

# DETECTION OF K-RAS MUTATIONS IN COLORECTAL CANCER AS SCREENING METHOD FOR TREATMENT SELECTION

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## INTRODUCTION

The **colorectal cancer** (CRC) corresponds to all those tumors located in the large intestine from the ileocecal valve to the rectum. In the recent years this disease has become a main health problem due to his high mortality and its increasing incidence. The principal treatment strategies are surgery (the only considerate curative), radiation therapy and chemotherapy, although in recent years a new treatment is being used: **monoclonal antibodies** (mAbs). The mechanism of action of these mAbs is that they recognize cellular components as antigens. The most used in CRC are those who recognize the **epidermal growth factor receptor** (EGFR) as Cetuximab and Panitumumab

The EGFR is found on the cell surface and is activated by binding its specific ligands. After the activation, this receptor suffers a conformational change from a monomeric form to an active homodimeric form. This stimulates the intrinsic activity of the intracellular protein tyrosine kinase which leads to the auto-phosphorylation of several tyrosine residues. The phosphorylation causes the activation of a cascade of proteins that initiate signaling transduction cascades of several signaling pathways, leading to DNA synthesis and cell proliferation. This can happen because these receptors are bound to a family of proteins called RAS.

The **family of proteins RAS** are G proteins (GTPases) which mediate between the extracellular ligand binding to EGFR and the intracellular transmission of signals to the nucleus. When these proteins are activated they incorporate into their structure a GTP that cause activation of BRAF that in turn activate MEK 1/2. These are the ones that activate by phosphorylation the transcription factors ERK 1/2. These are phosphorylated in the cytoplasm, translocated to the nucleus and bound DNA leading to the transcription of several genes important for cell proliferation. When the RAS proteins are mutated the signaling pathway is continuously activated and the mAbs lose their efficacy.

