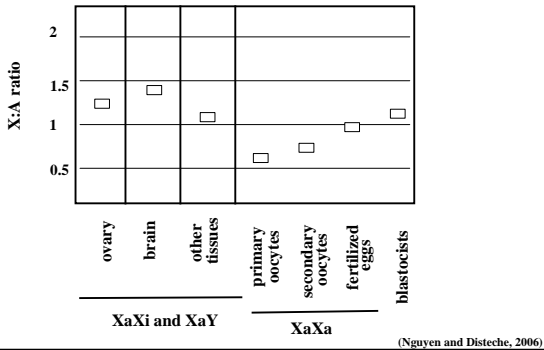


Equal global gene expression

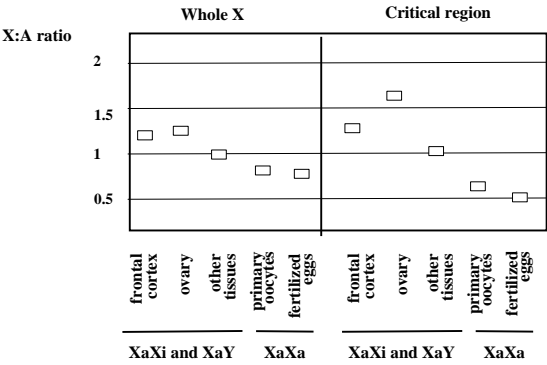
A:A ~ 1

X:A ~ 1

Global gene dosage of the active X chromosome
from analysis of expression micro array data in many tissues



Global gene dosage of the POF critical region

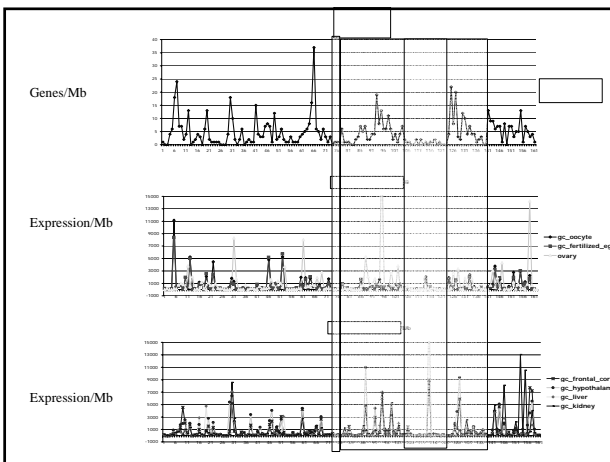


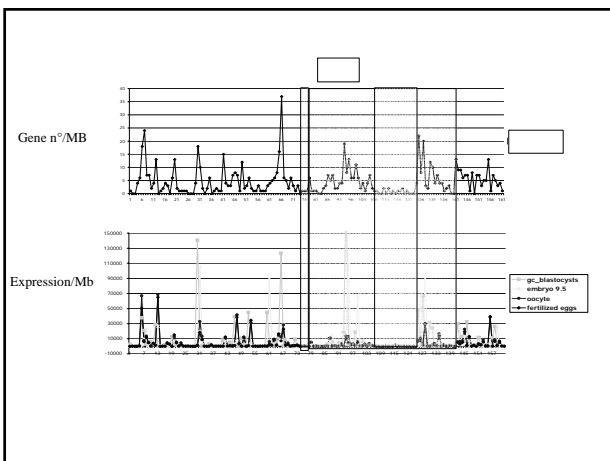
Dosage compensation in the POF critical region§

	X chromosome		Critical region		Rest of the chromosome	
	n [§]	X:A	n [§]	X:A	n [§]	X:A
ovary	365	1.30	171	1.62	194	1.01
oocyte	322	0.84	139	0.68	183	0.96
fertilized eggs	344	0.76	150	0.57	194	0.91
frontal cortex	373	1.25	181	1.36	192	1.15
hypothalamus	392	1.47	192	1.83	200	1.13
Others*	1377	0.97(0.30)	650	1.08 (0.26)	727	0.87 (0.34)

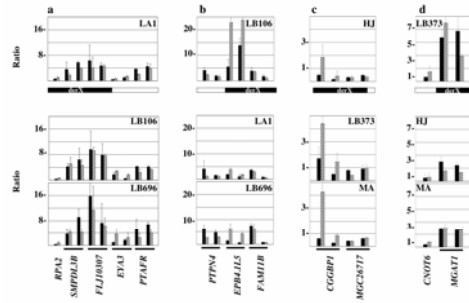
§ is in the mouse systemic region
n[§]: number of probes analyzed
*: average expression value of four somatic tissues: liver, kidney, lung, pancreas. In brackets, standard deviation
The average number of autosomal probes was 9864 for the mouse and 10629 for human tissues







Epigenetic modifications of the autosomal genes
translocated to the X chromosome
in POF patients



DIAPH2 L2-rare allele is associated with
Premature Ovarian Failure (POF)

(POF=161, Con=477)

SNP	Position	Alleles	Allele frequency i POF	Allele frequency i Controls	X-square p value
L2	95819273	C T	0,745 0,255	0,800 0,200	0,0394

The T allele is 5% more frequent in POF than in controls

Association of L2 subhaplotypes in North Italian POF cohort 1 (POF=161, Con=477)

SNPs	Haplotypes	Haplotypes frequency in POF	Haplotypes frequency in Controls	One-tail X-square p value (Bonferroni corrected)
L2+L3	TC TT	0,140 0,115	0,126 0,074	
L2+rs5949942	TT TG	0,180 0,080	0,115 0,080	0,0014 (0,0176) 0,5417
L2+rs1565828	TG TA	0,138 0,116	0,101 0,099	
L2+rs11092134	TA TT	0,155 0,100	0,123 0,077	
L2+rs6523076	TG TA	0,103 0,152	0,086 0,114	
L2+rs5966759	TG TA	0,164 0,091	0,130 0,070	

Association of the TT haplotype in North Italian POF cohort 2 (POF=161, Con=471)

SNPs	Haplotypes	Haplotypes frequency in POF	Haplotypes frequency in Controls	One-tail X-square p value
L2+rs5949942	TT	0,164	0,110	0,0058