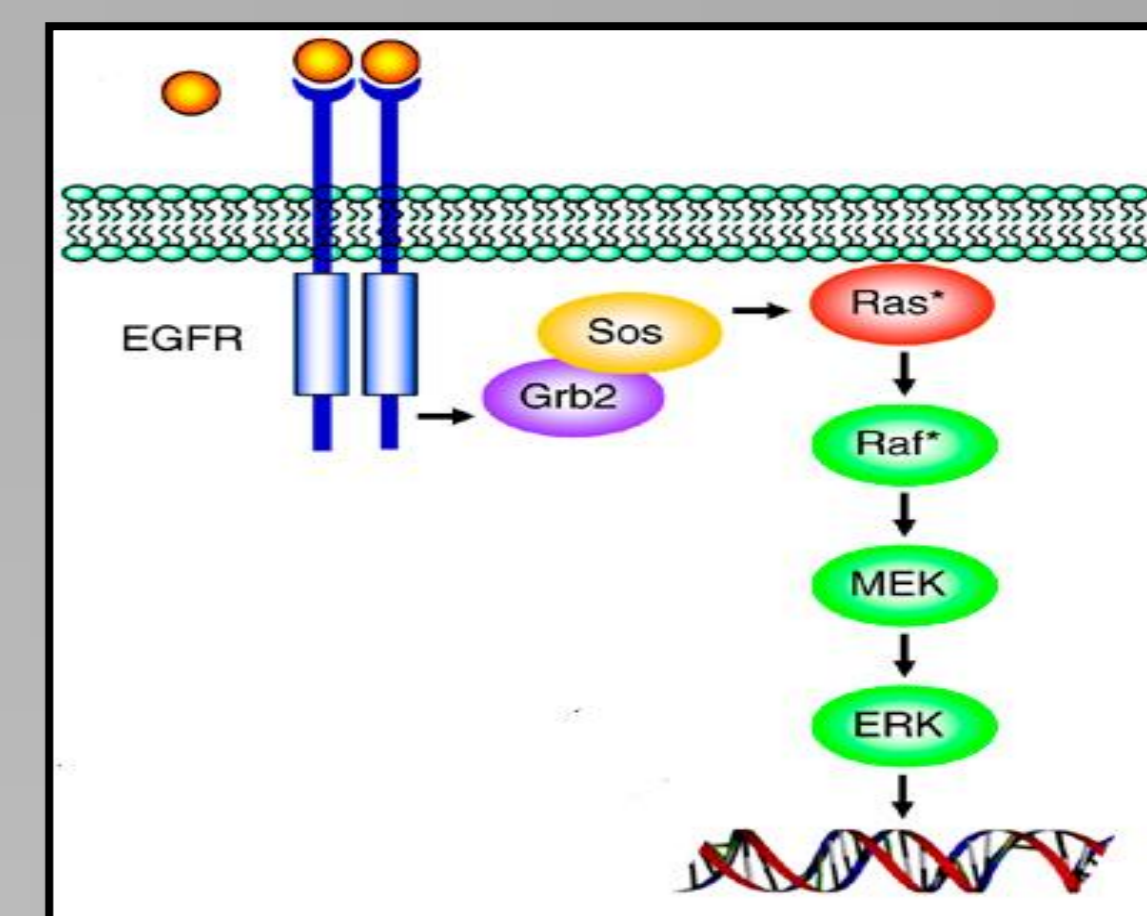


Figure 1. MAP kinases pathway.

## OBJECTIVES

The aim of this study is to assess the capacity of K-RAS status to predict possible resistances to treatment with mAbs.

## MATERIALS AND METHODS

### Study Subjects

Tumor samples were obtained from 83 patients with CRC who underwent surgery at the Cruces University Hospital between 2004 and 2007.

### DNA extraction

*Qiam DNA mini kit* (Qiagen) was used, following manufacturer's recommendations, to extract DNA from the tissue samples obtained after the surgical resection of the tumor.

### Polymerase Chain Reaction I + Gel Electrophoresis

With the aim of amplifying a specific region of DNA (codons 12 and 13 of the K-ras gene) for later sequencing, a PCR was made. *Kapa Taq Hot Start PCR kit* (Kapa Biosystems) was used following manufacturer's recommendations.

Specific primers were used to introduce a concrete aminoacid sequence that could be recognized by the restriction enzyme: the primer forward A (5'-TGT AAA ACG ACG GCC AGT AAA TAT ACT CTT GTG GAA GTA GTT GGA CCT-3') and the primer reverse B (5'-CAG GAA ACA GCT ATG ACC TCA AAG AAT GGT CCT GGA CC-3').

Finally PCR was tested by 3% agarose gel electrophoresis.

### Polymerase Chain Reaction II + Gel Electrophoresis

With the aim of enriching the number of mutated fragments cut in the previous RFLP process, a second PCR was performed. In this case a different primer was used instead of the primer reverse B: The primer reverse C (5'-CAG GAA ACA GCT ATG ACC TAA TAT GTC GAC TAA AAC AAG ATT TAC CTC-3').

This binds to the DNA sequence just before the cut produced by the restriction enzyme (Fig. 2). Finally PCR was tested by 3% agarose gel electrophoresis.

### Purification + Gel Electrophoresis

To extract the DNA from agarose gels a purification process was carried out using the *QIAquick Gel Extraction Kit* (Qiagen) and following manufacturer's recommendations.

Finally, purification was tested by 3% agarose gel electrophoresis.

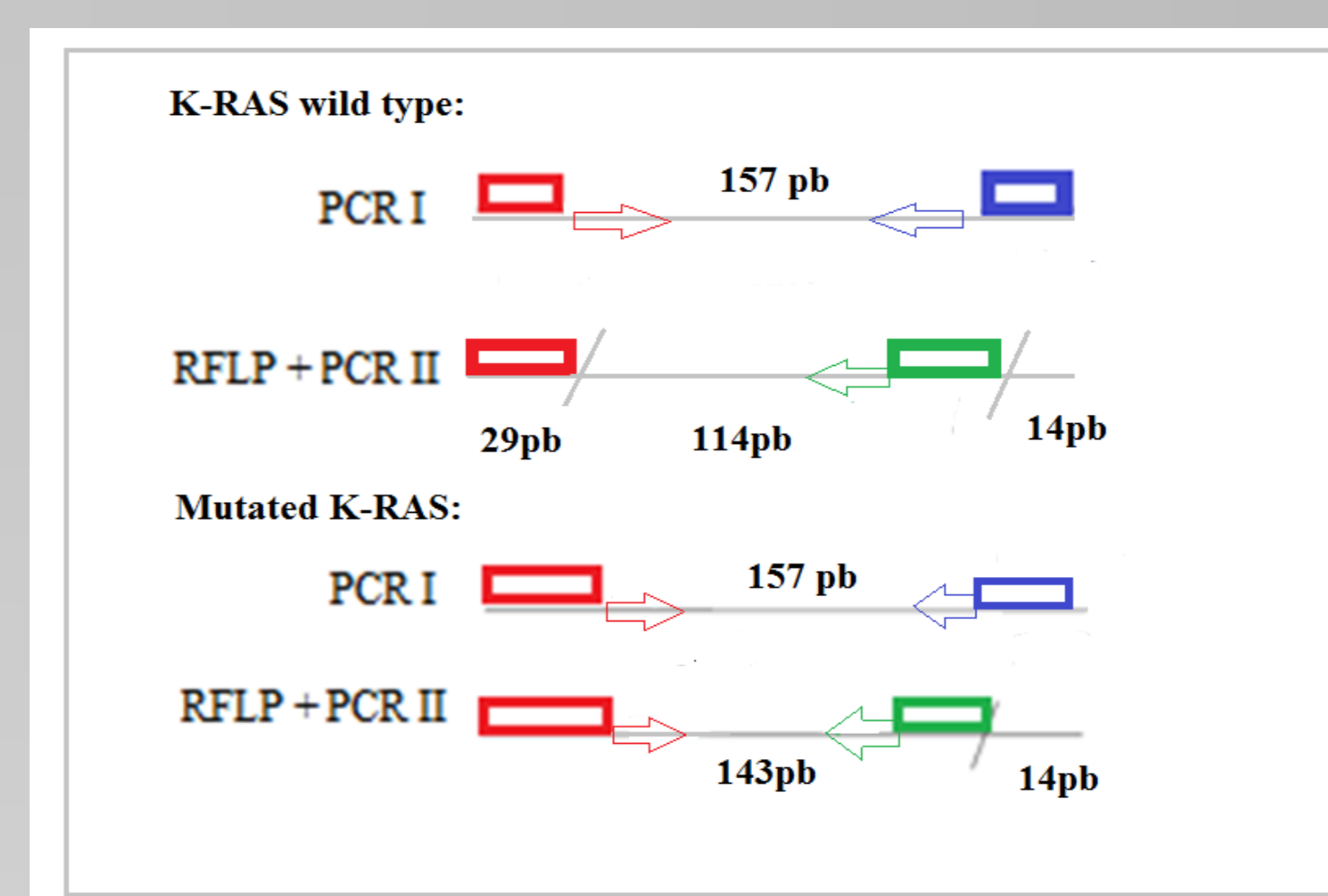


Figure 2. PCR I, II and RFLP.

### RFLP

The restriction enzyme *Mva* I is an endonuclease that recognizes these sequences: CC(A/T)GG and GG(T/A)CC. Later it binds to them and cuts creating different fragments of DNA. When the DNA amplified in the PCR I is mutated the enzyme only has one site to bind and cut, but when the DNA is wild type there is an extra site and cuts the fragment in 3 smaller fragments.

### Sequencing

To identify the DNA mutations the technique of automatic sequencing was used. The reaction was performed in 3 steps, first took out the sequencing reaction using the *Big Dye Terminator v3.1 Cycle Sequencing system* (Life technologies), below the purification using the Ethanol/ EDTA/ Sodium acetate protocol and finally a capillary electrophoresis was performed in the *ABI PRISM 3100 Avant Genetic Analyzer* (Life technologies).

## RESULTS AND DISCUSSION

### K-RAS MUTATIONS

Of 83 patients studied, 41% of cases had an oncogenic mutation of K-RAS gene. This mutation was in 20.5% of cases at codon 13 and in 79.5% of cases at codon 12. The most frequent mutations found were: GGT to GAT at codon 12 (glycine to aspartic acid), GGT to GTT also in codon 12 (glycine to valine) and GAC to GGC codon 13 (glycine to aspartic acid), figs. 3 and 4.

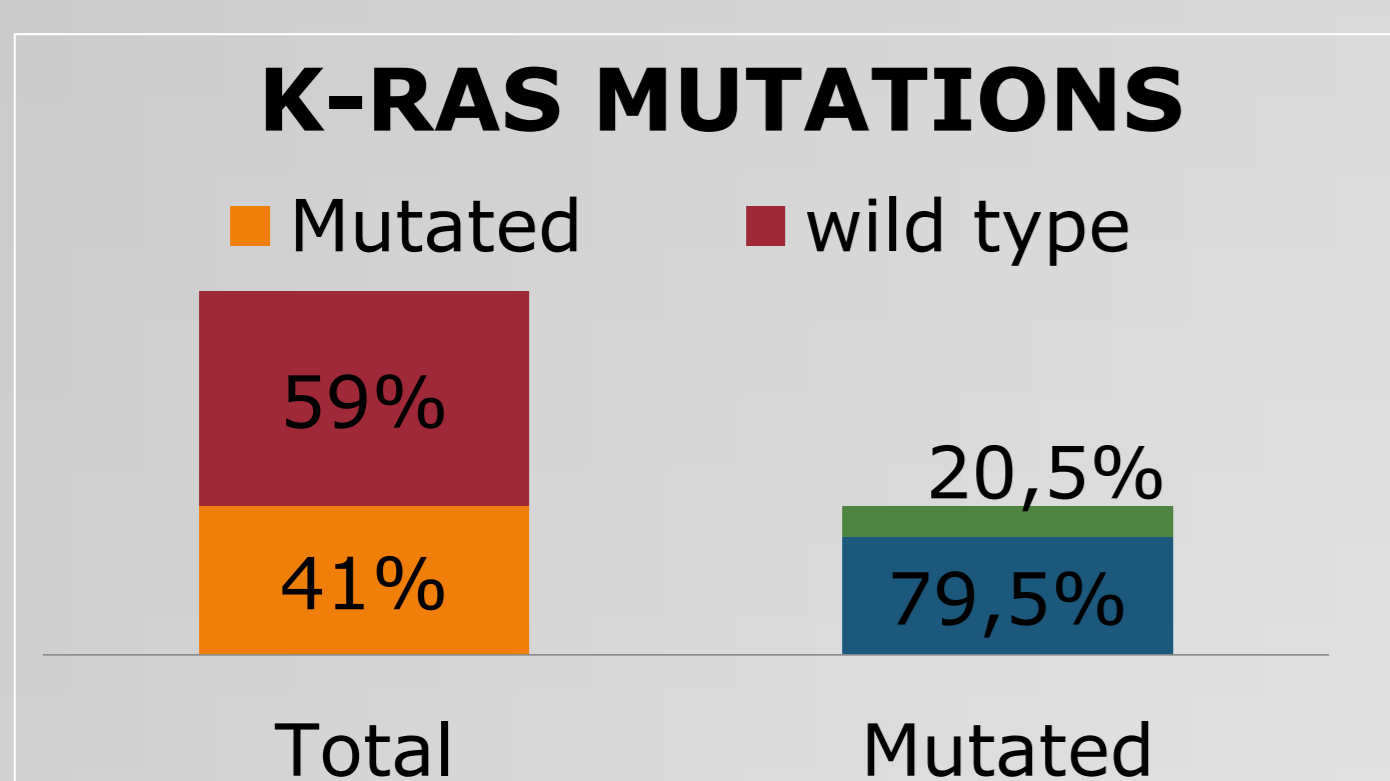


Figure 3. Distribution of k-ras mutations.

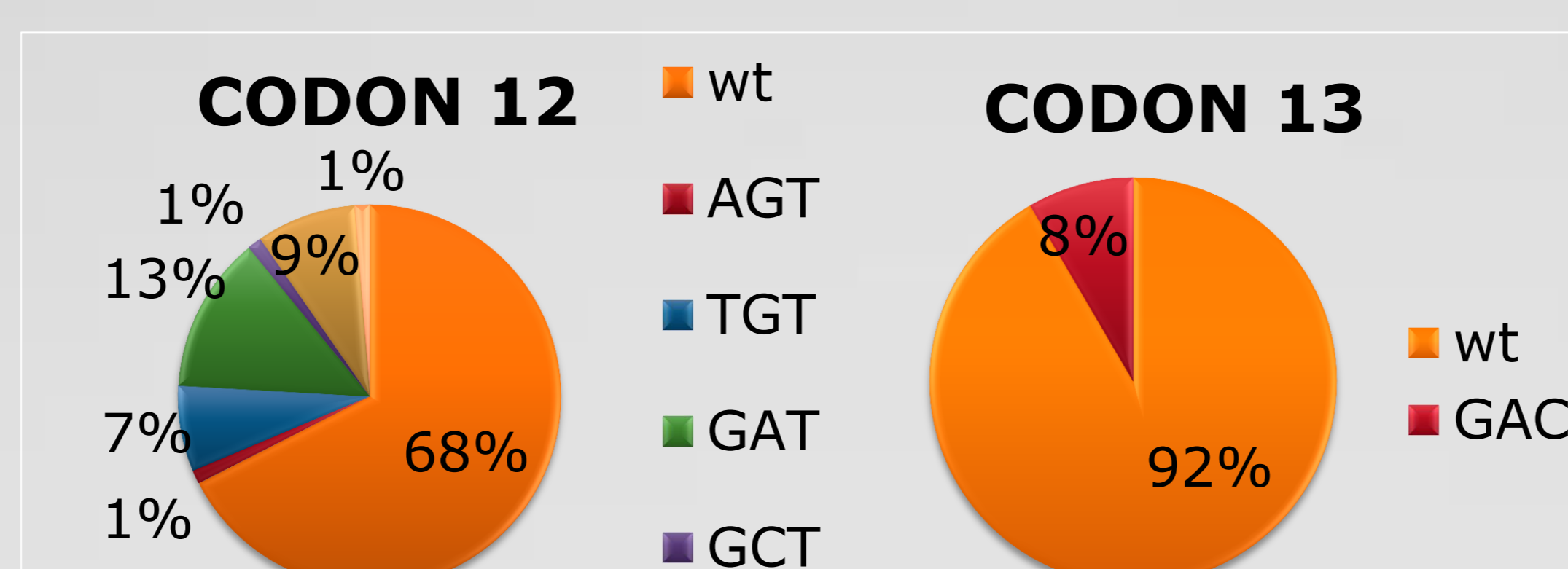


Figure 4. Distribution of k-ras mutations in codon 12 and 13.

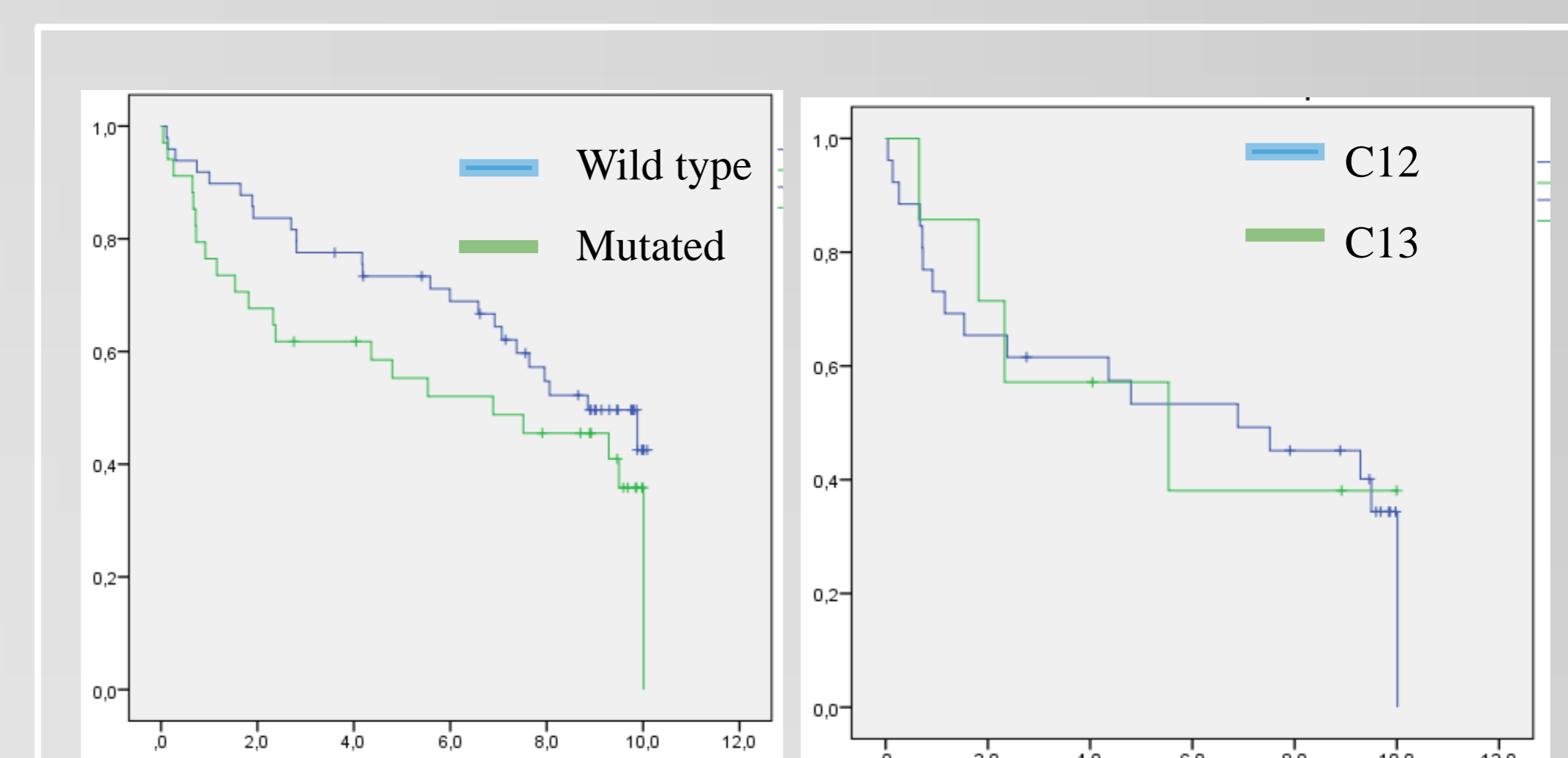


Figure 7. Relation between mutations and survival.

### MUTATIONS AND GENDER

Although the results seem to have a relationship between mutations of K-RAS gene and male gender, being more frequent in males than women, the results are not significant ( $p = 0.686$ ), fig.5.

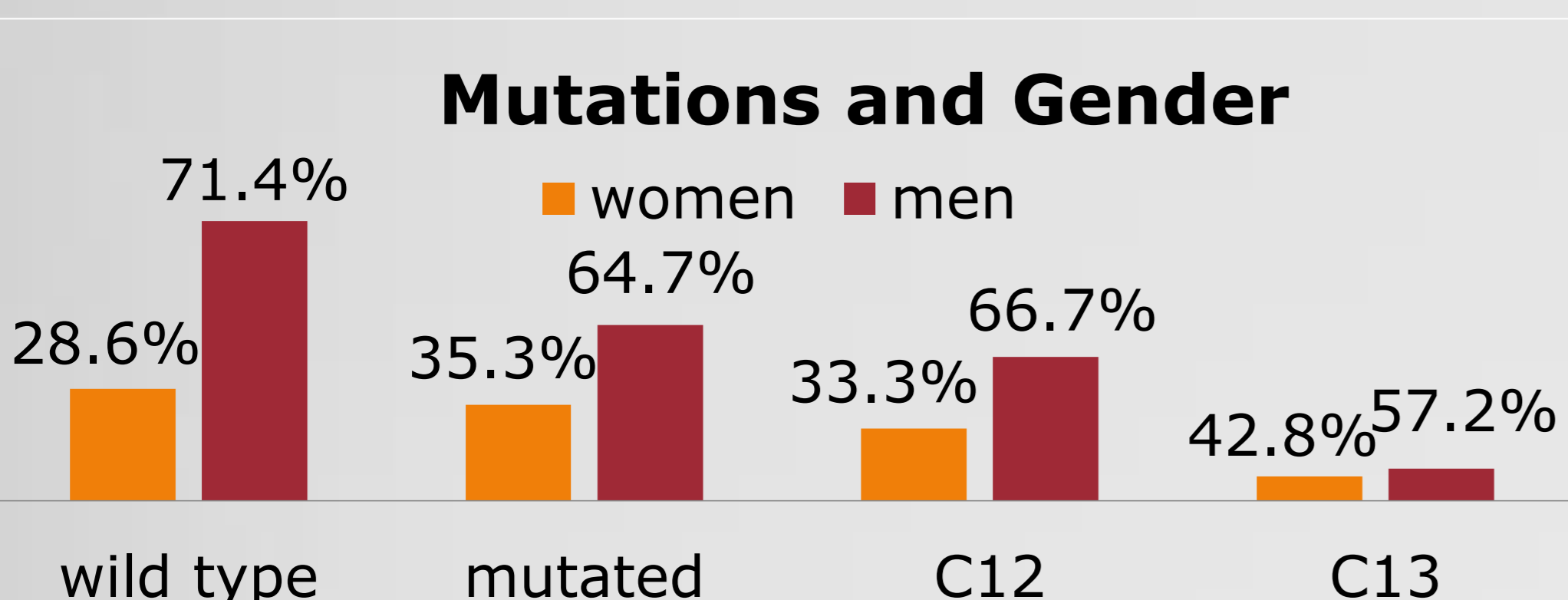


Figure 5. Relation between mutations and gender.

### MUTATIONS AND METASTASES

Although in this study, as in others, have been found that patients with mutations in the K-RAS gene have more frequent metastases, which means that tumors are more aggressive, especially at codon 13, the results are not significant ( $p = 0.609$ ), fig.6.

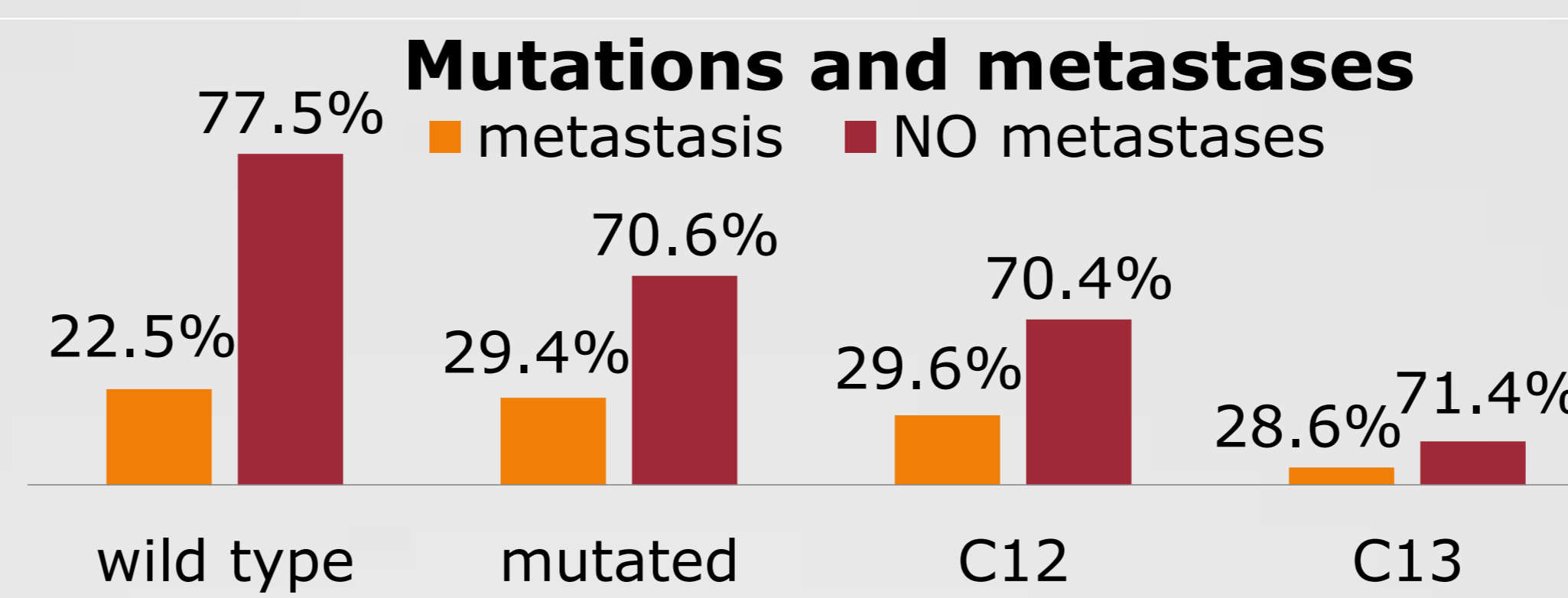


Figure 6. Relation between mutations and metastases.

### SURVIVAL AND METASTASES

According to the results, the survival is higher in subjects with K-RAS wild type. Furthermore the survival is higher in those with mutations at codon 13, although other studies probe that survival is higher at codon 12. Results obtained are not significant ( $p=0.157$  and  $p=0.984$ ), fig.7.

## CONCLUSION

In conclusion, the routine analysis of K-RAS mutations are recommended in all patients with metastatic CRC because allows to predict the possible resistance of the patients to the monoclonal antibodies that inhibit EGFR as Cetuximab or Panitumumab. Therefore, those with K-RAS mutated are not candidates for this type of treatment.

The mentioned measures will improve survival and minimize the costs of such treatment while avoiding exposing patients to unnecessary toxicity from drugs used and extending the time without the correct treatment.